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Establishment and Characterization of a Novel Polarized MDCK Epithelial Cellular Model for CFTR Studies

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Key Words

CFTR • Cystic fibrosis • Polarized epithelial cells • MDCK cells

Abstract

F508del is the most common mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that is responsible for the genetic disease Cystic Fibrosis (CF). It results in a major failure of CFTR to traffic to the apical membrane of epithelial cells, where it should function as a chloride (Cl^-) channel. Most studies on localization, processing and cellular trafficking of wild-type (wt) and F508del-CFTR have been performed in non-epithelial cells. Notwithstanding, polarized epithelial cells possess distinctly organized and regulated membrane trafficking pathways. We have used Madin-Darby canine kidney (MDCK) type II cells (proximal tubular cells which do not express endogenous CFTR) to generate novel epithelial, polarized cellular models stably expressing wt- or F508del-CFTR through transduction with recombinant lentiviral vectors. Characterization of these cell lines shows that wt-CFTR is correctly processed and apically localized, producing a cAMP-activated Cl^- conductance. In contrast, F508del-CFTR is mostly detected in its immature form, localized intracellularly and producing only residual Cl^- conductance. These novel cell lines constitute *bona fide* models and significantly improved resources to investigate the molecular mechanisms of polarized membrane traffic of wt- and F508del-CFTR in the same cellular background. They are also useful to identify/validate novel therapeutic compounds for CF.

Introduction

Cystic fibrosis (CF), a clinically complex disease known as the most common lethal inherited disorder among Caucasians, is caused by mutations in the cystic fibrosis transmembrane conductance regulator *(CFTR)* gene which encodes a membrane protein that functions as a cAMP-activated chloride (Cl^-) channel. CFTR is expressed in a wide variety of epithelial cells, including those lining sweat ducts, small pancreatic ducts, airways surface and submucosal gland, intestinal surface and crypts, and kidney tubules [1-3]

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A high number of different loss-of-function gene variants cause distinct cellular defects on the resulting protein. However, one single mutation, the deletion of phenylalanine residue at position 508 (F508del) of CFTR, occurs in about 90% of CF patients. It is believed that deletion of Phe-508 causes a protein folding defect [4-7] and the resulting abnormal CFTR conformation is recognized as such by the endoplasmic reticulum (ER) quality control that targets most of it for degradation via the ubiquitin-proteasome pathway [8, 9].

A limited amount of cAMP Cl^- conductance, however, is detected at the epithelia surface of F508del-homozygous patients and mice, suggesting that the ER retention is not absolute in native tissues [10-12]. Consistently, minor expression of F508del-CFTR can be detected at the cell surface in recombinant cells, CF primary airway cells, and CF native tissues using a large variety of anti-CFTR antibodies [13-17].

Polarized monolayers of human airway epithelial cells are thus the desirable model to study CFTR traffic and indeed there is a number of human airway epithelial cell lines from CF patients that are able to polarize in culture (for a review see [18]). These immortalized cell lines are of significant importance to study CFTR because the scarcity of material provided by native tissues and primary cell systems causes major limitations to the feasibility of the experiments that can actually be carried out [18]. However, they are often difficult to maintain in culture and to keep consistent and reproducible transepithelial resistance (R_{te}) values. Moreover, their CFTR expression levels have been observed to decrease with increasing number of passages [18].

Therefore, the majority of studies on the traffic and processing of wt- and F508del-CFTR have been conducted in heterologous, non-epithelial or non-polarized/ epithelial cellular systems. Because the efficiency of CFTR processing has also been shown to be widely cell-type and polarization dependent, with endogenous epithelial CFTR maturation patterns differing from those reported in overexpressing systems [19, 20] , our aim was to generate novel epithelial, polarized cellular models stably expressing wt- or F508del-CFTR.

We have thus herein established and characterized two novel cellular models by stably transducing MDCK type II cells with wt- or F508del-CFTR recombinant lentivirus. MDCK-type II cells were the model of choice due to their reported absence of endogenous CFTR protein expression and cAMP-stimulated Cl^- conductance [21].

These models are characterized here for their CFTR expression at the RNA and protein levels as well as regarding the intracellular localization of the respective transgene product and also in terms of their transepithelial electric properties. Results presented demonstrate that these novel epithelial/ polarized cell lines are *bona fide* models to investigate the differences in wt- and F508del-CFTR polarized expression and traffic on the same cellular background, being thus relevant tools to elucidate the basic cellular mechanisms of CF disease.

Materials and Methods

MDCK type II cells

Derived by SH Madin and NB Darby from the kidney tissue of an adult female cocker spaniel, MDCK cells were originally produced in September 1958 and ever since have been commonly used as a general model to study protein traffic in epithelial cells [22-24]. MDCK cells are easy to maintain, form highly polarized monolayers of epithelial cells when grown on filters, with microvilli in the apical surface and with the basolateral membrane interdigitating with the filter. Moreover, their membrane trafficking pathways have been thoroughly characterized (for a review see [24]). Cultured monolayers of MDCK cells display many features of *in vivo* epithelia and their growth on permeable substrates also allows measurement of electrophysiological parameters [25].

However, MDCK cells were previously shown to be heterogeneous in nature, with marked differences reported between type I and type II cultures. It is believed that the two MDCK strains consist in distinct cell populations derived from the same initial pool of cells, being strain I cells derived from the distal collecting-duct, while strain II cells are most probably derived from the proximal tubule [26]. Some authors, however, suggests that MDCK II may be of thick ascending limb origin [27]. MDCK type II cells used here were obtained from the ATCC (Rockville, MD, USA).

Viral transduction

MDCK type II cells were transduced to overexpress CFTR (Tranzyme Corporation from Birmingham, Alabama, USA). Cells were infected with HIV-based translentiviral recombinant vectors containing the human wt- or F508del-CFTR cDNAs, as previously described [28, 29]. Cells were maintained in DMEM/ F12 medium supplemented with 10% (v/v) fetal bovine serum (FBS) and 1% glutamine (both from Invitrogen, Carlsbad, CA, USA) at 37°C in a humidified incubator in 5% (v/v) CO_2 and selection was performed with 2 μg/ml of blasticidin S (Sigma, St Louis, MI, USA) for MDCK wt-CFTR cells and 4 μg/ml of puromycin (Invitrogen) for MDCK F50del-CFTR cells. Following transfection of MDCK II cells (that do not express endogenous CFTR) with HIV-based translentiviral vectors, clones were selected and characterized for CFTR expression (by reverse transcription polymerase chain reaction, RT-PCR and Western blotting, WB), subcellular localization (by immunofluorescence) and for transepithelial electric properties (through Ussing chamber measurements).

Cell culture conditions

Cells were grown on 24 mm polyester Transwell filter supports (Corning Costar, Cambridge, MA, USA), with an area of 4.7 cm^2 and a pore size of 0.4 μm. R_{te} was measured using an EVOMX Epithelial Ohmmeter with a STX2 electrode (WPI, Sarasota, FL, USA).

CFTR transcript analysis by RT-PCR

Total RNA was isolated using the RNeasy extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA concentration was determined by measurement of A_{260} and 1μg was treated with 1U of RNAse-free DNAse I (Invitrogen) for 15 min at room temperature to eliminate contamination with genomic DNA. DNAse-treated RNA was used for the synthesis of complementary DNA (cDNA). RNA was annealed to 100 pmol of random hexamers (Invitrogen), the mixture was incubated for 10 min at 60°C and then chilled on ice. Following the addition of 5x first strand buffer (Invitrogen), 0.1M DTT (Invitrogen), 25 mM dNTP mix (Amersham Biosciences, Uppsala, Sweden) and 20U RnaseOut (Roche, Basel, Switzerland), contents were incubated for 2 min at 42ºC. SuperScript II RNaseH- reverse transcriptase (RT) (200 units; Invitrogen) was then added and the final mixture was incubated for 60 min at 42°C for reverse transcription. The RT enzyme was inactivated by heating at 70°C for 15 min. The PCR amplification of the cDNA products was carried out in a reaction that contained 5 μl of cDNA, 10 x PCR buffer, 25 mM dNTP mix (Amersham), 10 pmol of each primer and 1U *Taq* polymerase (Perkin Elmer, Norwalk, CT, USA). Three sets of primers were used for the PCR amplification:

- "universal mammalian" CFTR primers amplifying from 5′ of exon 22 to 3′ of exon 23 (~ 200 bp fragment) [30]: forward primer 5′-CTAAGCCATGGCCACAAGCA-3′ (positions 4168-4187 of human CFTR mRNA, GenBank Accession Number M28668) and reverse primer 5′-CATTGCTTCTATCCTGTGTT-3′ (4353-4372),

- human CFTR primers amplifying a fragment spanning exons 8 to 10 (391 bp fragment) [31]: forward primer 5′-AATGTAACAGCCTTCTGGGAG-3′ (1318-1338 of human CFTR mRNA) and reverse primer 5′-GTTGGCATGCTTTGATGACGCTTC-3′ (1685-1708),

- β-actin primers amplifying a fragment spanning exon 8 to 10 (227 bp fragment) [32]: forward primer 5′-GCACTCTTCCAGCCTTCC-3′ (positions 852-869 of human β-actin mRNA, GenBank Accession Number BC014861) and reverse primer 5′-GCGCTCAGGAGGAGCAAT-3′ (1079-1062).

For the mammalian CFTR and human β-actin set of primers, cDNA samples were heated at 94°C for 5 min and then subjected to 35 amplification cycles of denaturation at 94°C for 1 min, primer annealing at 57°C for 1 min, and extension at 72°C for 2 min, followed by a final extension at 72°C for 12 min. For the human CFTR set of primers, the only difference was the annealing temperature, which was 60°C. DNA fragments were visualized after agarose gel electrophoresis by staining with ethidium bromide. The following negative controls were included: water instead of RNA and no RT.

Automatic sequencing

PCR products were purified with the JetQuick kit (Genomed, Lohne, Germany) and sequencing was performed on a 3100 Genetic Analyser (Applied Biosystems, Norwalk, CA, USA), using the ABI PRISM™ Dye Terminator Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's procedure.

Western blotting and treatment with glycosidases

Protein quantification, WB and treatment with glycosidases were performed as previously described [33, 34].

Antibodies

For immunofluorescence subcellular localization, all antibodies (Abs) were diluted in 0.5% (w/v) BSA in PBS and for WB in 5% (w/v) skimmed milk in PBS. The following Abs, previously shown to specifically recognize CFTR [35], were used: monoclonal (mAb) 24–1 (R&D Systems; Abington, UK) raised against amino acid (aa) residues 1377-1480 [15] 1:20, polyclonal (pAb) MPCT-1 (a kind gift from Dr. R. Dormer; University of Wales, Cardiff, UK) raised against the C-terminus [17] 1:100 and M3A7 mAb (Chemicon, Temecula, CA, USA) raised against aa residues 1370-1380 [36] 1:2000.

Other primary Abs were anti-Rab4 pAb (Stressgen, Victoria, BC, Canada) 1:250; anti-GM130 mAb (BD Transduction, San Jose, CA, USA) 1:250; anti-E-cadherin (BD) 1:100; anti-α/β-tubulin mAb (Amersham), 1:100; anti-cytokeratin18/19 mAb (Roche, Basel, Switzerland), 1:1500. Secondary Abs were: FITC-conjugated, anti-rabbit IgG (Amersham), 1:50; FITC-conjugated anti-mouse IgG (Roche), 1:60; TRITC-conjugated, anti-mouse IgG (Sigma), 1:600 and HRP-conjugated anti-mouse IgG (Amersham), 1:3000.

Subcellular localization by immunofluorescence

Cells grown on 8-well chamber slides (Nalge Nunc, Roskilde, Denmark) or on Transwell filters were incubated for 18h with 5 mM sodium butyrate, an inhibitor of histone deacetylases (HDACs) and thus a transcriptional activator of non-housekeeping genes [37], for enhancement of CFTR expression. Slides/filters were rinsed twice with cold PBS and fixed in ice-cold methanol for 10 min. After two washes with PBS, cells were permeabilized with 0.2% (v/v) Triton X-100 in PBS for 15 min, washed 3 times in PBS, and blocked with 1% (w/v) BSA/PBS for 30 min prior to incubation with the primary Ab for 90 min at room temperature. Cells were then washed three times with PBS and incubated with the secondary Ab for 30 min at room temperature. Slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) containing DAPI (Sigma). Immunofluorescence staining was observed and recorded on an Axioskop fluorescence microscope (Zeiss, Jena, Germany) equipped with a cooled CCD camera (Photometrics, Tucson, AZ, USA). Images were processed with the Power Gene 810/Probe software system (PSI, Chester, UK). Confocal images were obtained in an MRC-600 laser confocal imaging system with the Comos software package (BioRad, Hercules, CA, USA).

Transepithelial short- and open-circuit measurements

For short-circuit current measurements, filters were mounted in modified Ussing chambers (Jim's Instruments, Iowa City, IA, USA) and bathed on both sides with identical HEPES-buffered saline solutions that contained (mM) 130 NaCl; 5 sodium pyruvate; 4 KCl; 1 $CaCl_2$; 1 $MgCl_2$; 5 glucose and 5 HEPES-NaOH (pH 7.4). Bath solutions were gassed with room air. Solution temperature was maintained at 37°C. Short-circuit current (Isc) measurements were obtained by using an epithelial voltage clamp (VCC-600; Physiologic Instruments, San Diego, CA, USA). Forskolin (Fsk) (20 μM) was added to the bathing solution to stimulate cAMP-mediated Cl^- secretion. Cl^- secretory currents were identified on the basis of activation by forskolin and by sensitivity to basolateral glibenclamide (Glib) (200 μm).

For open-circuit measurements MDCK cells were grown for 3 – 12 days on permeable supports (Millipore MA, Germany) coated with rat-tail collagen (Roche, Germany). The resistances of the monolayers were checked every day using a volt-Ω-meter (Millicell-ERS, Millipore, Germany) and typically reached values between 600 and 1800 Ωcm^2. Apical and basolateral surfaces of the epithelium were perfused continuously at a rate of 10 ml/min (chamber volume 2 ml). The bath solution contained (mM): 145 NaCl; 0.4 KH_2PO_4; 1.6 K_2HPO; 5 D-glucose; 1 $MgCl_2$; 5 HEPES and 1.3 Ca-gluconate (pH 7.4). Bath solutions were maintained at 37°C. The transepithelial resistance (R_{te}) was determined by applying short (1 s) current pulses (I = 0.5 ìA).

Results

CFTR transcript analysis

Firstly, CFTR expression in the transduced MDCK cells was characterized by RT-PCR analysis with two different pairs of CFTR primers (mammalian and human-specific, respectively). The "universal mammalian" primers amplified a 200 bp fragment spanning the region between exons 22-23 and were selected due to their reported success in amplifying CFTR transcripts from seven different mammalian species [30]. The human-specific primers amplifying a fragment of 391 bp spanning exons 8-10 were previously described [31]. Samples were also amplified for the housekeeping gene β-actin as a control [32]. As shown in Fig.1A, CFTR transcripts were amplified by the two different sets of primers in the three cell lines tested: the parental MDCK II (PAR), MDCK wt-CFTR and MDCK F508del-CFTR, showing that the human-specific primers can also amplify canine CFTR transcripts. However, results show that for a similar intensity of the β-actin-specific band, significantly lower levels are obtained for the CFTR-specific band in parental cells in comparison to wt- or F508del-CFTR transfected cell lines (compare lane 2 to lanes 3 and 4 in Fig.1A).

Sequencing of these RT-PCR amplification products and nucleotide alignment analysis with human CFTR

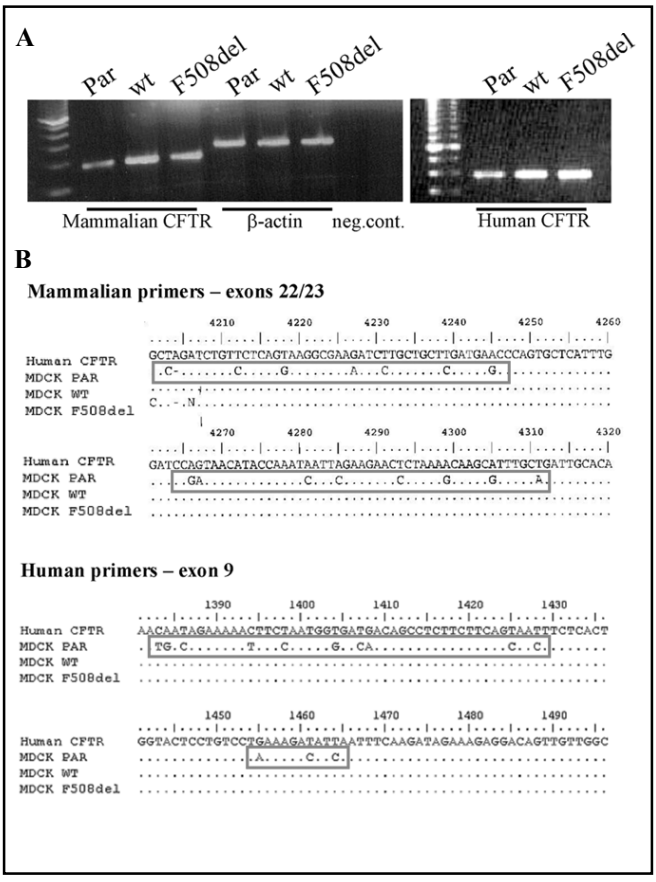

Fig. 1. CFTR transcript analysis in MDCK II cells. (A) RT-PCR analysis of CFTR transcripts in MDCK II cells. Total RNA was prepared from parental and CFTR-transfected MDCK II cells. Samples of 1.0 μg of total RNA were reverse-transcribed to cDNA and PCR-amplified with mammalian or human-specific CFTR primers. b-actin specific primers were used as a positive control for the RT-PCR and a reaction with no RNA was the negative control. (B) Sequence alignment of the RT-PCR products from parental and CFTR-transfected MDCK II cells with human CFTR mRNA (GenBank Accession Number M28668), using the BioEdit software package (Tom Hall, Ibis Therapeutics, Carlsbad, CA, USA). In boxes are marked the differences between the MDCK parental endogenous CFTR transcript and human CFTR.

(Fig.1B), revealed that in both wt- and F508del-CFTR transfected cell lines, transcripts correspond to CFTR of human origin (i.e., expressed from the transgene) whereas transcripts detected in the parental line correspond to canine CFTR, i.e., resulting from endogenous expression.

We have submitted the sequences of these canine CFTR transcripts to the EMBL Nucleotide Sequence database, as expressed sequence tags (EST) with accession numbers: AJ810177, (EST resulting from exons 22/23 amplification, clone CFTR22) and AJ8110178 (EST from exons 8/10, clone CFTR8).

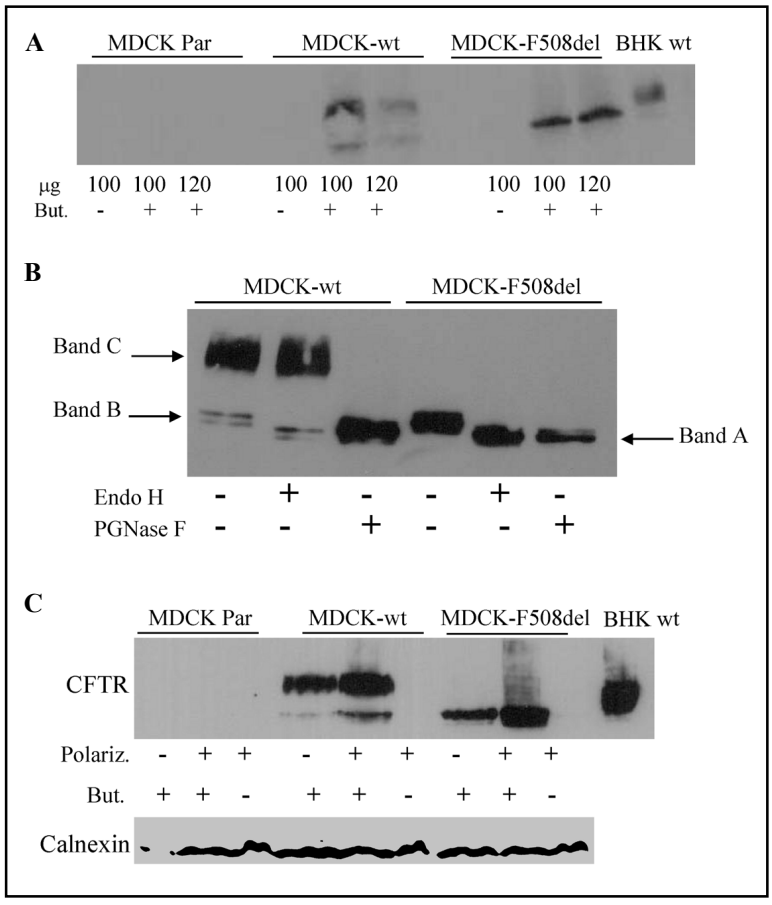

Fig. 2. Characterization of CFTR protein expression. (A) Western blot of total protein extracts from parental (PAR), wt-CFTR and F508del-CFTR MDCK cells. Cells were grown on plastic dishes and incubated with 5mM sodium butyrate for 18h (but.). Cell lysates were prepared (µg of protein indicated) and resolved on a SDS-polyacrylamide gel before electrophoretic transfer to nitrocellulose for immunodetection of CFTR, using M3A7 Ab. Wt-CFTR Baby hamster kidney cells (BHK) were used as a positive control. (B) Western blot analysis combined with digestion with Endo H and PGNase F of total protein extracts from wt-CFTR and F508del-CFTR MDCK cells. Cells were grown on plastic dishes and incubated with 5mM sodium butyrate for 18h to enhance expression. Cell lysates (150 µg) were treated with the endo H and PGNase F glycosidases, as indicated, proteins precipitated and resolved on a SDS-polyacrylamide gel before electrophoretic transfer to nitrocellulose for immunodetection of CFTR, using M3A7 Ab. (C) Western blot of total protein extracts (250 µg) from parental, wt-CFTR and F508del-CFTR cells. Cells were grown on plastic dishes (polariz -) or on filters (polariz +) and incubated with 5 mM sodium butyrate for 18h. Proteins were resolved on a SDS-polyacrylamide gel before electrophoretic transfer to nitrocellulose for immunodetection of CFTR and calnexin, which was used as an internal control. Wt-CFTR baby hamster kidney cells (BHK) were used as a positive control.

Expression of CFTR protein by MDCK cells

In order to assess expression of CFTR protein, total protein extracts from parental, wt-CFTR and F508del-CFTR MDCK cells were analysed by WB (Fig.2A). To enhance CFTR expression levels, cells were incubated with sodium butyrate, an inhibitor of HDACs and thus a transcriptional activator of non-housekeeping genes [37] (see Methods). Results in Fig.2A show that CFTR is not detected in MDCK parental cells and is only detected in transfected cell lines when incubated with butyrate. To confirm the nature of the bands detected, Western blot analysis combined with digestion with two glycosidases, Endo H and PGNase F was also performed (Fig.2B). Results show that in wt-CFTR transfected MDCK cells the protein is fully-glycosylated, as demonstrated by the presence of the typical two-band pattern: the mature, Endo H-resistant and PGNase F-sensitive band C and the immature, Endo H- and PGNase F-sensitive band B. In contrast, F508del-CFTR is only detected as the immature ER-characteristic form (band B) that is sensitive to both glycosidases [34].

Since expression of membrane proteins is known to depend on the degree of polarization of epithelia, we examined here whether expression of CFTR is altered in MDCK II cells under highly polarized conditions. MDCK cells were maintained on permeable filter supports for 10 days to induce polarization, and WB analysis (Fig.2C) shows that when these cell lines polarize, expression levels of wt- and F508del-CFTR are significantly increased in comparison to non-polarized cells.

Subcellular localization of CFTR

In order to evaluate the localization of CFTR in MDCK transfected cells, immunofluorescence microscopy was performed with two different anti-CFTR Abs (see Methods), namely: mAb 24-1 (Fig.3A-C) and pAb MPCT1 (Fig.3D-F). Results show that wt-CFTR exhibits membrane localization (Fig.3B and E, compare with Fig.4B), whereas F508del-CFTR is localized intracellularly (Fig.3C and F), in an ER-like staining pattern (compare with Fig.4A). No specific CFTR labelling was detected in MDCK II parental cells (Fig.3A and D).

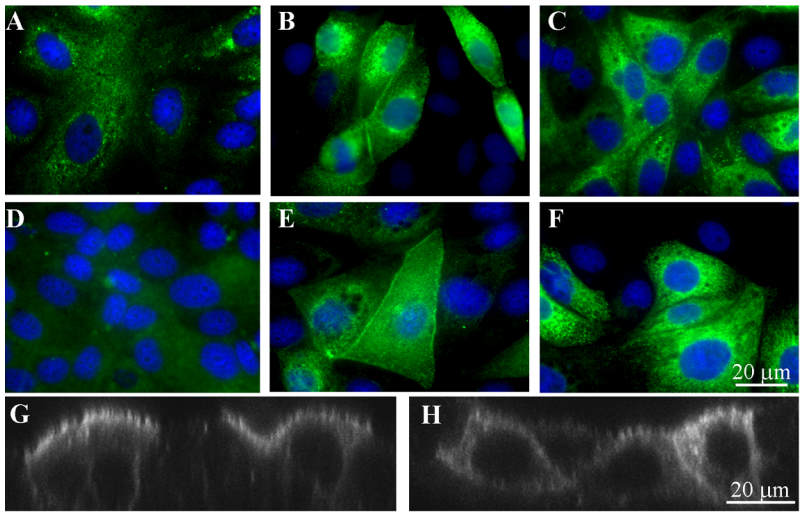

Fig. 3. Immunolocalization of CFTR in parental, wt-CFTR and F508del-CFTR MDCK cells. Cells were grown in 8-well chamber slides and analysed by immunofluorescence using the 24-1 mAb (A-C) or the MPCT1 (D-F) pAb against CFTR. F508del-CFTR (C and F) is detected strictly in the cytoplasm, while wt-CFTR (B and E) is located at the plasma membrane. No specific CFTR labelling was detected in MDCK II parental cells (A and D). Confocal fluorescence micrographs (z axis) of wt-CFTR (G) and F508del-CFTR (H) detected in polarized MDCK cells grown on filters and analysed by immunofluorescence using the anti-CFTR 24-1 mAb. Bar = 20µm.

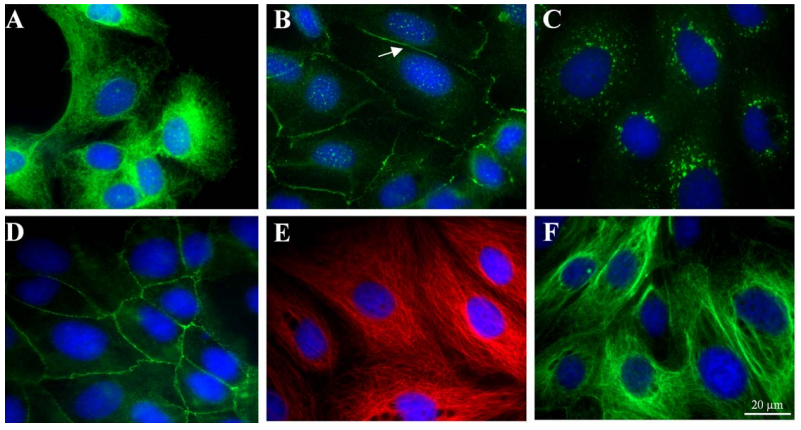

Fig.4. Immunolocalization of several organelle markers in parental MDCK cells. Cells were analysed by immunofluorescence using the following Abs: A- anti-calnexin for ER staining; B- anti-Rab4 for early endosomes (arrow shows perimembranar localization); C- anti-GM130 for Golgi; D- anti-E-cadherin for plasma membrane; E- anti-tubulin for microtubules and F- anti-cytokeratin 18/19 for intermediate filaments of epithelial cells. Bar = 20µm.

To characterize the localization of CFTR in polarized cells, MDCK wt and MDCK F508del cells were grown on filters, immunostained with 24-1 anti-CFTR mAb and analysed by confocal microscopy. Sections on the z plane of the cells, from the apical to the basal membrane were acquired. While wt-CFTR localized predominantly in the apical region (Fig.3G), F508del-CFTR showed mostly an intracellular, perinuclear distribution (Fig.3H). These results are consistent with those evidencing wt- and F508del-CFTR localization in non-polarized cells (Fig 3A-F).

Immunolocalization of several organelle markers was also performed, showing that non-polarized MDCK II cells present a diffuse ER staining pattern (Fig.4A), a perinuclear Golgi localization (Fig.4C) and a well-differentiated cytoskeleton network (panels E and F). E-cadherin was chosen as a membrane marker (Fig.4D), while Rab4, a marker for the early endosomes, exhibits a localization that seems almost juxtaposed to the membrane (Fig.4B).

CFTR functional analysis

Functional expression of CFTR in the transfected cell lines was assessed through transepithelial open- and short-circuit current measurements. Under short-circuit conditions, secretory CFTR currents were identified on the basis of activation by forskolin (Fsk, 20 µM) and inhibition by glibenclamide (Glib, 200 µM). Fsk-stimulated Cl^- secretion was clearly detected in wt-CFTR expressing cells (Fig.5A), while MDCK F508del-CFTR only evidenced residual Fsk response when grown at 37 °C (Fig.5B). However, when F508del-CFTR cells were incubated at 27ºC for 24h, the Fsk response and inhibition by Glib was increased. Open-circuit conditions (Fig.6) confirmed these results and showed a large increase in the transepithelial conductance (G_{te}) of wt-CFTR cells upon stimulation with 3-isobutyl-1-methylxantine (IBMX) and forskolin (I/F; 100 µM/2 µM) and just a minor increase in F508del-CFTR cells. Parental cells did not respond at all (Fig.6A). Amiloride sensitive Na^+ transport was not detected in any of the three cells lines and could not be

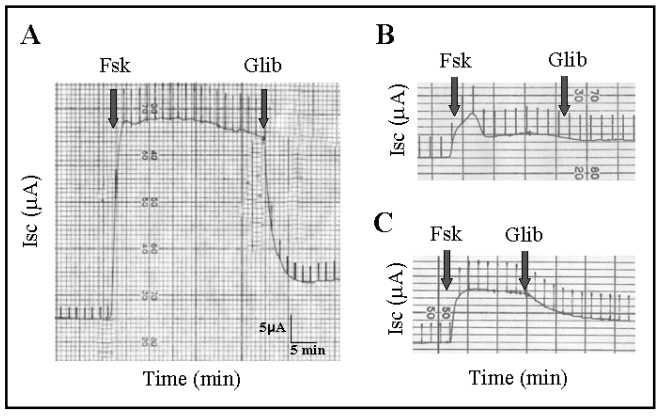

Fig. 5. Functional analysis of CFTR in short-circuit measurements. (A) Original recording of the short circuit current induced by forskolin (Fsk, 20 μM) and inhibited by glibenclamide (Glib, 200 μM) in MDCK wt-CFTR cells. B) A modest and transient increase in Isc was also detected upon addition of forskolin to F508del-CFTR MDCK cells which was enhanced following a 24h-incubation at 27ºC (C). Arrows indicate time of addition of forskolin or glibenclamide, respectively.

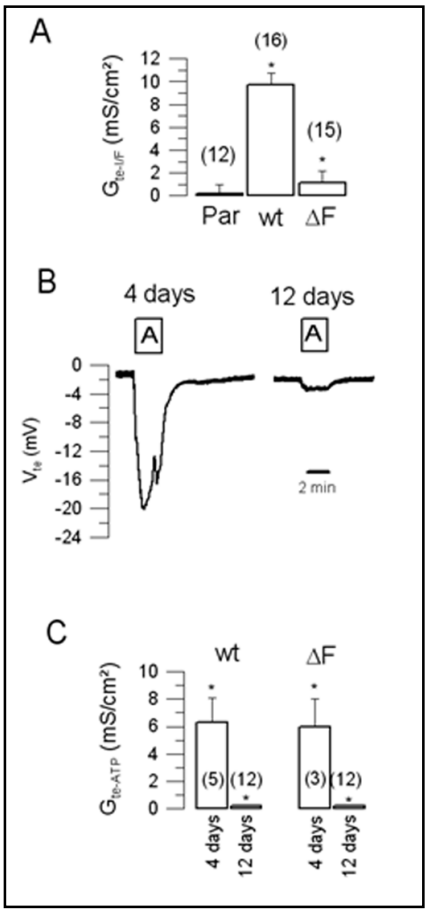

Fig. 6. Functional analysis of CFTR in open-circuit measurements. (A) Summary of the transepithelial conductances (G_{te}) activated by IBMX and forskolin (I/F; 100 μM/ 2 μM) under open-circuit conditions in parental (PAR), wt-CFTR (wt) and F508del-CFTR (ΔF) cells. (B) Original recording of the transepithelial voltage (V_{te}) and changes induced by ATP (A, 100 μM) in a early (4 days) and a late (12 days) culture of wt-CFTR cells. (C) Summary of the transepithelial conductances activated by ATP in early and late cultures of wt-CFTR and F508del-CFTR cells. *indicates significant effects of I/F and ATP, respectively (paired t- test). (n) = number of experiments.

induced by dexamethasone (5 days/ 0.1 μM) (data not shown). Moreover, we examined Ca^{2+}-activated transport by stimulation of luminal purinergic receptors with ATP (A, 100 μM). Large changes in transepithelial voltage and an increase in transepithelial conductance upon ATP stimulation were detected in early cultures (4 days) of all three cell lines, while Ca^{2+}-activated Cl^- conductance was progressively lost during continuous culture on permeable supports (12 days) (Fig.6B and C).

Discussion

It is well known that protein trafficking is largely dependent on the polarization status of the epithelium and yet there are multiple examples of CFTR trafficking studies performed in non-epithelial / non-polarized cells, mainly due to difficulties in culture of primary cultures and to the fact that CFTR expression is progressively lost by immortalized cell cultures [18]. Examples of studies carried out in non-epithelial / non-polarized cells include descriptions of a non-conventional biosynthetic trafficking pathway [38, 39]; a late quality control mechanism operating at early endosomes [40]; characterization of ER-to-proteasome sorting in yeast cells [41].

The need and importance of appropriate epithelial polarized cellular models to finely investigate both the processing mechanisms and the trafficking pathways of wt- and F508del-CFTR in the same background, led us to produce the two novel cellular models described here.

We had first attempted to produce the cell lines stably expressing wt- and F508del-CFTR by transfection using cationic liposomes. Expression of CFTR was characterized in such cell lines at the level of transcripts and protein, total and at the membrane. However, after several reproducible experiments, a rapid decrease in CFTR protein expression occurred in those stable lipofectin-transfected MDCK II cell lines (F. Mendes, unpublished results), similarly to what is above described for immortalized primary cultures.

Since virally-transduced cell lines are generally described to exhibit very stable transgene expression [42] such an approach was also adopted here. We therefore, used an HIV-based lentivirus to transduce MDCK II cells

with wt- and F508del-CFTR as transgenes and thus generate the stable expression systems herein described. Characterization of such cell lines at CFTR mRNA level evidenced that MDCK II parental cell line solely transduced with the lentiviral vector (i.e., with no transgene) already exhibits detectable levels of CFTR transcripts through the sensitive RT-PCR analysis employed here. This was unexpected since MDCK II cells had been previously reported to lack CFTR expression [21]. These apparent contradictory results suggest that either the sensitivity of our RT-PCR protocols is higher or that the viral transduction somehow activated the endogenous canine CFTR gene. Indeed, sequencing of the respective CFTR RT-PCR product from the parental MDCK cells confirmed that it is not of human origin. Additionally, we cannot exclude the possibility that the MDCK II cells used here for lentiviral transduction differed from those used by the other authors. Notwithstanding, since no CFTR protein could be detected in the MDCK parental cell line, at least using the anti-CFTR Abs tested here (see below), we still regard this MDCK II cellular system as a good parental line for CFTR transduction.

Results from CFTR protein analysis of MDCK wt-CFTR and MDCK F508del-CFTR cell lines evidenced that wt-CFTR is detected mostly as the fully-glycosylated form (band C), typical of post-ER cellular compartments, being thus correctly processed, whereas F508del-CFTR could only be detected as the immature, core-glycosylated protein (band B) that is characteristic of the ER. These biochemical results were confirmed by immunofluorescence subcellular localization data which revealed an apical membrane localization for wt-CFTR and most F508del-CFTR localized intracellularly in these cellular systems. Additionally, expression levels of both wt-CFTR and F508del-CFTR proteins in these MDCK II cells detected by WB at steady-state were shown to be increased when cells are polarized.

Comparison of CFTR expression results in the lentiviral transduced MDCK cells with those previously obtained for liposome-transfected MDCK cells, evidenced significantly higher expression levels of the CFTR transgene in the transduced cells and, most importantly, sustained expression after a relatively high number of passages (F. Mendes, unpublished results).

Functional analysis of the transduced MDCK cell lines by transepithelial open- and short-circuit current measurements clearly identified secretory CFTR currents (IBMX/forskolin-activated, glibenclamide-inhibited) in MDCK wt-CFTR cells, while MDCK F508del-CFTR cells only showed residual Cl^- currents in response to IBMX/forskolin when grown at 37°C. Parental cells did not respond at all. The Cl^- currents of MDCK F508del-CFTR cells were, however, increased following a 24h-incubation at 27ºC, consistently with the rescuing effect of low temperature described for F508del-CFTR in other cellular systems.

There was no evidence for Na^+ transport mediated by the epithelial Na^+ channel (ENaC) in these cell lines, as none of them evidenced amiloride-sensitive luminal currents, nor could these be detected following dexamethasone induction. This observation is not surprising since MDCK type II cells are derived from the kidney proximal tubule [26] (see Methods). As a consequence of the lack of expression of ENaC channels, the transepithelial voltage of the epithelium was close to zero mV in the absence of any stimulation. However, upon stimulation of luminal purinergic receptors with ATP, Ca^{2+}-activated Cl^- transport was clearly detected in early cultures (4 days) of all three cell lines, which was progressively lost with prolonged (12 days) culture of polarized cells. This progressive loss of Ca^{2+}-activated Cl^- secretion during prolonged cell culture has also been observed for other epithelial cell lines such as M1 (collecting duct cells) or 16HBE (bronchial epithelial cells), and appears to reflect changes during differentiation (unpublished observations, K Kunzelmann).

MDCK type I cells transfected with wt- and F508del-CFTR have been used previously [43, 44], but those systems have the disadvantage of expressing endogenous canine wt-CFTR protein in significant amounts [21, 45]. To circumvent the caveats of endogenous CFTR expression, the 27-kDa green fluorescent protein (GFP) tag was added at the N-terminus of CFTR [44]. However, this tag most plausibly disrupts the interaction of CFTR with other proteins that have been shown to be critical for its membrane traffic in epithelial cells, namely components of the SNARE machinery (reviewed in [46]). Recent studies carried out using such MDCK I cells include the characterization of GFP-tagged F508del-CFTR trafficking to the plasma membrane under low temperature [39] and the identification of the C-terminus of CFTR as a PDZ interacting apical membrane retention motif [39]. MDCK II cells expressing FLAG-CFTR were used to show that CFTR is not involved in the regulation of organelle pH nor on protein post-endocytic traffic [48] and that cAMP stimulates the apical recruitment of CFTR [49].

We believe that the novel cellular models produced here are more appropriate to investigate polarized

membrane trafficking pathways of CFTR. Moreover, they allow comparison of such pathways between wt- and F508del-CFTR in the same cellular background. These models can also be useful to confirm/discard previously described mechanisms shown to occur in non-polarized cells regarding biogenesis, degradation, processing and traffic of CFTR (reviewed in [46]) and for the development of pharmacological therapies, namely by high-throughput screening strategies, aimed at identifying new drugs for the treatment of CF.

We conclude that these novel cell lines constitute *bona fide* models that mimic wt- and F508del-CFTR traffic, processing and function occurring in native polarized epithelial systems and provide significantly improved resources for cellular studies focused on the pathology or treatment of devastating genetic disorder.

Abbreviations

aa (amino acid); Ab (antibody); CF (cystic fibrosis); CFTR (CF transmembrane conductance regulator); ENaC (epithelial Na^+ channel); endo H (endoglycosidase H); ER (endoplasmic reticulum); EST (expressed sequence tags); Fsk (forskolin); Glib (glibenclamide); G_{te} (transepithelial conductance); HDAC (histone deacetylase); IBMX (3-isobutyl-1-methylxantine); *I*sc (short circuit current); PAGE (polyacrylamide gel electrophoresis); PNGase F -or N-glycanase- (peptide N-glycosidase F); PCR (polymerase chain reaction); R_{te} (transepithelial resistance); RT-PCR (reverse transcriptase-PCR); SDS (sodium dodecyl sulphate); V_{te} (transepithelial voltage); WB (Western blot).

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