EGF and \(K^+\) channel activity control normal and cystic fibrosis bronchial epithelia repair

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1Centre de recherche, Centre hospitalier de l’Université de Montréal (CR-CHUM)-Hôpital-Dieu, and 2Département de médecine and 3GÉPROM, Département de physiologie, Université de Montréal, Montréal, Québec, Canada

Submitted 12 March 2008; accepted in final form 25 August 2008

Trinh NT, Privé A, Maillé É, Noël J, Brochiero E. EGF and \(K^+\) channel activity control normal and cystic fibrosis bronchial epithelia repair. *Am J Physiol Lung Cell Mol Physiol* 295: L866–L880, 2008. –Severe lesions of airway epithelia are observed in cystic fibrosis (CF) patients. The regulatory mechanisms of cell migration and proliferation processes, involved in the repair of injured epithelia, then need to be better understood. A model of mechanical wounding of non-CF (NuLi) and CF (CuFi) bronchial monolayers was employed to study the repair mechanisms. We first observed that wound repair, under paracrine and autocrine EGF control, was slower (up to 33%) in CuFi than in NuLi. Furthermore, EGF receptor (EGFR) activation, following wounding, was lower in CuFi than in NuLi monolayers. Cell proliferation and migration assays indicated a similar rate of proliferation in both cell lines but with reduced (by 25%) CuFi cell migration. In addition, cell migration experiments performed in the presence of conditioned medium, collected from NuLi and CuFi wounded bronchial monolayers, suggested a defect in EGF/EGFR signaling in CF cells. We (49) recently demonstrated coupling between the EGF response and \(K^+\) channel function, which is crucial for EGF-stimulated alveolar repair. In CuFi cells, lower EGF/EGFR signaling was accompanied by a 40–70% reduction in \(K^+\) currents and KvLQT1, ATP-sensitive potassium (\(K_{ATP}\)), and \(Ca^{2+}\)-activated \(K^+\) (\(K_{Ca3.1}\)) channel expression. In addition, EGF-stimulated bronchial wound healing, cell migration, and proliferation were severely decreased by \(K^+\) channel inhibitors. Finally, acute CFTR inhibition failed to reduce wound healing, EGF secretion, and \(K^+\) channel expression in NuLi. In summary, the delay in CuFi wound healing could be due to diminished EGFR signaling coupled with lower \(K^+\) channel function, which play a crucial role in bronchial repair.

airways; potassium channels; cystic fibrosis; wound repair

IN CYSTIC FIBROSIS (CF), mutations of the gene coding for cystic fibrosis transmembrane conductance regulator (CFTR) \(Cl^-\) channels (43) cause dysfunction of \(Cl^-\) secretion, resulting in imbalanced \(Cl^-\), \(Na^+\), and fluid transport with reduced mucociliary clearance (10). Accumulation of viscous mucus, obstructing the small airways, favors bacterial colonization and chronic inflammation. Persistence of infection and inflammation then leads to progressive damage of the pulmonary epithelium and remodeling of the small and large airways, which ultimately evoke respiratory failure.

Chronic bronchiectasis is the most commonly observed structural abnormality in CF patients. Analyses of CF lung morphology initially reveal alterations of the small airways, whereas inflammation and mucus plugging of the large airways occur later. Ultrastructural studies also disclose nonspecific ciliary abnormalities, goblet cell hyperplasia, squamous cell metaplasia in airways, as well as areas of alveolar epithelium injury and regeneration (11, 17, 47). Most of these morphological changes seem not to be specific to CF disease and could be secondary to chronic bacterial infection and inflammation. Indeed, the release of proteases by neutrophils or bacteria, such as *Pseudomonas aeruginosa* for example, contributes to tissue damage in CF (41, 47). Overproduction of proinflammatory cytokines by inflammatory and epithelial cells in CF lungs also plays a role in injury and remodeling of respiratory epithelia (5, 47). After injury, epithelial cells, macrophages, and fibroblasts release growth factors such as hepatocyte growth factor (HGF; Refs. 13, 37, 47), EGF (1, 3, 13, 38, 47), keratinocyte growth factor (KGF; Refs. 13, 37, 47), and transforming growth factor-\(\beta\) (TGF-\(\beta\); Refs. 3, 47). After binding to their respective receptors on the injured airways and alveolar epithelia, these factors induce mitogenic, motogenic, and/or morphogenic cellular responses involved in their repair. However, the relative roles of inflammatory mediators and growth factors in the complex balance between lung injury, repair, and remodeling are not completely understood. In addition, the elevated levels of growth factors, observed in CF lungs, seem insufficient to protect lung epithelium against injury and remodeling (47). Thus it is crucial to better understand the repair mechanisms of lung epithelia to develop strategies that promote CF tissue healing.

We (49) have recently confirmed the involvement, in primary cultured alveolar epithelial cells, of an autocrine EGF loop acting on both erbB1 and erbB2 EGF receptors (EGFR) in the control of wound healing as well as cell migration and proliferation. We (49) have also demonstrated that ATP-sensitive potassium (\(K_{ATP}\)) and KvLQT1 \(K^+\) channels, expressed at the basolateral membrane of alveolar cells (25, 26), play a role in repair processes. Indeed, basal and EGF-stimulated repair processes are, for the most part, dependent on the activity of these \(K^+\) channels. Furthermore, close coupling between EGF signaling and \(K^+\) channel activity and expression has been highlighted. It was the first evidence of \(K^+\) channel involvement in EGF-stimulated lung epithelia repair. Our results were consistent with previous studies showing that \(K_{ATP}\) channels, \(Ca^{2+}\)-activated \(K^+\) channels (\(K_{Ca3.1}\)), and channels from the voltage-dependent Kv family control the basal- and growth factor-mediated proliferation and migration of various cell types (4, 7, 21–23, 35, 40, 42, 45, 54, 56).

The aim of the present study was to evaluate the role of EGF signaling and \(K^+\) channel activity in the repair processes of...
normal and CF bronchial epithelia. We chose two cell lines, NuLi-1 and CuFi-1, derived from normal (NuLi) and CF (CuFi-1, Δ508/Δ508 genotype) human airway epithelial cells (HAE) (57). We first evaluated the repair capacity of NuLi and CuFi monolayers after wounding. We then determined the respective involvement of EGF/EGFR signaling and K⁺ channels in the repair processes. We observed that the delayed repair of CuFi monolayers was coupled with decreased EGF secretion and/or EGFR activation as well as reduced KATP, repair of CuFi monolayers was coupled with decreased EGF

**MATERIALS AND METHODS**

**Cell culture.** Repair processes of normal and CF bronchial epithelia were studied in two cell lines, NuLi-1 and CuFi-1, a generous gift from Dr. J. Zabner, University of Iowa (57). The NuLi-1 (normal lung) line was derived from HAE of normal genotype, whereas the CuFi-1 line was derived from a CF patient with Δ508/Δ508 genotype. The NuLi-1 and CuFi-1 bronchial epithelial cell lines were obtained by transformation of normal and CF primary HAE with a RT component of telomerase and human papillomavirus type 16 E6 and E7 genes.

For the wound healing, cell proliferation, and migration experiments, NuLi and CuFi cells, from passages 11 to 17, were seeded at a specified density on Petri dishes coated with human placental collagen type VI (C7521; Sigma-Aldrich, St. Louis, MO) and cultured in bronchial epithelial cell growth medium (BEBM; Lonza, Walkersville, MD) supplemented (BEGM) with EGF, hydrocortisone, bovine pituitary extract, transferrin, bovine insulin, triiodothyronine, epinephrine, retinoic acid, penicillin-streptomycin (0.25 μg/ml), gentamicin (0.05 μg/ml), and flouconazole (0.002 μg/ml). This normal BEGM contained 0.5 ng/ml EGF (BEGM-EGF0.5). Some wound healing, cell proliferation, and migration experiments were performed in the absence of EGF (BEGM not supplemented with EGF; BEGM-EGF-free).

For electrophysiology experiments, NuLi and CuFi were plated at 1 × 10⁵ cells/cm² on Costar Transwell permeant filters (Costar Transwell, Toronto, Ontario, Canada) coated with collagen type VI and grown in DMEM/F-12 (GIBCO, Grand Island, NY), this medium was supplemented with 5% fetal bovine serum (26140-079, GIBCO) and grown in DMEM/F-12 (GIBCO, Grand Island, NY). This medium was supplemented with 5% fetal bovine serum (26140-079, GIBCO) for the first 24 h and then replaced by Ultroser G (2%, 15950-017; Invitrogen, Burlington, Ontario, Canada). Suspension was collected after 1 h.

**CuFi monolayers were washed with BEGM-EGF0.5 to remove detached, injured cells, and fresh BEGM-EGF-free medium was added.**

**In some experiments (EGF0.5), 0.5 ng/ml EGF was added in the upper compartment.**

**The lower compartment was filled with BEGM-EGF0.5 or BEGM-EGF-free in the presence or absence of K⁺ channel modulators.**

After a 6-h period, the filters were washed with PBS, and migrating cells that passed through the lower face of the filters were fixed with paraformaldehyde-acetone solution and stained for 48 h with crystal violet (0.1% in 20% methanol). After some washes, nonmigrating cells of the upper compartment were scraped off with cotton-tipped applicators (Fisher, Nepean, Ontario, Canada). The filters were examined by microscopy at x40 enlargement, and cells that had migrated were counted in five different fields.

**In migration assays with conditioned media, bronchial epithelial cell suspensions were resuspended in BEGM-EGF-free and placed in the upper compartment of the filters.**

**The lower compartment was filled with the relevant wound (WCM) or unwounded BEGM-EGF-free conditioned media (see Wound healing) in the presence or absence of 5 μg/ml EGF Ab or CFTRinh-172 (C2992; Sigma-Aldrich, Oakville, Ontario, Canada).**

**Cell proliferation.** NuLi and CuFi cell proliferation was evaluated by [³H]thymidine incorporation assays (over an 18-h period) as well as by counting the cell number (at day 3 of culture).

**For [³H]thymidine incorporation assay, NuLi and CuFi cells were seeded at low density (75,000 cells/well) in 12-well plates (Costar, Corning, NY), coated with human placental collagen type VI (C7521, Sigma-Aldrich) and cultured for 48 h in BEGM-EGF0.5 (until 50% confluence).**

**The medium was then replaced by BEGM-EGF-free containing 0.5 μCi/ml [³H]thymidine (MP Biomedical, Irvine, CA) in the presence of K⁺ channel modulators and/or EGF0.5.**

**After an 18-h period of incorporation, the [³H]thymidine medium was removed by inversion and replaced by ice-cold TCA (5%, 5–10 min).**

TCA was then discarded, and the plates were washed in three successive ice-cold water baths. The cells were lysed with 0.1 N NaOH, transferred into scintillation tubes, and counted (Bq/cm²) in a beta counter (Tri-Carb 1600TR liquid scintillation analyzer, Canberra Packard). Protein content was measured by Bradford assay (Pierce, Rockford, IL). [³H]thymidine incorporation (counts per minute) was presented as percentage of NuLi incorporation (in BEGM-EGF-free).

**Cell proliferation was also evaluated by measuring cell number after 3 days of culture.**

**NuLi and CuFi cells were seeded at low density (150,000 cells) in 35-mm Petri dishes coated with human placental collagen type VI and cultured for 3 days in BEGM-EGF0.5.**

The cells were then separated with trypsin-EDTA (0.25%) and counted on a hemacytometer.
**Immunoblotting.** Total proteins were extracted from bronchial epithelial cells cultured on collagen-coated plastic dishes for 8 days in BEGM-EGF. Twenty-four hours before extraction, some of them were treated with EGF (25 ng/ml EGF, BEGM-EGF) for at least 2 hours at room temperature and then homogenized to ensure equivalent loading. The intensity of p-EGFR and p-erbB2 proteins was quantified with ImageJ software and normalized to the actin protein as loading control. 

**Ectopic expression of human KATP, KCa3.1, and KvLQT1 channels in CF bronchial epithelial cells**

For KvLQT1, Kir6.1 (KATP), and KCa3.1 K+ channel detection (26, 49), the cells were solubilized in lysis buffer [150 mM NaCl, 50 mM Tris-HCl, pH 7.6, 1% Triton X-100, 0.1% SDS, and protease inhibitor cocktail (Complete Mini EDTA-free protease inhibitor cocktail; Roche, Mannheim, Germany)] for 1 h on ice and centrifuged at 12,000 g for 15 min. The supernatants were collected, and protein content was measured. After denaturation at 95°C for 5 min in 2× sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.2% bromophenol blue, and 4% 2-mercaptoethanol), the proteins (20–60 µg for KvLQT1 and Kir6.1, 120 µg for KCa3.1) were separated by SDS-PAGE (7.5%) and transferred onto polyvinylidene difluoride membranes. The membranes were first blocked with 10% dried fat-free milk in TBST for 1 h (18 h for KCa3.1) at room temperature and then incubated with commercial polyclonal antibodies directed against either Kir6.1 (dilution 1:200, sc-11224; Santa Cruz Biotechnology), anti-KvLQT1 (dilution 1:1,000, sc-10645, Santa Cruz Biotechnology), or anti-KCa3.1 (dilution 1:300, APC-064; Alomone Labs, Jerusalem, Israel) in TBST plus 10% milk, overnight at 4°C (KCa3.1 Ab was incubated for 6 h at room temperature before overnight blotting at 4°C). After washing with TBST, the membranes were incubated with donkey anti-goat (for KvLQT1 and Kir6.1, Santa Cruz Biotechnology) and goat anti-rabbit (for KCa3.1, Cell Signaling Technology) IgG linked to horseradish peroxidase for 1 h. The anti-Kir6.1, anti-KCa3.1, and anti-KvLQT1 antibodies, respectively, recognized a ~50-kDa (Kir6.1, KCa3.1) and 75-kDa (KvLQT1) protein (26, 49).

**PCR amplification of K+ channels.** Total RNA from bronchial epithelial cells was purified with TRIzol reagent according to the manufacturer’s instructions (Invitrogen). Five micrograms of total RNA were reverse-transcribed to cDNA with Moloney murine leukemia virus (MMLV) RT (Invitrogen) in the presence of oligo(dT) primers. cDNAs were amplified with Taq polymerase (Invitrogen), employing specific primers designed from sequences of the following cloned K+ channels: human KvLQT1 (GenBank acc. no. NM_002182.2), KCa3.1 (GenBank acc. no. NM_002250.2), Kir6.1 (GenBank acc. no. NM_004982), and SUR2B (GenBank acc. no. NM_020297). KvLQT1 primers (sense: 5′-taaagaagaccaacactgct-3′, exon 11; antisense: 5′-cagatccttgcttcttga-3′, exon 15; 1 µM final concentration of each, 355 bp PCR product), KCa3.1 primers (sense: 5′-cggctgctgtcaacg-3′, exon 5; antisense: 5′-caccagcaggctgtcag-3′, exon 6; 1 µM, 337 bp PCR product), and SUR2B primers (sense: 5′-ttgccacctggactctc-3′; antisense: 5′-cgcgaacaaaagaacaaag-3′; 1 µM, 223 bp PCR product) served to explore the presence of these K+ channels in bronchial cells. The PCR products were separated on agarose gels, stained with ethidium bromide, and analyzed by Typhoon Gel Imager (25, 26).

**Electrophysiology.** The electrophysiological characteristics of bronchial epithelial layers were studied by short-circuit current (Isc) measurements in an Ussing chamber (25, 26, 49). First, bronchial epithelial cells were cultured on filters (4 cm2) Costar Transwell, coated with human collagen type VI for 6–8 wk until they reached air liquid interface (see Cell culture) and formed a polarized epithelium with high resistance (> 1,200 Ω·cm2). The cells were then treated or not for 24 h with EGF (25 ng/ml) at the basolateral side. After washing, the alveolar monolayers were mounted in a heated (37°C) Ussing chamber and perfused on the apical and basolateral sides with warm physiological solution. Some short-circuit experiments were also performed with acute EGF treatment (10 min). To evaluate the amount of active K+ channels at the basolateral membrane, Ileak was measured after establishment of a K+ gradient and permeabilization of the apical membrane with 7.5 µM amphotericin B. The apical-to-basolateral K+ gradient was created by bathing the apical side with high K+ physiological solution (containing in mM: 81 NaCl, 65.4 KCl, 0.78 Na2HPO4, 0.8 MgCl2, 1.8 CaCl2, 5 glucose, and 15 HEPES, pH 7.4), whereas for the basolateral side, 60 mM KCl was replaced by an equivalent amount of N-methyl-D-glucamine chloride (NMDGCl). Transepithelial potential difference was clamped to zero by an external voltage clamp amplifier (VCCMC2, Physiological Instruments) with KCl agar-calomel half-cells and Ag-AgCl electrodes, and the resulting Ileak was recorded continuously on a computer with a PowerLab system (ADInstruments, Toronto, Ontario, Canada) (25). Membrane resistance was verified with 1-mV pulses every minute.

**Statistics.** The data are represented as means ± SE collected from at least 4 different passages (n > 4). Groups were compared by paired t-test or the one-group t-test with StatView software (SAS Institute, Cary, NC). A probability of P < 0.05 was considered to be significant (P values were reported for each experiment).

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**RESULTS**

**Wound healing of normal and CF bronchial monolayers.** The repair processes of normal and CF bronchial epithelia were studied in our model of mechanical wound injury (49), adapted to the normal and CF bronchial cell lines NuLi and CuFi, respectively. Wound healing was then compared in NuLi and CuFi monolayers, in various EGF conditions (Fig. 1). First, the wound healing of NuLi and CuFi monolayers was followed over an 18-h period in BEGM-EGF (6.5-fold (Fig. 1A). We observed 33.7% ± 2.3% of repair for NuLi monolayers at 6 h after injury. Wound healing was complete after 18 h (99.5% ± 2%, n = 44). Wound repair was slower in CuFi monolayers (22.5% ± 1.9% at 6 h, i.e., a 1/2 decrease compared with NuLi,
n = 46; P < 0.0001). We also noted a delay (1/3 reduction compared with NuLi) in CuFi repair after 18 h, suggesting that the mechanism of repair is defective in CuFi cells.

The absence of EGF in culture medium (BEGM-EGFfree) reduced wound healing of NuLi monolayers by one-half (51.8% ± 4.1% of repair at 18 h, n = 43, Fig. 1B, instead of 99.5% in BEGM-EGF0.5, Fig. 1A). We then decided, in subsequent wound-healing experiments, to study bronchial wound repair in the presence of EGF at 6 h after injury and in the absence of EGF at 18 h postinjury.

Similar to findings in the BEGM-EGF0.5 condition, wound healing in BEGM-EGFfree was significantly lower in CuFi (38.8% ± 3.2% repair at 18 h, n = 43; Fig. 1B) than in NuLi (P < 0.0001). An EGF Ab was then added to BEGM-EGFfree to titrate EGF, potentially released from injured cells. This EGF Ab induced a dramatic decrease in wound repair, reaching 17.5% ± 4.9% and 20.2% ± 5% at 18 h after injury in NuLi and CuFi, respectively (Fig. 1B, BEGM-EGFfree + Ab). It has to be noted that wound repair in the complete absence of exogenous or secreted EGF (Fig. 1B, BEGM-EGFfree) or BEGM not supplemented with EGF (BEGM-EGFfree; Ab, 5 ug/ml) was similar in NuLi and CuFi monolayers, indicating that the EGF-independent signaling mechanisms contributing to wound repair are not affected in CuFi cells. However, in the presence of secreted EGF (BEGM-EGFfree in the absence of EGF Ab; Fig. 1B), stimulation of wound healing was higher for NuLi than for CuFi cells (P < 0.0001), suggesting that EGF secretion was lower in CF cells. These results indicated that lower EGF secretion and/or EGF signaling could contribute to the delay in CF monolayer repair.

EGFR activity in normal and CF bronchial monolayers during repair. Since bronchial wound repair was dependent on EGF, EGFR expression and EGFR activation (phosphoprotein) were evaluated by immunoblotting (Fig. 2A). The level of erbB1 (EGFR, Fig. 2A) receptor protein expression was evaluated first. We observed that EGFR expression was not affected by elevation of EGF concentration (EGF0.5 to EGF25; P = 0.9), whereas 24-h treatment with EGF Ab induced a small (14%) but nonsignificant (P = 0.26) decrease in EGF. Comparison of EGFR expression in NuLi and CuFi extracts revealed a 20% reduction of EGFR in CuFi cells (P < 0.02).

EGFR activation was then evaluated by quantifying the ratio of p-EGFR to EGF signals (p-EGFR/EGFR, presented as percentage of this ratio measured in NuLi extracts in the EGF0.5 condition). As seen in Fig. 2A, EGFR was activated (2.5-fold increase of p-EGFR/EGFR in NuLi) by 10-min exposure to 25 ng/ml EGF (EGF25). In contrast, the presence of 5 ug/ml EGF Ab for 24 h in BEGM-EGFfree (EGF Ab) severely lowered the NuLi p-EGFR845 level. EGFR activation was also evaluated in bronchial monolayers during repair. In this experiment, proteins were extracted 30–60 min after mechanical injury of the monolayers. Interestingly, EGFR activation was enhanced by 51.6% ± 18% in injured (wound) compared with intact NuLi monolayers (EGF0.5). These results indicated activation of the EGF/EGFR autocrine loop during bronchial monolayer repair.

Comparison of EGFR activation in normal and CF monolayers revealed that the p-EGFR/EGFR level (Fig. 2A) was lower in CuFi than in NuLi cells in basal conditions (EGF0.5; P = 0.04) as well as after EGF stimulation (10 min, EGF25; P = 0.04). Finally, EGF845 activation in wounded bronchial monolayers was also lower in CuFi than in NuLi cells (Fig. 2A, wound; P = 0.01). These data indicated that lower EGF secretion and/or EGFR activation during repair could be postulated in CF bronchial cells.

erbB2 and p-erbB2 levels were also evaluated. Unlike erbB1, erbB2 protein expression was found to be similar in
NuLi and CuFi cells as well as in EGF$^{0.5}$ or EGF$^{25}$ conditions. We also discerned a 2.8-fold increase in the p-erbB2$^{877}$ level after EGF activation (EGF$^{25}$). On the other hand, p-erbB2 stimulation in wounded monolayers was not detectable (data not shown). Finally, the p-erbB2/erbB2 level was lower in CuFi than in the NuLi in EGF$^{0.5}$ as well as in the EGF$^{25}$ condition, indicating that erbB2 signaling could also be reduced in CF cells.

**Role of EGF in bronchial cell migration and proliferation.**

Since cell migration and proliferation are involved in epithelial regeneration after injury, we also analyzed these processes in NuLi and CuFi cells in various EGF conditions. Migration of NuLi and CuFi cells was evaluated over a 6-h period in a Boyden-type migration chamber, with BEGM-EGFfree in both the upper and lower compartments. The number of migrating NuLi cells (Fig. 3A, column 1) was higher than the number of CuFi migrating cells (75.8% ± 3.9% of NuLi cell migration, n = 37; P < 0.001; Fig. 3A, column 6). This reduced cell migration of CF cells could explain the delay in CF monolayer repair.

The impact of wounded conditioned BEGM-EGFfree medium (WCM), collected from NuLi (NuLi-WCM) and CuFi (CuFi-WCM) monolayers 1 h after injury, applied at the lower side of the migration chamber, was then tested. The WCM collected from NuLi-injured cells (NuLi-WCM) increased the number of NuLi migrating cells by 43.9% (7.5% (n = 27; P < 0.0002; Fig. 3A, column 2), a value similar to that observed after the addition of exogenous EGF (0.5 ng/ml, column 5). In addition, the stimulatory effect of NuLi-WCM was completely abolished by the presence of EGF Ab (column 3). On the other hand, the conditioned medium collected from noninjured NuLi monolayers did not significantly (NS) stimulate NuLi cell migration (5.8% ± 4.5%, n = 9, NS, data not shown). These results confirm the autocrine activation of cell migration through EGF receptor activation after EGF secretion postinjury.

The WCM collected from CuFi (CuFi-WCM) also stimulated the migration of CuFi cells (Fig. 3A, column 7; P = 0.0003). However, the number of CuFi-migrating cells in the presence of CuFi-WCM (column 7) remained significantly
lower than the number of NuLi-migrating cells measured in the presence of NuLi-WCM (column 2). NuLi-WCM had a higher stimulatory effect on CuFi cell migration (column 9; \( P \leq 0.002 \)). Conversely, CuFi-WCM was less effective on NuLi cell migration (column 4). These results confirmed higher EGF secretion/EGFR signaling in injured NuLi than CuFi cells.

NuLi and CuFi cell proliferation was then compared by evaluating the increase in cell number over a 3-day period of culture (Fig. 3B); 150,000 NuLi or CuFi cells were seeded at day 0. After 3 days of culture in BEGM-EGF-free, the number of cells, counted after detachment with trypsin, was not significantly different in NuLi vs. CuFi. Cell proliferation was also estimated by \([3H]\)thymidine incorporation assay over an 18-h period (Fig. 3C). As with cell count measurements, \([3H]\)thymidine incorporation in CuFi (106.5% \( \pm \) 12% of NuLi, \( n = 20 \)) was not lower than in NuLi cells (Fig. 3C). The presence of EGF (0.5 ng/ml) during the assay elicited a nonsignificant increase in \([3H]\)thymidine incorporation in NuLi and CuFi cells (Fig. 3C).

Our migration and proliferation assays indicate that a decrease in cell migration, rather than cell proliferation, could explain the delay in CuFi monolayer repair.

Evidence of KvLQT1, \( K_{ATP} \), and \( KCa3.1 \) channels in bronchial cells. We (49) recently demonstrated coupling between EGF signaling and \( K^{+} \) channels as well as the involvement of \( K^{+} \) channel activity in EGF-stimulated wound repair of primary cultured alveolar type II cells. Since EGF signaling seems to be downregulated in CuFi cells, we decided to compare \( K^{+} \) function in NuLi and CuFi bronchial epithelia. We first defined the identity of \( K^{+} \) channels, which was not determined yet in NuLi and CuFi cells. As illustrated in Fig. 4A, cDNA transcripts of Kir6.1 and SUR2B (forming a \( K_{ATP} \) channel) as well as KvLQT1 and KCa3.1 were detected in bronchial cells. KvLQT1, KCa3.1, and \( K_{ATP} \) proteins were also revealed by Western blotting (Fig. 4B). We explored the presence of \( K_{ATP} \), KvLQT1, and KCa3.1 activities in bronchial monolayers. NuLi cells were cultured on permeant filters at the air-liquid interface and mounted in an Ussing chamber to measure transepithelial \( I_{sc} \). In these experiments, the apical membrane was permeabilized with amphotericin B and an apical-to-basolateral \( K^{+} \) gradient was established to estimate the presence of active \( K^{+} \) channels at the basolateral membrane. Clofilium (Fig. 4C; an inhibitor of KvLQT1 channels) or glibenclamide (Fig. 4D; an inhibitor of \( K_{ATP} \) channels) applied at the basolateral membrane revealed
3.1 (Figs. 4C, D, and E). Total K+ currents through the basolateral membrane of NuLi and CuFi were compared first. We recorded K+ currents (I_KATP; Fig. 5, B, and D) and KATP (glibenclamide-sensitive K+ current, I_KATP; Fig. 5, C and E) currents. Clofilium-sensitive K+ current and glibenclamide-sensitive K+ current through the NuLi basolateral membrane were 54.6 ± 11 μA/cm² (n = 7; Fig. 5B) and 38 ± 7 μA/cm² (n = 6; Fig. 5C), respectively. The total of these two currents (54 ± 38 = 92 μA/cm²) was similar to total K+ current (99 μA/cm²; Fig. 5A) in the absence of the inhibitors. Consistently, we observed (Fig. 4E) that clofilium and glibenclamide, applied in combination, completely blocked total basolateral K+ currents. These results indicate that KvLQT1 and KATP channels contribute to the major part of basolateral K+ current through NuLi epithelia. In CuFi cells, KvLQT1 currents (26.6 ± 4.5 μA/cm²; n = 7; Fig. 5B) and KATP currents (19.1 ± 2.4 μA/cm²; n = 6; Fig. 5C) were lower (P < 0.05) than in normal NuLi cells. We also compared KCa3.1 current after activation by 1-EBIO, which was found to be higher in NuLi (69.1 ± 13, n = 4) than in CuFi (21.3 ± 13, n = 4) epithelia. Taken together, our electrophysiological results demonstrated lower KvLQT1, KATP, and KCa3.1 currents through the basolateral membrane of CuFi than NuLi.

Comparison of K+ currents in normal and CF bronchial monolayers. We then quantified and compared K+ currents through the basolateral membrane of NuLi and CuFi cells in an Ussing chamber, after apical membrane permeabilization and establishment to an apical-to-basolateral K+ gradient (Fig. 5). Total K+ currents through the basolateral membrane of NuLi and CuFi were compared first. We recorded 99 ± 11 and 67 ± 6 μA/cm² K+ currents through the basolateral membrane of NuLi and CuFi monolayers, respectively, i.e., a one-third decrease in CF cells (P < 0.0001; n = 16; Fig. 5A).

Similarly to the experiments reported in Fig. 4, C and D, clofilium and glibenclamide were applied at the basolateral membrane to specifically inhibit KvLQT1 (clofilium-sensitive K+ current, I_KvLQT1; Fig. 5, B and D) and KATP (glibenclamide-sensitive K+ current, I_KATP; Fig. 5, C and E) currents. Clofilium-sensitive K+ current and glibenclamide-sensitive K+ current through the NuLi basolateral membrane were 54.6 ± 11 μA/cm² (n = 7; Fig. 5B) and 38 ± 7 μA/cm² (n = 6; Fig. 5C), respectively. The total of these two currents (54 ± 38 = 92 μA/cm²) was similar to total K+ current (99 μA/cm²; Fig. 5A) in the absence of the inhibitors. Consistently, we observed (Fig. 4E) that clofilium and glibenclamide, applied in combination, completely blocked total basolateral K+ currents. These results indicate that KvLQT1 and KATP channels contribute to the major part of basolateral K+ current through NuLi epithelia. In CuFi cells, KvLQT1 currents (26.6 ± 4.5 μA/cm²; n = 7; Fig. 5B) and KATP currents (19.1 ± 2.4 μA/cm²; n = 6; Fig. 5C) were lower (P < 0.05) than in normal NuLi cells. We also compared KCa3.1 current after activation by 1-EBIO, which was found to be higher in NuLi (69.1 ± 13, n = 4) than in CuFi (21.3 ± 13, n = 4) epithelia. Taken together, our electrophysiological results demonstrated lower KvLQT1, KATP, and KCa3.1 currents through the basolateral membrane of CuFi than NuLi.
Since we (49) have shown previously that alveolar KvLQT1 and K<sub>ATP</sub> currents are stimulated by acute (10 min) EGF exposure, we also evaluated the impact of EGF on K<sup>+</sup> currents in bronchial cells (Fig. 5, D and E). This acute EGF treatment stimulated KvLQT1 currents in NuLi cells (54.6 ± 11.4 and 77.1 ± 11.9 μA/cm<sup>2</sup>, in the absence and presence of EGF, n = 7; P = 0.009), whereas it had a small (NS) effect in CuFi cells (26.6 ± 4.5 and 37.7 ± 7.4 μA/cm<sup>2</sup>, n = 7; P = 0.13). EGF also upregulated glibenclamide-sensitive K<sub>ATP</sub> current of NuLi (37.9 ± 7.2 and 66.1 ± 11.8 μA/cm<sup>2</sup>), before and after EGF addition, respectively, n = 6; P = 0.0036) and CuFi epithelia (19.1 ± 2.4 and 54.7 ± 13.1 μA/cm<sup>2</sup>), before and after EGF addition, respectively, n = 6; P = 0.037). On the other hand, acute EGF treatment had no effect on either NuLi or CuFi KCa3.1 current (data not shown). Finally, we tested the impact of 24-h EGF treatment on bronchial cells and noted that neither KvLQT1 nor K<sub>ATP</sub> K<sup>+</sup> currents were affected by this treatment (data not shown).

Comparison of K<sup>+</sup> channel expression in normal and CF bronchial monolayers. Because we observed lower K<sup>+</sup> currents in CuFi, we explored a possible decrease in K<sup>+</sup> channel expression in these CF cells. Comparison of K<sup>+</sup> channel expression in NuLi and CuFi cells measured by immunoblotting (Fig. 6) revealed lower expression of KvLQT1, Kir6.1, and KCa3.1 proteins in CF cells in all EGF conditions tested. Indeed, the KvLQT1 expression level in CuFi cells was 36.8% ± 5.5% (EGF<sub>0</sub>, n = 8; P = 0.0003), 33.8% ± 4.6% (EGF<sub>0</sub>, n = 8; P = 0.0001), and 30.2% ± 9.5% (EGF<sub>25</sub>, n = 7; P = 0.02) lower than in NuLi cells. A severe decline of Kir6.1 expression was also apparent in CuFi cells (compared to NuLi cells) in all EGF conditions tested (decreases of 69.7% ± 5.5%, 53.9% ± 13%, and 44.5% ± 14% with EGF<sub>0</sub>, EGF<sub>0.5</sub>, and EGF<sub>25</sub>, respectively, n = 5). KCa3.1 channel protein expression was tested only in BEGM-EGF<sub>0</sub> (Fig. 6C). Similar to KvLQT1 and Kir6.1 proteins, KCa3.1 was also reduced in CuFi cells (55.6% ± 14.8% of NuLi cells, n = 4).

Experiments in the Ussing chamber disclosed that chronic treatment with EGF (for 24 h) failed to increase K<sup>+</sup> currents. Consistent with this finding, we verified that KvLQT1 and K<sub>ATP</sub> expression was not modified in bronchial monolayers treated for 24 h with EGF (25 ng/ml; Fig. 6, A and B). Similarly, K<sup>+</sup> channel expression was not affected by the absence of EGF (EGF 0; Fig. 6, A and B). These results indicated that EGF signaling could regulate bronchial K<sup>+</sup> channels through changes of channel activity and/or surface membrane expression rather than variation of total protein expression.

Impact of K<sup>+</sup> channel modulators on bronchial wound healing, cell migration, and proliferation. We (49) recently demonstrated that the repair processes of alveolar epithelia are
dependent on K⁺ channel activity. Their potential role in wound healing of normal and CF bronchial monolayers was now evaluated using K⁺ channel inhibitors. In the presence of EGF (BEGM-EGF0.5; Fig. 7, left), wound repair of NuLi monolayers (31.5% ± 5.5% at 6 h after injury, in the absence of inhibitors) was significantly reduced by clofilium (21% ± 4.2% of repair, n = 12; P = 0.0011), glibenclamide (21.5% ± 4.1% of repair, n = 12; P = 0.016), and TRAM-34 (22% ± 3.3% of repair, n = 12; P = 0.016). It is noteworthy that the inhibitory effects of these compounds were similar. A combination of two or three inhibitors decreased bronchial repair more severely (11% ± 1.5% of repair in their presence). The impact of these pharmacological agents was then tested on CuFi cell monolayers (Fig. 7, right). Similarly to Fig. 1A, we observed that wound healing of CuFi (24.6% ± 3.6%) was lower than that of NuLi (compare Fig. 7, left and right). In addition, wound repair in CuFi is reported as percentage of K⁺ channel protein expression measured in NuLi cells. *P < 0.05.

Fig. 7. Effect of K⁺ channel inhibitors on bronchial wound healing. NuLi and CuFi bronchial epithelial cell monolayers were injured mechanically, and the percentage of wound healing measured after 6 h (in the presence of BEGM-EGF0.5) was compared in control monolayers (ctl) and monolayers treated with clofilium (an inhibitor of KvLQT1 channels, 5 μM, n = 12), glibenclamide (an inhibitor of KATP channels, 100 μM, n = 12), TRAM-34 (an inhibitor of KCa3.1 channels, Tram, 5 μM, n = 9), or combination of clofilium and glibenclamide (clofi + glib, n = 9), clofilium and TRAM-34 (clofi + Tram, n = 9), or the 3 inhibitors together (n = 9). *P < 0.05. These results showed that the major part of bronchial repair was dependent on K⁺ channel activity.
healing of CuFi was significantly inhibited by clofilium \( (P < 0.0001) \), glibenclamide \( (P = 0.008) \), TRAM-34 \( (P < 0.0001) \), and a combination of two (clofi + glib or clofi + Tram) or three (clofi + glib + Tram) of these inhibitors \( (P < 0.001) \). The results indicated that EGF-stimulated wound healing of NuLi and CuFi monolayers depends on \( K^+ \) channel activity.

We also tested the effect of pinacidil, a \( K_{ATP} \) activator, on NuLi and CuFi wound repair. Unfortunately, pinacidil failed to increase NuLi and CuFi wound healing in either BEGM-EGF\(^{0.5} \) or BEGM-EGF\(^{free} \) (data not shown). It has to be noted, however, that in these conditions, EGF secreted by injured/reparing cells already stimulated \( K^+ \) channels and wound healing. Accordingly, cell migration of intact NuLi and CuFi cells measured in the Boyden-type chamber in BEGM-EGF\(^{free} \) was efficiently stimulated by pinacidil, i.e., a 149\% \( \pm 17\% \) increase in NuLi \((n = 6; P = 0.03; \text{Fig. 8A, column 2}) \) and 134.7\% \( \pm 10\% \) in CuFi \((n = 6; P = 0.02; \text{Fig. 8A, column 6}) \) cell migration. This stimulation was similar to that observed after the addition of EGF (BEGM-EGF\(^{0.5} \); \text{Fig. 8A, columns 3 and 7; } n = 22).

EGF-stimulated cell migration was highly sensitive to \( K^+ \) channel inhibitors. Indeed, combined treatment with glibenclamide, clofilium, and TRAM-34 reduced NuLi and CuFi cell migration to 35.5\% \( \pm 7.9\% \) (\text{Fig. 8A, column 4}) and 20.8\% \( \pm 2.2\% \) (\text{Fig. 8A, column 8}) of NuLi cell migration in control conditions, demonstrating that \( K^+ \) activity is crucial for EGF-stimulated bronchial cell migration.

The impact of \( K^+ \) channel inhibitors was then tested on NuLi and CuFi cell proliferation measured in BEGM-EGF\(^{0.5} \). The addition of \( K^+ \) channel inhibitors (glibenclamide, clofilium, and TRAM-34) severely reduced \([3H]\)thymidine incorporation (\text{Fig. 8B}; \( n = 6; P < 0.0009 \)). Pinacidil failed to increase NuLi and CuFi cell proliferation (data not shown).

Effect of CFTR inhibition on wound healing, cell migration, and \( K^+ \) channel expression. The role of CFTR channel activity in NuLi bronchial wound healing was also evaluated. We saw that the presence of the CFTR inhibitor (CFTR\(^{inh-172} \), 10 \( \mu \)M) during repair induced a slight, nonsignificant decrease in NuLi wound repair (36.5\% \( \pm 5.5\% \) and 27.2\% \( \pm 4\% \) of repair in the absence or presence of CFTR\(^{inh-172} \), \( n = 7, \text{NS} \)). This negative control also confirmed that the observed inhibitory effect of glibenclamide (an inhibitor of \( K_{ATP} \) and CFTR channels) was specific to \( K_{ATP} \) channels. As expected, CFTR\(^{inh-172} \) had no significant impact (21.6\% \( \pm 2.9\% \) and 17.7\% \( \pm 2.3\% \) of repair in the absence and presence of CFTR\(^{inh-172} \), \( n = 7, \text{NS} \)) on NuLi cell migration, which did not express functional CFTR channels. It should be mentioned that this compound is toxic (several cells were detached after the 6-h period of treatment), probably explaining the slight decline in NuLi and CuFi monolayer repair. A lower concentration of CFTR\(^{inh-172} \) (5 \( \mu \)M) was less toxic. Subsequent experiments, reported in \text{Fig. 9}, were then performed in the presence of 5 \( \mu \)M CFTR\(^{inh-172} \). At this concentration, we recorded 28.9\% \( \pm 4.1\% \) and 24.3\% \( \pm 4.2\% \) of NuLi repair in the absence and presence of CFTR\(^{inh-172} \) \((n = 6, \text{NS}; \text{Fig. 9A, NuLi}) \). Pretreatment with CFTR\(^{inh-172} \) 24 h before the wound assay, followed by another treatment at time \( 0 \), did not elicit a greater inhibitory effect on NuLi repair (23.8\% \( \pm 3.3\% \) of repair, \( n = 6, \text{NS} \)). Similarly, we observed a slight, nonspecific inhibition of CuFi wound healing in the presence of CFTR\(^{inh-172} \) (\text{Fig. 9A, CuFi}).

NuLi cell migration was not affected by CFTR\(^{inh-172} \) (\text{Fig. 9B, column 2}). We also evaluated a possible impact of CFTR inhibition on EGF secretion and signaling, estimated by migration assay in the presence of conditioned medium. In these experiments, we compared the stimulatory effect on NuLi cell migration of WCM from NuLi monolayers treated or not during repair with CFTR\(^{inh-172} \). As illustrated in \text{Fig. 9B} (columns 3 and 4), CFTR\(^{inh-172} \) was ineffective, suggesting that EGF secretion by injured/reparing cells was not reduced after CFTR inhibition.

Because \( K^+ \) channel expression was reduced in CuFi cells, we evaluated whether CFTR current inhibition, with CFTR\(^{inh-172} \)
172, could decrease K⁺ channel expression in NuLi cells. We found (Fig. 9C) that KvLQT1, K_ATP, and KCa3.1 channels were not affected by CFTRinh-172, indicating that 24-h inhibition of CFTR currents failed to downregulate NuLi K⁺ channel expression to the level in CuFi cells.

**DISCUSSION**

The regulatory mechanisms that control the repair processes of injured CF bronchial epithelia need to be better understood. Using a model of normal (NuLi) and CF (CuFi) bronchial epithelia, we demonstrated that EGF signaling is a crucial component of bronchial repair. Indeed, wound healing and bronchial cell migration were highly dependent on EGF, probably secreted by bronchial cells during repair. Furthermore, we observed that wound healing of non-CF monolayers was faster than that of CF monolayers. This delay in CuFi repair probably arose from reduced cell migration. Our results also suggested lower EGF secretion by injured/repairing CuFi cells as well as decreased EGFR activation in CuFi cells during repair. Consistently, we discerned EGFR activation (elevated p-EGFR level) in wounded NuLi monolayers. Similarly, EGFR activation was seen at the wound edge of 16HBE14o- cells (39). We also demonstrated that conditioned medium collected from injured bronchial monolayers stimulated cell migration to a level similar to that recorded after exogenous EGF addition. The stimulatory effect of the conditioned medium was abolished in the presence of EGF Ab. These data, favoring an autocrine EGF loop in the control of bronchial epithelial repair, are in agreement with previous studies showing the contribution of EGF signaling in airway repair and remodeling (3, 18, 39, 47, 53).

Comparison of wound repair rates in normal NuLi and CF CuFi established that wound healing of CuFi monolayers was delayed compared with NuLi in the presence of secreted and/or exogenous EGF. This result indicated that the response to EGF could be defective in CF cells. Our NuLi and CuFi cell migration and proliferation assays disclosed a decrease in CuFi cell migration and proliferation in response to EGF. The role of the EGF/EGFR pathway in the repair processes of normal and CF bronchial epithelia was evaluated in a mechanical wounding model of human bronchial NuLi and CuFi monolayers. Our results showed that bronchial wound healing was stimulated by exogenous EGF (2-fold increase in BEGM-EGF0.5 compared with BEGM-EGFfree). EGF also stimulated bronchial cell migration, whereas bronchial cell proliferation was not regulated by EGF in the conditions of this assay. Stimulation of wound repair by EGF could then probably be secondary to an increment of cell migration rather than cell proliferation.

Titration of EGF with an EGF Ab in BEGM-EGFfree dramatically reduced bronchial repair. This result indicated stimulation of wound healing after EGF secretion by epithelial cells during repair. Consistently, we discerned EGFR activation (elevated p-EGFR level) in wounded NuLi monolayers. Similarly, EGFR activation was seen at the wound edge of 16HBE14o- cells (39). We also demonstrated that conditioned medium collected from injured bronchial monolayers stimulated cell migration to a level similar to that recorded after exogenous EGF addition. The stimulatory effect of the conditioned medium was abolished in the presence of EGF Ab. These data, favoring an autocrine EGF loop in the control of bronchial epithelial repair, are in agreement with previous studies showing the contribution of EGF signaling in airway repair and remodeling (3, 18, 39, 47, 53).
cell migration, whereas NuLi and CuFi cell proliferation was similar. These results suggested that the observed delay in CuFi wound healing could be consecutive to decreased cell migration rather than cell proliferation. This delay in CF epithelial repair is consistent with the findings of Hajj and colleagues (15), demonstrating delayed and abnormal regeneration of CF tracheal epithelia in a xenograft model. They reported that the two first repair steps, i.e., cell migration and proliferation, were not defective in CF epithelia compared with the controls. In fact, higher cell proliferation rates were found in CF-repairing epithelia (15). In contrast, our proliferation assay, performed on intact subconfluent cultures, showed similar proliferation levels of NuLi and CuFi cells. Hajj and colleagues (15) also reported epithelial remodeling, delayed differentiation/reepithelialization, as well as altered proinflammatory and matrix metalloproteinase (MMP) responses in their CF model. More precisely, IL-8, MMP-7, MMP-9, and tissue inhibitor of metalloproteinase (TIMP-1) levels were elevated in CF epithelia. As detailed below, our findings suggested that EGF signaling and K^+ function could also be affected in CF cells.

**EGF expression, activation, and ligands in CF cells.** ErbB1 (and, at a lower level, erbB2) receptors were detected in NuLi and CuFi cell extracts. The presence of these receptors was consistent with the reported distribution of erbB receptors in airways (1, 3, 38, 51, 52). Several studies have shown that EGF expression could be affected in lung pathologies characterized by injury and remodeling. In asthmatic airways, for example, higher EGF expression has been reported (3, 39). In CF, Vojnov et al. (52) predominantly detected EGF in proliferative areas (mainly basal cells) of CF lung sections. Another study demonstrated that EGFR staining intensity did not differ in airway and alveolar tissues from CF and control lungs. Surprisingly, EGFR staining was reduced in the CF peribronchial submucosal region, which was characterized by a higher number of proliferative cells (16). In our study, total erbB1 (EGFR) expression was slightly (20%) lower in CF (CuFi) than in normal (NuLi) cell extracts, whereas total erbB2 receptor expression was similar in NuLi and CuFi. Our results, like those of Hardie et al. (16), support the hypothesis that EGFR ligands (EGF, TGF-α, etc.) and/or EGFR activation, rather than total EGF expression level, could be regulated in injured CF airways. Indeed, we noted that erbB1 and erbB2 activation, estimated by their normalized phosphorylation levels, was significantly lower in CuFi than in NuLi cells. Interestingly, p-EGFR stimulation in wounded CuFi monolayers was reduced compared with wounded NuLi monolayers. This defect could be due to the decreased synthesis of pro- or mature EGF and/or reduced EGF secretion by injured/remodeling cells. Lower expression at the plasma membrane of activable EGFR and/or defective EGFR tyrosine kinase activity cannot be disregarded. Cell migration data obtained in the presence of WCM favor lower EGF secretion by CF cells during repair. Indeed, we observed that CuFi cell migration, stimulated by WCM from CuFi (CuFi-WCM), was lower than NuLi cell migration in the presence of NuLi-WCM. In the presence of NuLi-WCM, CuFi cell migration was significantly enhanced. Conversely, CuFi-WCM was less effective on NuLi cell migration, confirming that EGF secretion by repairing CuFi cells could be lower.

We are aware that our results, suggesting lower EGFR signaling and/or EGF secretion during CF bronchial epithelia repair in vitro, are not necessarily representative of the complex mechanisms involved in airway repair in vivo. Indeed, growth factor signaling in vivo involves several types of molecules, such as TGF-β, HGF, KGF, or EGF, secreted by different cell types, including epithelial and endothelial cells, fibroblasts, and macrophages. These factors act together to control epithelial repair and remodeling. Elevated TGF-β, KGF, and VEGF levels have been detected in the airways, sputum, and serum of CF patients (47). To our knowledge, there is actually no clear evidence in the literature of up- or downregulation of EGF secretion by CF airway epithelial cells. As pointed out in a review by Shute et al. (47), elevated levels of some growth factors (TGF-β, KGF, and VEGF) in CF airways seem insufficient to protect lung epithelia against the progression of injury and remodeling. For this reason, we thought that it was necessary to better understand the regulatory mechanisms of airway epithelia remodeling and to identify some alternative pathways involved in tissue repair.

**Evidence of KvLQT1, K_ATP, and KCa3.1 channels in bronchial epithelia.** Many types of K^+ channels are expressed in airway and lung epithelial cells. Among them, KvLQT1 is expressed in the upper and lower airways (9, 14, 31) as well as in alveoli (26). A calcium-activated K^+ channel of intermediate conductance, KCa3.1, is also detected in tracheal, bronchial, and alveolar cells (6, 26, 32). Evidence of KvLQT1 and KCa3.1 mRNA and proteins in human bronchial NuLi and CuFi cells is thus consistent with their tissular distribution in the lungs. These channels have been mostly studied in airways since they play a crucial role in Cl^- secretion (6, 28, 30, 48). We recently reported the presence of another K^+ channel, a K_ATP channel (25), which is involved in ion and fluid absorption through the alveolar epithelium (25, 26). In the current study, we demonstrated this K_ATP channel in NuLi and CuFi bronchial cells.

Short-circuit experiments in the Ussing chamber showed that KvLQT1, K_ATP, and KCa3.1 channels were functional in NuLi and CuFi cells. However, a major part of basolateral K^+ current (measured through apically permeabilized bronchial epithelia) was driven by KvLQT1 and K_ATP channels. Indeed, the addition of clofilium-sensitive (55 μA/cm²) and glibenclamide-sensitive (38 μA/cm²) currents amounted to total K^+ currents (99 μA/cm²) measured in the absence of the inhibitors (Fig. 5). Consistently, these two inhibitors, employed in combination, reduced basolateral I_sc to zero (Fig. 4E). In basal conditions, intracellular calcium concentration ([Ca^{2+}]_i) is probably too low to activate KCa3.1 channels. Nevertheless, it was possible to activate them with the 1-EBIO opener; this current was reversed by TRAM-34 (Fig. 4F).

The membrane localization of K^+ channels in lung epithelia revealed multiple types of K^+ channels at both apical and basolateral membranes. Until recently, basolateral K^+ channels of airways and alveolar epithelia, which control the membrane potential of these cells, were studied more extensively than apical K^+ channels. However, recent evidence has revealed the presence of apical K^+ channels in airway epithelial cells. Indeed, KvLQT1 currents have been reported at the apical membrane of Calu-3 cells (33). The same group also identified two-pore K^+ channels at the same membrane (8). In the human bronchial cell line 16HBE14o-, Bernard et al. (2)
identified KCa3.1 currents at both apical and basolateral membranes. In addition, multiple Kv channels have been localized at the apical membrane of alveolar cells and could be involved in K+ secretion (34). Our short-circuit experiments have shown evidence of KvLQT1, KATP, and KCa3.1 currents through the basolateral membrane of bronchial NuLi and CuFi cells. The presence of apical channels was not investigated in this study. Nevertheless, in our wound-healing experiments and migration/proliferation assays performed on nonpolarized bronchial cells, K+ channel modulators could act on K+ channels expressed at apical and/or basolateral membranes in polarized epithelia.

Coupling between EGF signaling and K+ channel function. Coupling mechanism between K+ channel activity and growth factor signaling has been reported in multiple cell types. Indeed, the open probability of 4-aminopyridine-sensitive Kv channels from myeloblastic cells was increased with EGF (4). Similarly, 4-aminopyridine-sensitive K+ channels from corneal epithelial cells were activated by EGF (42). This growth factor also stimulated calcium-activated K+ channels from VSMC (19, 23) and mucus airway cells (20). Consistently, we observed a stimulation of K+ currents by EGF in bronchial monolayers (Fig. 5, D and E). Indeed, we found that acute application (10 min) of EGF significantly increased KvLQT1 current through the basolateral membrane of NuLi cells. It has to be noted that this treatment did not significantly enhance CuFi KvLQT1 current, indicating defective EGF responsiveness of KvLQT1 channels in these CF cells. Furthermore, basolateral KATP current was significantly stimulated by EGF in both NuLi and CuFi cells. On the other hand, KCa3.1 current, preactivated with 1-EBIO, was not further elevated by the addition of EGF.

In alveolar type II cells, we (49) previously found activation of KvLQT1 and KATP currents after acute (10 min) and chronic (24 h) treatment with EGF. Our immunoblotting experiments also revealed an increase in alveolar KvLQT1 and KATP protein expression after 24-h treatment with EGF (49). In NuLi and CuFi, only acute EGF was effective, whereas 24-h EGF treatment failed to stimulate K+ currents and protein expression. This short-term effect of EGF is probably due to modulation of K+ channel activity. However, rapid membrane insertion of new K+ channels cannot be excluded.

Comparison of K+ channel expression and K+ currents in NuLi and CuFi cells. Because KATP and KvLQT1 channel activity was coupled to EGF and lower EGF/EGFR signaling was observed in CuFi cells, we hypothesized that ion transport by K+ channels could be reduced in CuFi monolayers. Our results showed that total basolateral currents as well as KvLQT1, KATP, and KCa3.1 currents were lower in CuFi than in NuLi (Fig. 5). Consistently, KvLQT1, KATP, and KCa3.1 channel expression was severely reduced (by 40–70%) in CuFi compared with NuLi cells (Fig. 6). This is the first demonstration of a decrease in K+ channel expression and activity, coupled with diminished EGF signaling in CF cells. It is not certain, however, whether this decline in CuFi cells could be directly attributed to the CF genotype/phenotype or could be due to another genotype difference between the two patients from whom NuLi and CuFi cells were derived. It would be interesting, in future experiments, to further evaluate K+ channel expression/activity as well as EGFR activation in other non-CF and CF models.

Role of K+ channels in the control of bronchial repair processes. As detailed earlier, K+ channel activity seemed to be coupled to EGF signaling. Moreover, it has been shown in several cell types that stimulation of proliferation and/or migration processes by growth factors depends on this K+ channel activation (21, 23, 42). Until recently, the main recognized K+ channel function in epithelia was to participate in transepithelial ion transport. However, a detailed review of the literature revealed that K+ channel activity also controls the mitogenic and motogenic properties of several cells (4, 21, 29, 35, 40, 45). We recently hypothesized that lung epithelia repair could be regulated by K+ channels. In a previous study, we (49) demonstrated that EGF-stimulated alveolar repair processes were controlled, to a large extent, by KATP and KvLQT1 channels. We then evaluated the role of KATP, KvLQT1, and KCa3.1 channels in wound healing, migration, and proliferation of normal and CF bronchial epithelia. We observed that KATP, KvLQT1, and KCa3.1 channel inhibitors (glibenclamide, clofilium, and TRAM-34) elicited a similar inhibitory effect (26–33%) on NuLi and CuFi wound healing measured in the presence of EGF (Fig. 7). These pharmacological agents had no influence in the absence of EGF (BEGM-EGF26), indicating that K+ channels controlled EGF-dependent wound repair. Glibenclamide, clofilium, and TRAM-34 in combination evoked a larger (68%) decrease in repair, suggesting that their actions were additive. Combined application of these three K+ channel inhibitors also reduced bronchial cell migration and proliferation in the presence of EGF (Fig. 8). Indeed, the number of migrating cells was four (NuLi)- to sevenfold (CuFi) lower after K+ channel inhibition. Similarly, cell proliferation was also affected by the K+ channel inhibitors. A concomitant decrease in cell migration and proliferation could be responsive to the wound repair inhibition observed in the presence of K+ channel blockers.

In polarized bronchial epithelia cultured on filters (as in the Ussing chamber experiments), KATP and CFTR channels are expressed at the basolateral and apical sides, respectively. In these conditions, basolateral or apical application of glibenclamide specifically blocks KATP or CFTR channels. However, in wound-healing experiments (performed on plastic supports), the inhibitory effect of glibenclamide was attributed to KATP and/or CFTR channels. We then tested the impact on NuLi wound healing of the specific inhibitor of CFTR, CFTRinh-172. The absence of significant repair inhibition with this agent confirmed that the action of glibenclamide was due to the KATP channel.

It should be noted that TRAM-34, an inhibitor of KCa3.1 channels, induced a decrease in wound repair similar to that elicited by glibenclamide or clofilium. This result could be surprising since basolateral K+ permeability was mainly driven by KvLQT1 and KATP rather than KCa3.1 channels. In fact, it has been shown that [Ca2+]i elevation could activate K+ efflux through KCa3.1 channels during cell migration and proliferation processes (44–46, 55). The regulatory pathways that link K+ channels to migration and proliferation processes are not clearly identified. However, it has been postulated that changes in K+ currents could also control these processes by altering membrane potential, [Ca2+]i, cell volume, and/or growth factor-mediated mitogenic and motogenic signaling.
A common stimulatory pathway of EGF and K⁺ channels could explain the absence of wound-healing stimulation by the KᵥATP activator pinacidil. Indeed, as demonstrated by the inhibitory effect of EGF Ab (in BEGM-EGFfree), the presence of EGF, released by injured/repairing cells, already stimulated wound repair (and probably K⁺ channels) in basal conditions. In that case, further addition of pinacidil could be ineffective. Accordingly, during the cell migration experiments on intact cells, conditions where the EGFR pathway was not basically activated, pinacidil enhanced cell migration to a level similar to that noted in the presence of exogenous EGF or conditioned medium from wounded monolayers (Fig. 8). Similarly to combined treatments with K⁺ channel inhibitors, it might be more effective to employ activators of KᵥATP, KvLQT1, and KCa3.1 channels in combination. Unfortunately, the presence of 1-EBIO was toxic over a long period (cell detachment occurred). In addition, no KvLQT1 activator is commercially available.

Impact of CFTR inhibition on wound healing, EGF signaling, and K⁺ channels. Of course, the absence of functional CFTR channels in CuFi cells could be directly responsible for the observed delay in repair. It has been shown, for example, that CFTR blockers inhibit the proliferation of MDCK cells (27). In addition, in utero cfr gene transfer in CFTR-deficient (knockout) mice accelerated lung secretory cell proliferation and differentiation. On the other hand, the rate of intestinal cell migration and proliferation was enhanced in CFTR null mice (12). Thus the possible role of CFTR channels in proliferation, migration, and repair processes is not clearly defined in airway cells. Our wound-healing experiments did not demonstrate a significant impact of acute CFTR current inhibition by CFTRinh-172 (Fig. 9). Indeed, we observed a similar slight decrease in NuLi and CuFi wound repair that could be attributed to a toxic effect of CFTRinh-172 (several cells were detached after 6-h treatment with 10 μM CFTRinh-172). A less toxic concentration (5 μM) was also ineffective when applied during repair or repeated two times (24-h pretreatment before wounding, followed by additional treatment during repair). No inhibition of NuLi and CuFi cell migration was apparent. We also evaluated the possibility that the absence of CFTR current could affect EGF secretion by injured bronchial cells. However, conditioned medium collected from injured NuLi monolayers (NuLi-WCM) treated or not during repair with CFTRinh-172 similarly stimulated NuLi cell migration. Finally, our results revealed that KvLQT1, KᵥATP, and KCa3.1 channel expression was not affected by CFTR channel inhibition. In summary, wound healing, cell migration, EGF secretion, and K⁺ channel activity/expression in CuFi was not affected by CFTR channel inhibition. It might be interesting to evaluate the expression are not sensitive to a short-term (max 24 h) inhibition of CFTR with CFTRinh-172 (10–20 μM) was also ineffective when applied during repair or repeated two times (24-h pretreatment before wounding, followed by additional treatment during repair). No inhibition of NuLi and CuFi cell migration was apparent. We also evaluated the possibility that the absence of CFTR current could affect EGF secretion by injured bronchial cells. However, conditioned medium collected from injured NuLi monolayers (NuLi-WCM) treated or not during repair with CFTRinh-172 similarly stimulated NuLi cell migration. Finally, our results revealed that KvLQT1, KᵥATP, and KCa3.1 channel expression was not affected by CFTR channel inhibition. In summary, wound healing, cell migration, EGF secretion, and K⁺ channel activity/expression in CuFi was not affected by CFTR channel inhibition. It might be interesting to evaluate the expression are not sensitive to a short-term (max 24 h) inhibition of CFTR with CFTRinh-172 (10–20 μM). Unfortunately, these conditions could not be reproduced with NuLi and CuFi monolayers, which were severely damaged by the high doses of CFTRinh-172.

In addition to its Cl⁻ channel function, CFTR protein could interact with several other proteins, including ion channels, via PDZ domains (24). As part of the multiprotein complex, the CFTR could also interfere with several signaling pathways. However, to our knowledge, there is no clear evidence of an EGF responsiveness defect in the absence of functional CFTR. In fact, it remains uncertain whether all specific CF phenotypes are related to Cl⁻ transport dysfunction or to the absence of CFTR protein. It might thus be interesting to evaluate the repair of CF epithelia from various genotypes. In addition, it could be useful to establish whether CFTR correction (by transfection of wild-type CFTR by adenovirus vector in CuFi cells; Ref. 57) could lead to normal wound-healing, EGF/EGF signaling, and/or K⁺ channel activity/expression in CuFi.

In conclusion, our results demonstrated, for the first time, that EGFR and K⁺ channel activation could play a crucial role in normal and CF bronchial epithelial repair. Furthermore, the delay in CuFi monolayer repair was coupled with decreased cell migration, lower EGF secretion, and/or EGFR activation as well as reduced K⁺ current and channel expression.

ACKNOWLEDGMENTS

We thank Dr. Joseph Zahnner for providing the NuLi and CuFi cell lines. We acknowledge the editorial assistance of Ovid Du Silva, Research Support Office, Research Centre, Centre hospitalier de l’Université de Montréal (CHUM).

GRANTS

This work was supported by the Cystic Fibrosis Foundation (E. Brochiero) and research credits from the Fonds de la Recherche en Santé du Québec (FRSQ-GEPROM) (J. Noël and E. Brochiero) and CHUM Research Centre Foundation (E. Brochiero). J. Noël is a Senior Research Scholar of FRSQ.

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