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Impact of the cystic fibrosis mutation F508del-CFTR on renal cyst formation and growth

[1]Hongyu Li, [1]Wanding Yang, [2,3]Filipa Mendes, [2,3]Margarida D. Amaral and [1]David N. Sheppard

[1]School of Physiology and Pharmacology, University of Bristol,
Medical Sciences Building, University Walk, Bristol BS8 1TD, UK,
[2]University of Lisboa, Faculty of Sciences, BioFIG-Center for Biodiversity, Functional and Integrative Genomics, 1749-016 Lisboa, Portugal and
[3]Department of Genetics, National Institute of Health, Av. Padre Cruz, 1649-016 Lisboa, Portugal

Running Title: Impact of F508del-CFTR on renal cysts

Address Correspondence to: D.N. Sheppard, Ph.D.
University of Bristol
School of Physiology and Pharmacology
Medical Sciences Building
University Walk
Bristol BS8 1TD
United Kingdom
Tel: +44 117 331 2290
Fax: +44 117 331 2288
E-mail: D.N.Sheppard@bristol.ac.uk

ABSTRACT

In autosomal dominant polycystic kidney disease (ADPKD), cystic fibrosis transmembrane conductance regulator (CFTR), the protein product of the gene defective in cystic fibrosis (CF), plays a crucial role in fluid accumulation, which promotes cyst swelling. Several studies have identified individuals afflicted by both ADPKD and CF. Two studies suggested that CF mutations might attenuate the severity of ADPKD, whereas a third found no evidence of a protective effect. In this study, we investigated the impact of the commonest CF mutation F508del-CFTR on the formation and growth of renal cysts. As a model system, we used Madin Darby canine kidney (MDCK) epithelial cells engineered to express wild-type and F508del human CFTR. We grew MDCK cysts in collagen gels in the presence of the cAMP agonist forskolin, measured transepithelial resistance and Cl^- secretion with the Ussing chamber technique and assayed cell proliferation using non-polarized MDCK cells. When compared with untransfected MDCK cells, cells expressing wild-type CFTR generated substantial numbers of large cysts, which grew markedly over time. By contrast, MDCK cells expressing F508del-CFTR formed very few tiny cysts that failed to enlarge. Interestingly, treatment of F508del-CFTR cysts with the CFTR corrector VRT-325 and the CFTR corrector-potentiator VRT-532 increased the number, but not size of F508del-CFTR cysts, possibly because VRT-325 inhibited strongly cell proliferation. Based on its effects on transepithelial resistance, Cl^- secretion and cell proliferation, we conclude that the F508del-CFTR mutation disrupts cyst formation and growth by perturbing strongly fluid accumulation within the cyst lumen without compromising epithelial integrity.

Keywords: chloride ion channel / autosomal dominant polycystic kidney disease / epithelial ion transport / Madin Darby canine kidney (MDCK) epithelial cells / small-molecule CFTR modulators

INTRODUCTION

The ATP-binding cassette (ABC) transporter cystic fibrosis transmembrane conductance regulator (CFTR; (43)) is expressed in epithelial tissues throughout the body, lining ducts and tubes (64). Located in the apical membrane, CFTR functions to control the quantity and composition of epithelial secretions by (i) forming a small conductance anion-selective channel with complex regulation (13, 53) and (ii) regulating the activity of ion channels and transporters in epithelial cells (26, 47). The pivotal role that CFTR plays in transepithelial ion transport is dramatically highlighted by the common, life-shortening genetic disease cystic fibrosis (CF). In CF, malfunction of CFTR causes ducts and tubes to become blocked by thick, tenacious mucus leading to the wide-ranging manifestations of the disease, which include severe chronic lung disease and exocrine pancreatic dysfunction (64).

The lack of major changes in renal function in CF patients (64) has led to speculation that CFTR has a limited role in renal physiology (57). However, both wild-type CFTR and a functional isoform comprising the N-terminal half of CFTR (i.e. membrane-spanning domain 1, nucleotide-binding domain 1 and the regulatory domain; (36) see also (50)) are differentially expressed along the length of the nephron (6, 36, 54) serving roles in ion transport and receptor-mediated endocytosis (18, 21). While CFTR's contribution to renal physiology remains to be fully elucidated, it is now widely recognized that CFTR plays an important role in the pathogenesis of autosomal dominant polycystic kidney disease (ADPKD), the most common single gene disorder to affect kidney function (56, 66). In ADPKD, mutations in the polycystin proteins lead to the formation of epithelial cysts containing a fluid-filled cavity surrounded by a single layer of immature renal epithelial cells (9, 56, 66). The formation and growth of multiple ADPKD cysts progressively destroys kidney function leading to severe renal failure (56, 66).

The observation that fluid accumulation within ADPKD cysts involves cAMP-stimulated transepithelial Cl^- movements reminiscent of those found in secretory epithelia affected by CF (55, 64) stimulated a search for evidence of a role for CFTR. Immunocytochemical studies localized CFTR to the apical membrane of ADPKD cysts (3, 16). Functional studies identified Cl^- currents with properties identical to CFTR in ADPKD epithelial cells (16) and demonstrated that cAMP-stimulated fluid secretion by ADPKD epithelia was inhibited by CFTR antisense oligonucleotides (8). Taken together, the data argue that CFTR plays a crucial role in fluid accumulation by ADPKD cysts.

Of note, three clinical studies identified individuals afflicted by both ADPKD and CF (38, 41, 67). In two studies from Torres and colleagues (38, 67), individuals with both ADPKD and CF had reduced kidney volumes with fewer smaller cysts, normal blood pressure and no liver disease when compared with family relatives with ADPKD alone. These data suggest that CF has a protective effect on kidney function, reducing the severity of ADPKD. By contrast, Persu et al. (41) found no evidence for a protective effect of CF on ADPKD severity either in individuals homozygous or heterozygous for CFTR mutations. The authors speculate that the protective effect of CF might depend on the class of CFTR mutation (65) harbored by individuals with ADPKD. However, both Persu et al. (41) and Xu et al. (67) studied individuals homozygous for F508del-CFTR, the commonest CF mutation (64). This mutation causes a temperature-sensitive folding defect that (i) disrupts the intracellular transport of CFTR to the apical membrane (5, 10, 11), (ii) attenuates protein stability at the cell surface (31) and (iii) impedes CFTR channel gating (7).

Thus, the aim of this study was to investigate the impact of the F508del-CFTR mutation on renal cyst formation and growth. For this project, we used Madin Darby canine kidney (MDCK) cells engineered to express high levels of wild-type and F508del human CFTR (34). MDCK cells are a valuable model system to investigate renal cyst formation and growth (for discussion, see (28, 55)). To investigate the consequences of the F508del-CFTR mutation for renal cyst formation and growth, we grew MDCK cysts in collagen gels in the presence of the cAMP agonist forskolin, measured transepithelial resistance and Cl^- secretion with the Ussing chamber technique and assayed cell proliferation using non-polarized MDCK cells. We discovered that the F508del-CFTR mutation disrupts cyst formation and growth by perturbing strongly fluid accumulation within the cyst lumen without compromising epithelial integrity.

MATERIALS AND METHODS

Cells and cell culture

For this study, we used three types of MDCK cells: (i) untransfected MDCK cells, (ii) MDCK cells stably expressing wild-type human CFTR and (iii) MDCK cells stably expressing the commonest CF mutation, F508del-CFTR. Wild-type and F508del- human CFTR were stably expressed in MDCK cells using the HIV-based translentiviral recombinant vectors containing CFTR cDNAs developed by Tranzyme Corporation (Birmingham, AL) (22, 34). Cells were cultured in MDCK media (a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 nutrient medium supplemented with 10% fetal bovine serum (FBS), 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin; all from Invitrogen Ltd., Paisley, UK) at 37 ºC in a humidified atmosphere of 5% CO_2. To select for wild-type CFTR expression, 2 μg ml^{-1}

123 blasticidin S (Sigma-Aldrich Company Ltd., Gillingham, UK) was added to MDCK media, while
124 to select for F508del-CFTR, 4 μg ml^{-1} puromycin (Invitrogen Ltd.) was used.
125
126 **Cyst growth**
127 To grow cysts, MDCK cells were cultured in collagen gels in the presence of the cAMP
128 agonist forskolin using a modification of the method of Grantham et al. (14). Individual wells of
129 a 24-well plate containing 0.4 ml of ice-cold PureCol (~3.0 mg ml^{-1} collagen; Cohesion
130 Technologies Inc., Palo Alto, CA) supplemented with 10% (v/v) 10X minimum essential
131 medium, 10 mM Hepes, 27 mM $NaHCO_3$, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin
132 (pH 7.4 with NaOH) were seeded with ~800 MDCK cells. After gelation of the PureCol, 1.5 ml
133 of MDCK media containing either 1 or 10% FBS and forskolin (10 μM) was added to each well
134 of the 24-well plate. (1% FBS was used to prevent the small-molecules VRT-325 and VRT-532
135 binding to protein; for further information, see below). Plates were maintained at 37 ºC in a
136 humidified atmosphere of 5% CO_2 and the MDCK media containing forskolin changed every 2
137 days.
138
139 Three days after seeding collagen gels with MDCK cells, cysts were detected at 100x
140 magnification using an inverted microscope with phase contrast optics (Leica, model DMIL,
141 Milton Keynes, UK). To study cyst formation by different MDCK cells, photographs of cysts
142 were taken at day 6 using a Nikon Coolpix 995 camera (Nikon UK Ltd., Kingston upon Thames,
143 UK). To investigate cyst growth by different MDCK cells, photographs of individual cysts were
144 taken at 2-day intervals between day 6 and 12. To identify individual cysts, each cyst was
145 assigned a unique reference number using a grid placed below the 24-well plate.

To test the effects of small-molecule CFTR modulators on cyst formation, drugs were added to MDCK media in the continuous presence of forskolin (10 μM). For studies of the thiazolidinone CFTR inhibitor $CFTR_{inh}$-172 (32), $CFTR_{inh}$-172 (10 μM) was added from day 0 onwards. However, addition of the CFTR corrector VRT-325 (59) and the CFTR corrector-potentiator VRT-532 (59, 63) at day 0 was cytotoxic. Therefore, VRT-325 (6 μM) and VRT-532 (10 μM) were added to MDCK media containing forskolin (10 μM) at day 3 and 24 h later the media replaced with fresh MDCK media with forskolin (10 μM). On day 5, individual cysts were photographed to determine the effects of VRT-325 and VRT-532 on cyst formation. To test the effects of $CFTR_{inh}$-172 on cyst growth, MDCK cysts were incubated with MDCK media containing forskolin (10 μM) and $CFTR_{inh}$-172 (10 μM) between day 6 and day 12; media was changed every 2 days and photographs of individual cysts taken at these time points.

To determine cyst numbers, we counted all cysts in each well that had a diameter larger than 50 μm on either day 3 or 6 with data expressed as number of cysts per well. To calculate cyst volumes, cyst diameter was measured using images that had been magnified by identical amounts. By assuming that cysts are spherical in shape, we calculated cyst volume ($4/3 \times \pi \times r^3$).

Ussing chamber experiments

To grow MDCK cells as polarized epithelia, cells were seeded onto permeable filter supports (Millicell-PCF culture plate inserts, 0.4 μm pore size, 12 mm diameter, Millipore Corp., Fisher Scientific UK, Loughborough, UK) at a density of 3×10^5 cells per 0.6 cm^2. Every second day after seeding, we changed the MDCK media and measured transepithelial resistance (R_t)

using an epithelial voltohmmeter (EVOM; World Precision Instruments, Stevenage, UK). On day 8, we used MDCK epithelia for experiments. To rescue the cell surface expression of F508del-CFTR, MDCK epithelia expressing F508del-CFTR were either incubated at 27 °C for 24 h (10) or treated with the CFTR corrector VRT-325 (6 μM) (59) in MDCK media containing 1% FBS at 37 °C for 24 h before being returned to drug-free MDCK media 4 h before experiments were commenced.

CFTR-mediated Cl^- currents in MDCK epithelia were recorded using identical conditions to our previous study (28) with the exception that the basolateral membrane was not permeabilized with nystatin. MDCK epithelia were mounted in modified Ussing chambers (Warner Instrument Corp., Dual Channel Chamber; Harvard Apparatus Ltd., Edenbridge, UK). To magnify the size of CFTR-mediated Cl^- currents, we imposed a large Cl^- concentration gradient across MDCK epithelia. The basolateral membrane was bathed in a solution containing (in mM): 140 NaCl, 5 KCl, 0.36 K_2HPO_4, 0.44 KH_2PO_4, 1.3 $CaCl_2$, 0.5 $MgCl_2$, 10 Hepes and 4.2 $NaHCO_3$, pH 7.2 with Tris ([Cl^-], 149 mM). The composition of the solution bathing the apical membrane was identical to that of the basolateral solution with the exception that (in mM): 133.3 Na gluconate + 2.5 NaCl and 5 K gluconate replaced 140 NaCl and 5 KCl, respectively, to create a transepithelial Cl^- concentration gradient ([Cl^-], 14.8 mM). To compensate for Ca^{2+} buffering by gluconate, the apical solution contained 5.7 mM Ca^{2+}. All solutions were maintained at 37 ºC and bubbled continuously with 5% CO_2.

After canceling voltage offsets, we clamped transepithelial voltage (referenced to the basolateral solution) at 0 mV and recorded I_{sc} continuously using an epithelial voltage-clamp

amplifier (Warner Instrument Corp., model EC-825, Harvard Apparatus Ltd.), digitizing data as described previously (28). The resistance of the filter and solutions, in the absence of cells, was subtracted from all measurements. Under the experimental conditions that we used (i.e. I_{sc} activation by the cAMP agonist forskolin and inhibition by the thiazolidinone CFTR inhibitor $CFTR_{inh}$-172 (32)), flow of current from the basolateral to the apical solution corresponds to Cl^- movement through open CFTR Cl^- channels and is shown as an upward deflection.

Cell proliferation assays

To study the proliferation of MDCK cells, we used cells grown in MDCK media containing either 1 or 10% FBS. On day –1, 3.5 x 10^4 MDCK cells were seeded in individual wells of a 12-well plate containing MDCK media with 1 or 10% FBS and on day 0, forskolin (10 μM) and small-molecule CFTR modulators were added to the MDCK media. MDCK media containing forskolin and small-molecules was changed every 2 days for a total period of 6 days. To determine the number of cells per well, MDCK cells were harvested using trypsin (0.25% w/v), centrifuged at 1200 rpm for 5 min and resuspended in 1 ml of MDCK media before counting using a hemocytometer. The viability of MDCK cells was determined by staining with trypan blue (0.2% v/v).

Reagents

The CFTR modulators VRT-325 and VRT-532 were generous gifts of Professor R.J. Bridges (Rosalind Franklin University of Medicine and Science, Chicago, IL) and Cystic Fibrosis Foundation Therapeutics Incorporated (Bethesda, MD). $CFTR_{inh}$-172 was purchased from Calbiochem (Merck Chemicals Ltd., Nottingham, UK) and genistein from LC Laboratories

(Woburn, MA). All other chemicals were of reagent grade and supplied by the Sigma-Aldrich Company Ltd.

Forskolin was dissolved in methanol; all other drugs were dissolved in DMSO. Stock solutions were stored at -20 ºC and diluted with either MDCK media or salt solutions to achieve final concentrations immediately before use. Drug concentrations were selected for study based on published literature (e.g. (59)) and previous work (e.g. (28)). Precautions against light-sensitive reactions were observed when using genistein, VRT-325 and VRT-532. DMSO (0.4% v/v) was without effect on cyst growth, cell proliferation and I_{sc} (28).

Statistics

Results are expressed as means ± SEM of n observations. To test for differences between groups of data, we used either a two-way analysis of variance (ANOVA) or Student's t-test. Differences were considered statistically significant when $P < 0.05$. All tests were performed using SigmaStat™ (version 3.5, Systat Software Inc., Richmond, CA).

RESULTS

F508del-CFTR impedes renal cyst formation and growth

MDCK cells form cysts when grown in collagen gels in the presence of cAMP agonists (14, 28). To investigate the effects of F508del-CFTR on renal cyst formation and growth, we used MDCK cells engineered to express high levels of wild-type and F508del human CFTR (34), hereafter termed MDCK-wt-CFTR and MDCK-F508del-CFTR, respectively. As a control, we studied untransfected MDCK cells, which express canine CFTR (35).

We began by investigating the effects of F508del-CFTR on renal cyst formation. To address this question, we overlaid collagen gels inoculated with untransfected, MDCK-wt-CFTR and MDCK-F508del-CFTR cells with MDCK media containing the cAMP agonist forskolin (10 μM). In previous work (28), we demonstrated that growth of MDCK cysts from day 0 onwards in the presence of the thiazolidinone CFTR inhibitor $CFTR_{inh}$-172 (32) decreases both cyst volume and number. Therefore, as a further control, we grew MDCK-wt-CFTR cysts in the continuous presence of $CFTR_{inh}$-172 (10 μM) from day 0 onwards. Figure 1, *A* – *H* shows images of cysts from the different MDCK cell lines at day 6 and Figure 1, *I* and *J* quantifies cyst number and volume. When compared to untransfected MDCK cells, MDCK-wt-CFTR cells formed substantial numbers of large cysts (number, 3.6-fold greater; size 1.4-fold, larger) (Fig. 1, *A* – *D*, *I* and *J*). By contrast, when MDCK-wt-CFTR cells were grown in the continuous presence of $CFTR_{inh}$-172 (10 μM) although the number of cysts almost doubled, their size did not differ from that of untransfected MDCK cells (Fig. 1, *A*, *B*, *E*, *F*, *I* and *J*). For two reasons, we were intrigued by the cysts formed by MDCK-F508del-CFTR cells. First, these cells generated very few cysts (number, 0.8-fold smaller than untransfected MDCK cells); mostly they formed solid masses of cells (Fig. 1, *A*, *B*, *G*, *H* and *I*). Second, MDCK-F508del-CFTR cysts were greatly reduced in size compared with untransfected MDCK cysts (size, 0.8-fold smaller than untransfected MDCK cells) (Fig. 1, *A*, *B*, *G*, *H* and *J*).

Next, we investigated the effects of F508del-CFTR on renal cyst growth. In previous work (28), we demonstrated that the volume of renal cysts increases markedly over the period day 6 to day 12. Therefore, in the present study, we compared renal cyst growth by different MDCK cell lines over the same time interval. Figure 2*A* shows images of individual cysts at day

6 and day 12, while Figure 2*B* quantifies cyst growth by different MDCK cell lines. Both untransfected and MDCK-wt-CFTR cysts grew noticeably over the 6-day period (Fig. 2). However, by day 12 the volume of MDCK-wt-CFTR cysts was 4.4-fold larger than that of untransfected MDCK cysts because of their larger initial volume and faster rate of growth (Fig. 2*B*). Treatment of MDCK-wt-CFTR cysts with $CFTR_{inh}$-172 (10 μM) for 6 days attenuated markedly cyst volume with the result that cyst volumes did not differ statistically from those of untransfected MDCK cysts ($P > 0.05$) (Fig. 2); similar results were observed for MDCK-wt-CFTR cysts grown in the continuous presence of $CFTR_{inh}$-172 (10 μM) from day 0 to day 12 (Fig. 2*B*). Strikingly, between day 6 and day 12, the volume of MDCK-F508del-CFTR cysts failed to increase; at all time points the volume of these cysts was minuscule compared with those of MDCK-wt-CFTR cysts (Fig. 2). Taken together, our data suggest that expression of wild-type human CFTR in MDCK cells promotes cyst formation and growth. By contrast, F508del-CFTR did not support cyst formation and growth, suppressing these processes more markedly than the action of the CFTR inhibitor $CFTR_{inh}$-172 on MDCK-wt-CFTR cysts.

The CFTR corrector VRT-325 and the CFTR corrector-potentiator VRT-532 increase cyst number, but not cyst volume

CFTR correctors and potentiators are small-molecules that rescue the cell surface expression and function of F508del-CFTR (4, 61). Because small-molecule inhibitors of CFTR diminish renal cyst formation and growth ((28, 69), present study), we were interested to learn whether CFTR correctors and potentiators might restore cyst formation and growth to MDCK-F508del-CFTR cells. To test this hypothesis, we used the CFTR corrector VRT-325 (59) and the CFTR potentiator VRT-532 (59), a small-molecule that also rescues the F508del-CFTR

trafficking defect (63), indicating that it is a dual-acting molecule (termed a CFTR corrector-potentiator). As a control, we tested the effects of VRT-325 and VRT-532 on untransfected MDCK cells.

Figure 3, *A* and *B* shows images of MDCK-F508del-CFTR and untransfected MDCK cysts grown in the presence of either VRT-325 (6 μM) and VRT-532 (10 μM) together or VRT-532 (10 μM), alone on day 5 and Figure 3, *C* and *D* quantifies their effects on cyst number and volume. Incubation of untransfected MDCK cysts with either treatment was without effect on cyst number, but decreased noticeably cyst volume (Fig. 3, *C* and *D*). Interestingly, treatment of MDCK-F508del-CFTR cysts with either VRT-532 (10 μM) alone or together with VRT-325 (6 μM) increased cyst number 1.5-fold, but was without effect on the volume of MDCK-F508del-CFTR cysts (Fig. 3, *C* and *D*). We interpret these data to suggest that the CFTR corrector VRT-325 and the CFTR corrector-potentiator VRT-532 restore cyst formation, but not cyst growth, by F508del-CFTR.

Effects of heterologous CFTR expression and small-molecules on cAMP-stimulated Cl^- secretion

Renal cyst growth involves both the proliferation of cyst-lining epithelial cells and the accumulation of fluid within the cyst lumen (17, 55, 68, 70). To investigate the contribution of fluid accumulation to differences in cyst formation and growth by the three MDCK cell lines, we measured transepithelial resistance (R_t) to evaluate epithelial integrity and recorded cAMP-stimulated short-circuit current (I_{sc}) to quantify transepithelial Cl^- secretion. To enhance the

magnitude of cAMP-stimulated I_{sc}, we clamped transepithelial voltage at 0 mV and imposed a large Cl^- concentration gradient across the epithelium.

Table 1 reports values of transepithelial resistance (R_t) for different MDCK epithelia recorded under current-clamp conditions before recording I_{sc}. The R_t value of MDCK-wt-CFTR epithelia was 0.7-fold lower than that of untransfected MDCK epithelia, whereas that of MDCK-F508del-CFTR epithelia did not differ from untransfected MDCK epithelia (Table 1). Figure 4, *A* and *B* demonstrates that addition of forskolin (10 μM) to the apical and basolateral solutions generated cAMP-stimulated I_{sc} in each of the different MDCK epithelia. The magnitude of cAMP-stimulated I_{sc} decreased in the rank-order: MDCK-wt-CFTR epithelia >> untransfected MDCK epithelia > MDCK-F508del-CFTR epithelia (Fig. 4*B*). However, rescue of the cell surface expression of F508del-CFTR either by incubating epithelia at reduced temperature (27 °C for 24 h; hereafter termed low temperature-rescued) (10) or by treating epithelia with VRT-325 (6 μM at 37 °C for 24 h; hereafter termed VRT-325-rescued) (59) decreased R_t and increased the magnitude of cAMP-stimulated I_{sc} generated by MDCK-F508del-CFTR epithelia to a level comparable with that of untransfected MDCK epithelia (Table 1 and Fig. 4*B*).

Following the activation of cAMP-stimulated I_{sc}, we added sequentially small-molecule CFTR modulators to the apical solution bathing different MDCK epithelia. Figure 4, *A* and *C* demonstrates that the CFTR corrector-potentiator VRT-532 (10 μM) enhanced modestly cAMP-stimulated I_{sc} in untransfected and MDCK-F508del-CFTR epithelia, but had little or no effect on cAMP-stimulated I_{sc} in MDCK-wt-CFTR, low temperature-rescued MDCK-F508del-CFTR and VRT-325-rescued MDCK-F508del-CFTR epithelia. Likewise, addition of genistein (50 μM), the

best-studied CFTR potentiator (4, 19), to the apical solution bathing different epithelia enhanced modestly or was without effect on cAMP-stimulated I_{sc} (Fig. 4*A* and data not shown). To verify that cAMP-stimulated I_{sc} was mediated by CFTR Cl^- channels in the apical membrane, we used the thiazolidinone CFTR inhibitor $CFTR_{inh}$-172 (32). Figure 4, *A* and *C* demonstrates that $CFTR_{inh}$-172 (10 μM) inhibited cAMP-stimulated I_{sc} in each of the epithelia tested. Inhibition by $CFTR_{inh}$-172 (10 μM) was potent in untransfected, MDCK-F508del-CFTR and low temperature-rescued MDCK-F508del-CFTR, but less marked in MDCK-wt-CFTR and VRT-325-rescued MDCK-F508del-CFTR epithelia. Taken together, the data suggest that Cl^- secretion by the different MDCK cell lines explains some of the differences in cyst size and number. However, the data do not account for all the effects of VRT-325 and VRT-532 on cyst formation and growth.

The CFTR corrector VRT-325 inhibits the proliferation of MDCK cells

Cyclic AMP-stimulated cell proliferation is a key element of renal cyst growth (17, 68). We therefore speculated that the reduction in volume of untransfected cysts treated with VRT-325 and VRT-532 might be caused by these small-molecules retarding cell proliferation. To test this idea, we examined the time course of cell proliferation over a 6-day period in the presence of forskolin (10 μM) and small-molecules using MDCK media containing 10 or 1% FBS.

Figure 5*A* shows the time course of cell proliferation by the different MDCK cell lines using MDCK media containing 10% FBS and the effects of $CFTR_{inh}$-172 on MDCK-wt-CFTR cells. For each of the different cell lines, the number of MDCK cells increased dramatically over the 6-day period. The time course of cell proliferation was very similar for untransfected and

MDCK-wt-CFTR cells. Consistent with previous results (28), in the presence of 10% FBS, $CFTR_{inh}$-172 (10 μM) only weakly attenuated the proliferation of MDCK-wt-CFTR cells (Fig. 5*A*). Interestingly, Figure 5*A* reveals that the growth of MDCK-F508del-CFTR cells was slower than that of the other MDCK cell lines, although this difference is only statistically significant on comparison with either untransfected or MDCK-wt-CFTR cells at day 4.

Finally, we tested the effects of the CFTR corrector VRT-325 and the CFTR corrector-potentiator VRT-532 on the proliferation of untransfected and MDCK-F508del-CFTR cells using MDCK media containing 1% FBS. Figure 5, *B* and *C* demonstrates that VRT-532 (10 μM) was without effect on MDCK cell proliferation. By contrast, VRT-325 (6 μM) either alone or together with VRT-532 (10 μM) dramatically inhibited the proliferation of untransfected and MDCK-F508del-CFTR cells (Fig. 5, *B* and *C*). Taken together, these data suggest that the CFTR corrector VRT-325 fails to promote cyst growth because it inhibits cell proliferation.

DISCUSSION

In this study, we investigated the consequences of the commonest CF mutation F508del-CFTR on renal cyst formation and growth using MDCK cells engineered to express wild-type and F508del- human CFTR. F508del-CFTR attenuated renal cyst formation and growth more strongly than blockade of wild-type human CFTR with $CFTR_{inh}$-172, an efficacious CFTR inhibitor. Based on its impact on transepithelial resistance, Cl^- secretion and cell proliferation, we conclude that F508del-CFTR disrupts cyst formation and growth by perturbing strongly fluid accumulation within the cyst lumen without compromising epithelial integrity.

In ADPKD, mutations in the polycystin proteins stimulate the development and growth of renal cysts, in part, by activating the cAMP signaling pathway. Elevation of the intracellular concentration of cAMP stimulates the proliferation of ADPKD epithelial cells (17, 68) and fluid accumulation within the cyst lumen mediated by CFTR (16, 55). Interestingly, Ikeda et al. (20) demonstrated that polycystin-1 is a negative regulator of the cell surface expression of CFTR. This suggests that mutation of polycystin-1 promotes fluid accrual within the cyst lumen, in part, by enhancing the apical membrane expression of CFTR in ADPKD epithelial cells. Taken together, the data argue that cyst growth in ADPKD might be retarded by inhibiting CFTR-mediated fluid accumulation.

Hanaoka & Guggino (17) first tested the idea that inhibitors of CFTR might retard ADPKD cyst growth, demonstrating that diphenylamine-2-carboxylate (DPC; (33)) and glibenclamide (52), two non-specific CFTR inhibitors, diminished ADPKD cyst growth, whereas 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), which inhibits other types of epithelial Cl^- channels, but not CFTR, when added to the outside of cells (46), was without effect. Building on these data, we found that cyst growth by MDCK cells is slowed by agents that inhibit either directly or indirectly CFTR-mediated transepithelial Cl^- secretion, but not by blockers of other types of apical membrane Cl^- channels (28). Our data further revealed that inhibition of cyst growth by CFTR blockers is correlated with blockade of cAMP-stimulated Cl^- transport, not cell proliferation (28). This provides an explanation for why $CFTR_{inh}$-172 diminished strongly cyst size, but not number ((28), present study). This agent potently inhibits the CFTR Cl^- channel, but only modestly slows cell proliferation (28, 32). Of note, Yang et al. (69) demonstrated that the $CFTR_{inh}$-172 analog tetrazolo-$CFTR_{inh}$-172 reduces cyst formation and kidney enlargement in vivo, providing compelling proof of concept data for the use of CFTR inhibitors in ADPKD.

The present study demonstrates that the CF mutation F508del impedes dramatically MDCK cyst formation and growth. The principal mechanism by which F508del causes CFTR dysfunction is protein misfolding. Molecular chaperones of the ER quality control mechanism recognize structural perturbations caused by F508del, leading to the retention of the mutant protein in the ER and its rapid targeting for degradation by the ubiquitin-proteasome pathway (for review, see (2)). As a result, the vast majority of F508del-CFTR is neither processed through the Golgi apparatus, where wild-type CFTR is matured, nor delivered to its correct cellular location, the apical membrane of polarized epithelia. Thus, the simplest interpretation of our data is that the absence of F508del-CFTR from the apical membrane prevents fluid accumulation within the cyst lumen, thwarting cyst formation and growth by MDCK-F508del-CFTR cells.

However, two alternative explanations of our results are suggested by previous work. First, the effects of some ion transport inhibitors on cell proliferation and cyst growth (28) argue that the F508del-CFTR mutation might hinder cell proliferation. Although the effects of F508del-CFTR on volume-regulated anion channels (60), which regulate cell cycle progression (49), make this an attractive possibility, our data do not support this idea. F508del-CFTR had little or no impact on MDCK cell proliferation. Second, the F508del-CFTR mutation might adversely affect tight junction formation with the result that fluid secreted into the cyst lumen might leak out through the paracellular pathway preventing cyst expansion. In support of this idea, F508del-CFTR disrupts the organization and function of tight junctions in human airway epithelia (27). However, our data show that the R_t of MDCK-F508del-CFTR epithelia exceeded greatly that of MDCK-wt-CFTR epithelia. These data suggest that the F508del mutation does not perturb the integrity of tight junctions in MDCK epithelia.

When compared with untransfected MDCK cells, MDCK-F508del-CFTR cells formed fewer, smaller cysts, grew more slowly and generated less cAMP-stimulated I_{sc}. These data are surprising because both cell lines express similar levels of canine CFTR and because CF is a recessive genetic disorder (64). One possible explanation of the data is that F508del human CFTR inhibits the processing and trafficking of canine CFTR by its impact on the ER and peripheral protein quality control mechanisms (2, 39). Alternatively, F508del human CFTR might alter the function of canine CFTR. Some studies suggest that CFTR displays cooperativity, whereby the behavior of one channel influences that of another channel. For example, Krouse and Wine (25) demonstrated that the kinetics of CFTR channel gating do not obey binomial statistics in multichannel patches. The authors interpreted their data to suggest that the open probability of CFTR is influenced by channel density and increases when individual channels are packed tightly together. Consistent with this idea, when CFTR proteins are tethered together by cytoskeletal proteins containing PDZ domains, channel activity is potentiated (42, 62). These data highlight the intricate control mechanisms that regulate CFTR activity in epithelial cells and suggest a plausible explanation for the different behavior of untransfected and MDCK-F508del-CFTR cells observed in this study.

To rescue cyst formation and growth by MDCK-F508del-CFTR cells, we used the CFTR corrector VRT-325 (59) and the CFTR corrector-potentiator VRT-532 (59, 63). Interestingly, these small-molecules increased the number, but not the size of MDCK-F508del-CFTR cysts and reduced the volume of untransfected cysts. In part, these data are explained by the marked inhibition of cell proliferation by VRT-325, which has been reported to cause cell detachment, when used at elevated concentrations (30). However, the reduction in size of untransfected cysts

raises the possibility that VRT-325 and VRT-532 might directly or indirectly inhibit fluid accumulation by MDCK cysts. In support of the latter idea, there is evidence that VRT-325 modulates the intracellular transport of different membrane proteins (58) raising the possibility that it might have non-specific effects on MDCK-F508del-CFTR cells. In support of the former idea, we previously showed that under certain circumstances CFTR potentiators inhibit CFTR-mediated transepithelial Cl^- transport by altering either the expression (44) or function of CFTR (for review, see (29)). Furthermore, Kim Chiaw et al. (23) demonstrated that VRT-325 inhibits anion transport by low-temperature rescued F508del-CFTR and diminishes the ATP affinity of purified reconstituted F508del-CFTR. Thus, restoration of cyst formation and growth by MDCK-F508del-CFTR requires the judicious selection of small-molecules that do not inhibit CFTR function nor cell proliferation.

When tested as CFTR potentiators, the modest effects of VRT-532 and genistein on cAMP-stimulated I_{sc} in MDCK epithelia likely result from the rundown of CFTR Cl^- currents. We do not favor the idea that changes in the activity of basolateral membrane ion channels and transporters are responsible because rundown is observed both when the basolateral membrane is intact and when it is permeabilized with nystatin ((28), present study). Current rundown might reflect differences in the complement of protein kinases (e.g. AMP kinase; (15)) and phosphatases (e.g. PP2A and PP2C; (12)) that deactivate CFTR Cl^- channels between MDCK cells and other epithelial cells used to investigate small-molecule CFTR modulators (e.g. Fischer rat thyroid epithelia (40) and human bronchial epithelia (59)). However, it is also feasible that MDCK cells might lack ion channels, transporters and/or interacting proteins found in other epithelial cells, which comprise additional components of the cellular mechanism for cAMP-stimulated transepithelial anion transport (48). Of note, the F508del-CFTR Cl^- channel is highly

susceptible to rundown, much more so than wild-type CFTR (e.g. see (45)). This hallmark of F508del-CFTR is likely linked to the deleterious impact of the F508del mutation on the thermal stability of CFTR (1). This defect is a key target for CF therapy development.

The magnitude of CFTR-mediated anion flow across the apical membrane of an epithelium is determined both by the number of CFTR channels in the apical membrane and the activity of individual channels (for discussion, see (51)). Small-molecule CFTR inhibitors, such as glibenclamide and $CFTR_{inh}$-172 exert their effects by blocking current flow and slowing channel gating, respectively, of individual CFTR channels ((24, 52); for review, see (29)). Based on the effects of the F508del-mutation on renal cyst formation and growth in vivo and in vitro ((38, 67), present study), an alternative, potentially more effective, strategy to retard cyst growth and enlargement would be to eliminate the apical membrane expression of CFTR. In support of this idea, Nofziger et al. (37) demonstrated that long-term incubation of MDCK epithelia with peroxisome proliferator-activated receptor-γ (PPARγ) agonists (e.g. pioglitazone) attenuated sharply CFTR-mediated transepithelial Cl^- secretion by decreasing CFTR mRNA levels. Future studies should explore further the therapeutic potential of small-molecules that attenuate CFTR expression in the treatment of ADPKD.

In conclusion, the goal of this study was to investigate the effects of the commonest CF mutation, F508del-CFTR on renal cyst formation and growth. Using MDCK cells expressing recombinant wild-type and F508del human CFTR, we demonstrated that F508del-CFTR retards robustly cyst formation and growth in excellent agreement with the clinical studies of individuals with both ADPKD and CF by Torres and colleagues (38, 67). By showing that F508del-CFTR

exerts its effects by perturbing strongly fluid accumulation within the cyst lumen without compromising epithelial integrity, our data provide an explanation for why patients with ADPKD and CF have less severe ADPKD. Taken together, our data and those of Torres and colleagues (38, 67) raise the possibility that the F508del-CFTR mutation might be a modifier gene in ADPKD. With continued improvements in the treatment of CF patients and hence, their life expectancy, the effects of this modifier gene will likely increase.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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AUTHOR CONTRIBUTIONS

Hongyu Li, designed experiments, acquired and analyzed data, interpreted data and wrote the manuscript; Wanding Yang, acquired and analyzed data; Filipa Mendes, supplied reagents; Margarida D. Amaral, supplied reagents; David N. Sheppard, designed experiments, interpreted data and wrote the manuscript; all authors reviewed and approved the final version of the manuscript.

The current address of Hongyu Li is Department of Applied Sciences, London South Bank University, London SE1 0AA, UK and that of Filipa Mendes is Chemical and

Radiopharmaceutical Sciences Unit, Technical University of Lisboa, Instituto Superior Técnico, IST/ITN, Estrada Nacional 10, 2686-953 Sacavém, Portugal.

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FIGURE LEGENDS

Figure 1: Heterologous expression of human CFTR in MDCK cells alters cyst formation and growth *A – H*: Images of MDCK cysts grown in collagen gels in the presence of forskolin (10 μM) on day 6 after seeding gels with MDCK cells. *A*, *B*: Cysts formed by untransfected MDCK cells; *C*, *D*: cysts formed by wild-type human CFTR expressing MDCK (MDCK-wt-CFTR) cells; *E*, *F*: cysts formed by MDCK-wt-CFTR cells grown in the continuous presence of $CFTR_{inh}$-172 (C172; 10 μM); *G*, *H*: cysts formed by F508del human CFTR expressing MDCK (MDCK-F508del-CFTR) cells. The vertical lines in *E* and *G* are grid lines used to identify individual cysts. In *A*, *C*, *E* and *G*, the bar is 0.5 mm, while in *B*, *D*, *F* and *H*, it is 50 μm. *I*, *J*: Effects of CFTR expression on cyst number and volume. Data are means ± SEM (number, n = 15 – 37 wells; volume, n = 11 – 48 cysts, except MDCK-wt-CFTR where n = 256 cysts; number of individual experiments = 5); **, $P < 0.01$ vs. untransfected cells; *, $P < 0.05$ vs. untransfected cells; ## $P < 0.01$ vs. MDCK-wt-CFTR cells.

Figure 2: Time course of cyst growth by MDCK cells heterologously expressing human CFTR *A*: Images of cysts formed by untransfected, MDCK-wt-CFTR and MDCK-F508del-CFTR cells on day 6 and day 12 after seeding gels. Cysts were grown in the continuous presence of forskolin (10 μM). C172 denotes MDCK-wt-CFTR cysts treated with $CFTR_{inh}$-172 (10 μM) between days 6 and 12. Bar = 100 μm. *B*: Relationship between cyst volume and time. Data are means ± SEM (untransfected, n = 13 cysts; MDCK-wt-CFTR, n = 99 cysts; MDCK-wt-CFTR treated with $CFTR_{inh}$-172 (10 μM) from (i) day 0, n = 20 cysts or (ii) day 6, n = 71 cysts; MDCK-F508del-CFTR, n = 11 cysts; number of individual experiments = 5); **, $P < 0.01$ vs.

819 untransfected; ##, $P < 0.01$ vs. MDCK-wt-CFTR. Where not shown error bars are smaller than
820 symbol size.
821
822 **Figure 3: VRT-325 and VRT-532 increase F508del-CFTR cyst number, but not volume** *A*:
823 Day 5 images of multiple cysts formed by MDCK-F508del-CFTR cells grown in the presence of
824 forskolin (10 μM) alone (Control, left) or forskolin (10 μM) and VRT-532 (10 μM) (VRT-532,
825 right). *B*: Day 5 images of individual cysts formed by untransfected (UT) and MDCK-F508del-
826 CFTR (F508del) cells grown in the presence of forskolin (10 μM) alone (Control, left), forskolin
827 (10 μM), VRT-325 (6 μM) and VRT-532 (10 μM) (VRT-325 + VRT-532, middle) or forskolin
828 (10 μM) and VRT-532 (10 μM) (VRT-532, right) using MDCK media containing 1% FBS to
829 prevent drug binding to protein. In *A* and *B*, the scale bars are 500 and 100 μm, respectively. *C*,
830 *D*: Effects of the CFTR corrector VRT-325 and the CFTR corrector-potentiator VRT-532 on the
831 number and volume of cysts formed by untransfected and MDCK-F508del-CFTR cells. Data are
832 means ± SEM (number, untransfected, 4 – 10 wells, MDCK-F508del-CFTR, 4 – 10 wells;
833 volume, untransfected, 34 – 43 cysts; MDCK-F508del-CFTR, 37 – 44 cysts, except VRT-325 +
834 VRT-532, where n = 120 cysts; number of individual experiments = 3); *, $P < 0.05$ vs. control
835 untransfected MDCK cells; # $P < 0.05$ vs. control MDCK-F508del-CFTR cells. For further
836 information, see the Methods.
837
838 **Figure 4: Effects of expression of human CFTR and CFTR modulators on cAMP-**
839 **stimulated short-circuit current in MDCK epithelia** *A*: Representative recordings show the
840 effects of VRT-532 (10 μM), genistein (50 μM) and $CFTR_{inh}$-172 (C172; 10 μM) on cAMP-
841 stimulated I_{sc} from different MDCK epithelia (UT, untransfected). Cyclic AMP-stimulated I_{sc}

842 was activated by addition of forskolin (10 μM) to both the apical and basolateral sides of
843 epithelia, other drugs were added to the apical side only; continuous lines indicate the period of
844 exposure to test drugs. Dotted lines indicate zero current. To augment cAMP-stimulated I_{sc} in
845 MDCK-F508del-CFTR epithelia, the cell surface expression of F508del-CFTR was rescued by
846 (i) incubating MDCK-F508del-CFTR epithelia at 27 °C for 24 h or (ii) treating MDCK-F508del-
847 CFTR epithelia with VRT-325 (6 μM) for 24 h at 37 °C. *B*: Magnitude of cAMP-stimulated I_{sc}
848 in different MDCK epithelia. Data are means ± SEM (n = 7 – 9); **, $P < 0.01$ vs. untransfected
849 epithelia; ##, $P < 0.01$ vs. MDCK-F508del-CFTR epithelia cultured at 37 °C. *C*: Magnitude of
850 cAMP-stimulated I_{sc} potentiated by VRT-532 (10 μM) and inhibited by $CFTR_{inh}$-172 (10 μM).
851 The dashed line indicates the control value before the addition of small-molecules. Data are
852 means ± SEM (VRT-532, n = 7 – 9, except MDCK-wt-CFTR, where n = 4; $CFTR_{inh}$-172, n = 8 –
853 9, except VRT-325-rescued MDCK-F508del-CFTR, where n = 6); #, $P < 0.05$ vs. control
854 response (i.e. forskolin-treated epithelia for VRT-532); ##, $P < 0.01$ vs. control response (i.e.
855 forskolin-treated epithelia for VRT-532 and genistein-treated epithelia for $CFTR_{inh}$-172).
856
857 **Figure 5: Effects of expression of human CFTR and CFTR modulators on the proliferation**
858 **of MDCK cells** Data show the relationship between cell proliferation and time for untransfected,
859 MDCK-wt-CFTR and MDCK-F508del-CFTR cells treated with small-molecule CFTR
860 modulators. Experiments were performed using MDCK media containing forskolin (10 μM) and
861 either 10% FBS (*A*) or 1% FBS (*B*, *C*). In *A*, MDCK-wt-CFTR cells were treated with $CFTR_{inh}$-
862 172 (C172; 10 μM), whereas in *B* and *C* untransfected and MDCK-F508del-CFTR cells were
863 treated with VRT-325 (6 μM), VRT-532 (10 μM) and VRT-325 (6 μM) + VRT-532 (10 μM).

Data are means ± SEM (n = 4); **, $P < 0.01$ vs. control (untreated) cells. Where not shown error bars are smaller than symbol size.

867 **TABLES**

868 **Table 1: Transepithelial resistance of MDCK epithelia**

869

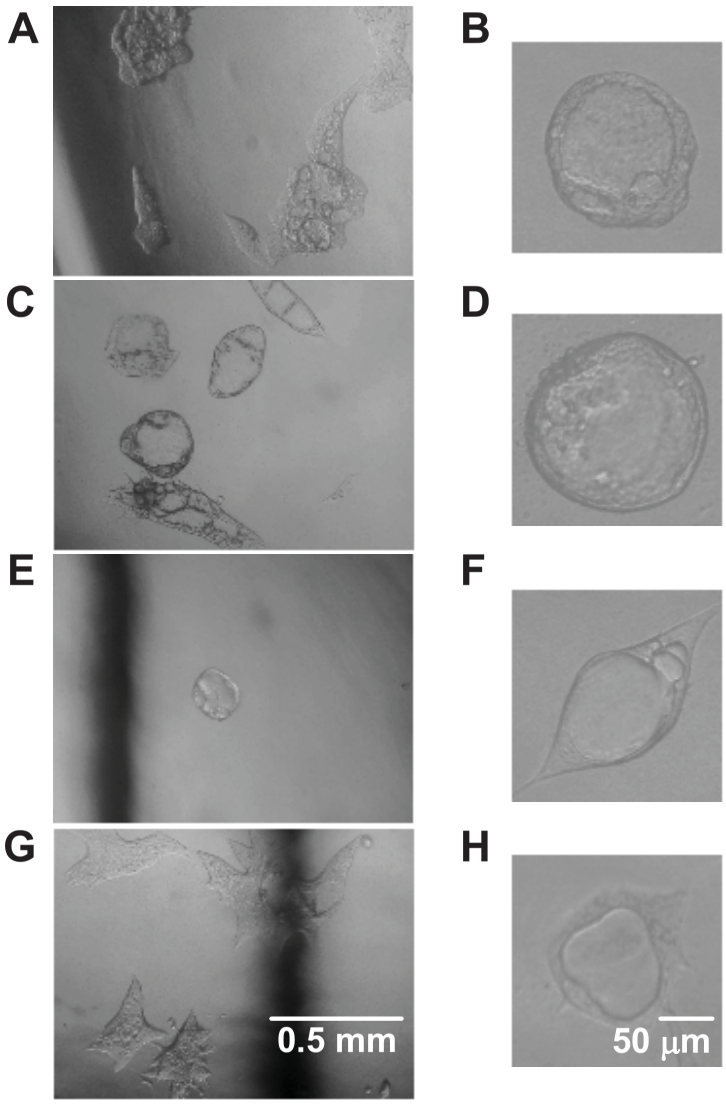
Epithelium	R_t (kΩ cm^2)
Untransfected	2.93 ± 0.54
MDCK-wt-CFTR	0.86 ± 0.22**
MDCK-F508del-CFTR	3.66 ± 0.79
MDCK-F508del-CFTR incubated at 27 °C	2.23 ± 0.18
MDCK-F508del-CFTR treated with VRT-325	1.71 ± 0.31

870

871 Values of transepithelial resistance (R_t) for different MDCK epithelia were measured under
872 current-clamp conditions at the start of experiments after series resistance compensation and the
873 resistance of the filter in the absence of cells was subtracted. To rescue the cell surface
874 expression of F508del-CFTR, MDCK-F508del-CFTR epithelia were (i) incubated at 27 °C for 24
875 h or (ii) treated with the CFTR corrector VRT-325 (6 μM) for 24 h at 37 °C. Data are means ±
876 SEM (n = 7 – 9); **, $P < 0.01$ vs. untransfected MDCK epithelia.

877

878


A
B
C
D
E
F
G
H
0.5 mm
50 μm

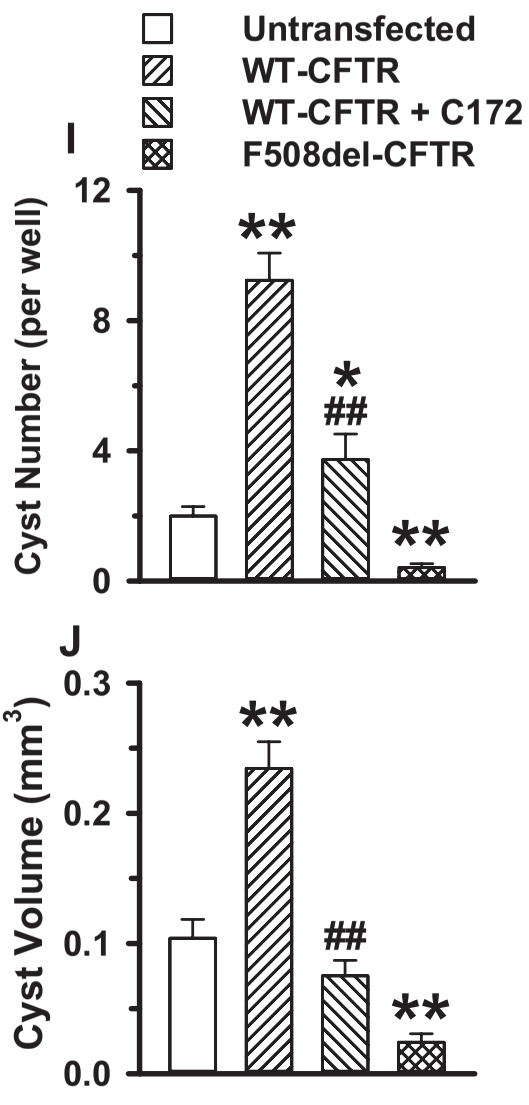
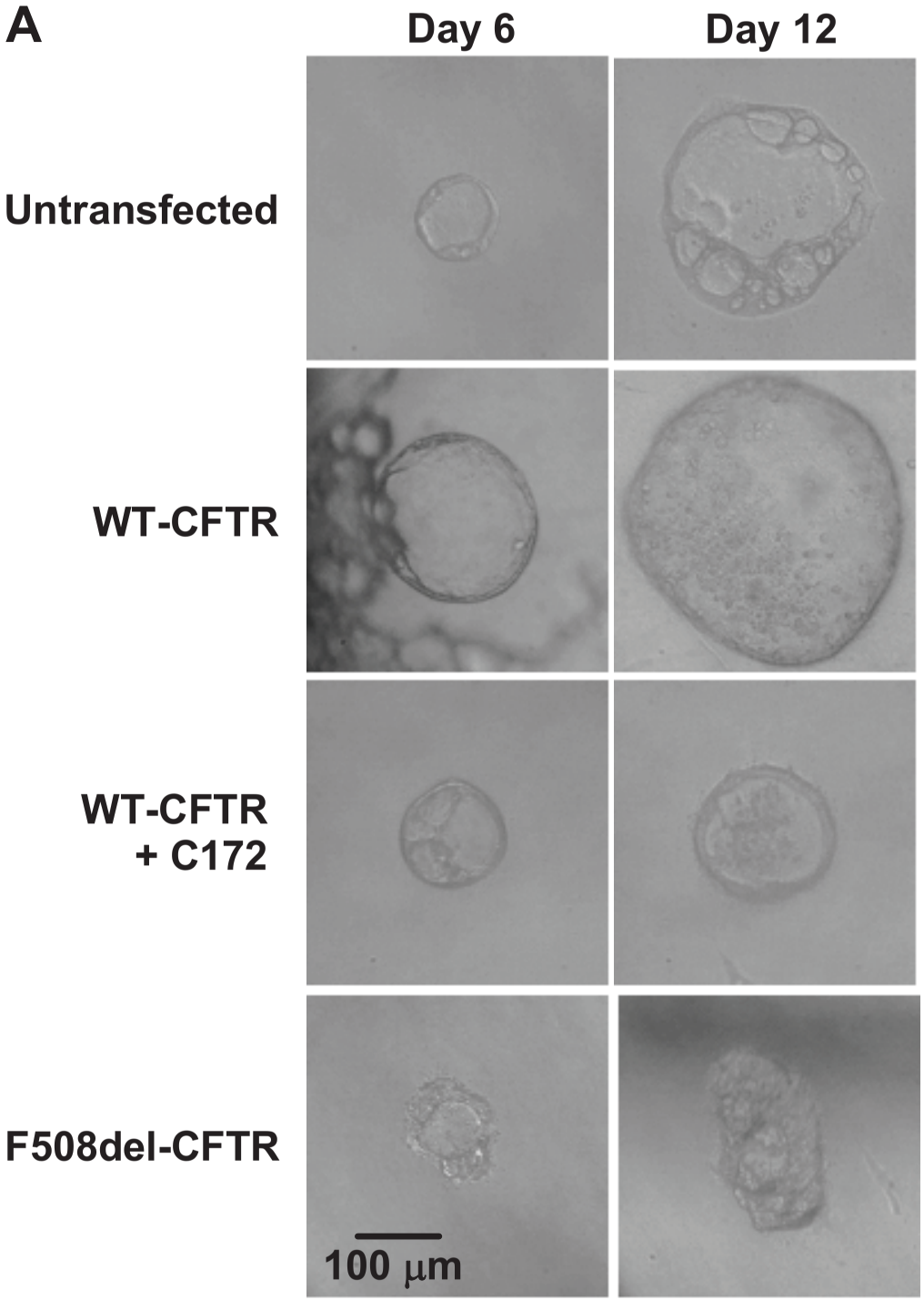

Untransfected
WT-CFTR
WT-CFTR + C172
F508del-CFTR
I
Cyst Number (per well)
12
8
4
0
**
*
##
**
J
Cyst Volume (mm3)
0.3
0.2
0.1
0.0
**
##
**

Fig. 1

Figure 2


A
Day 6
Day 12
Untransfected
WT-CFTR
WT-CFTR
+ C172
F508del-CFTR
100 μm

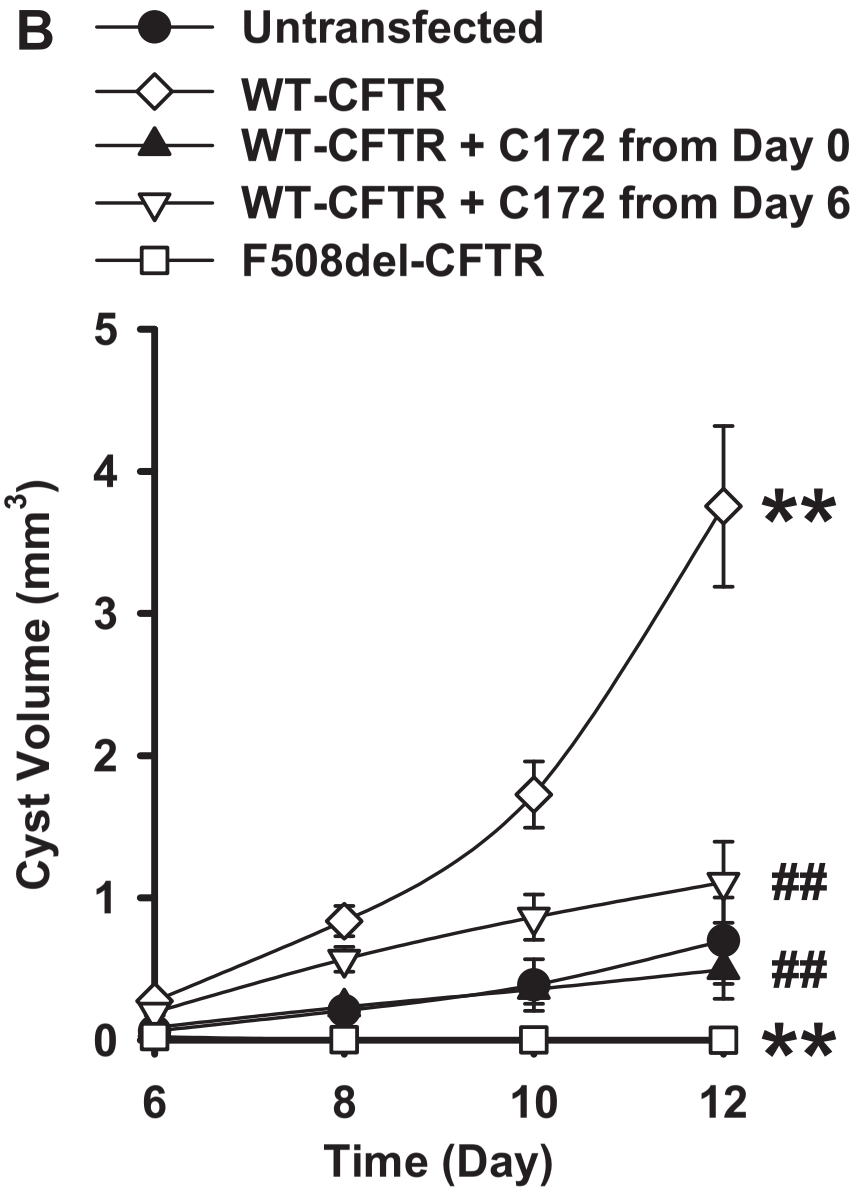
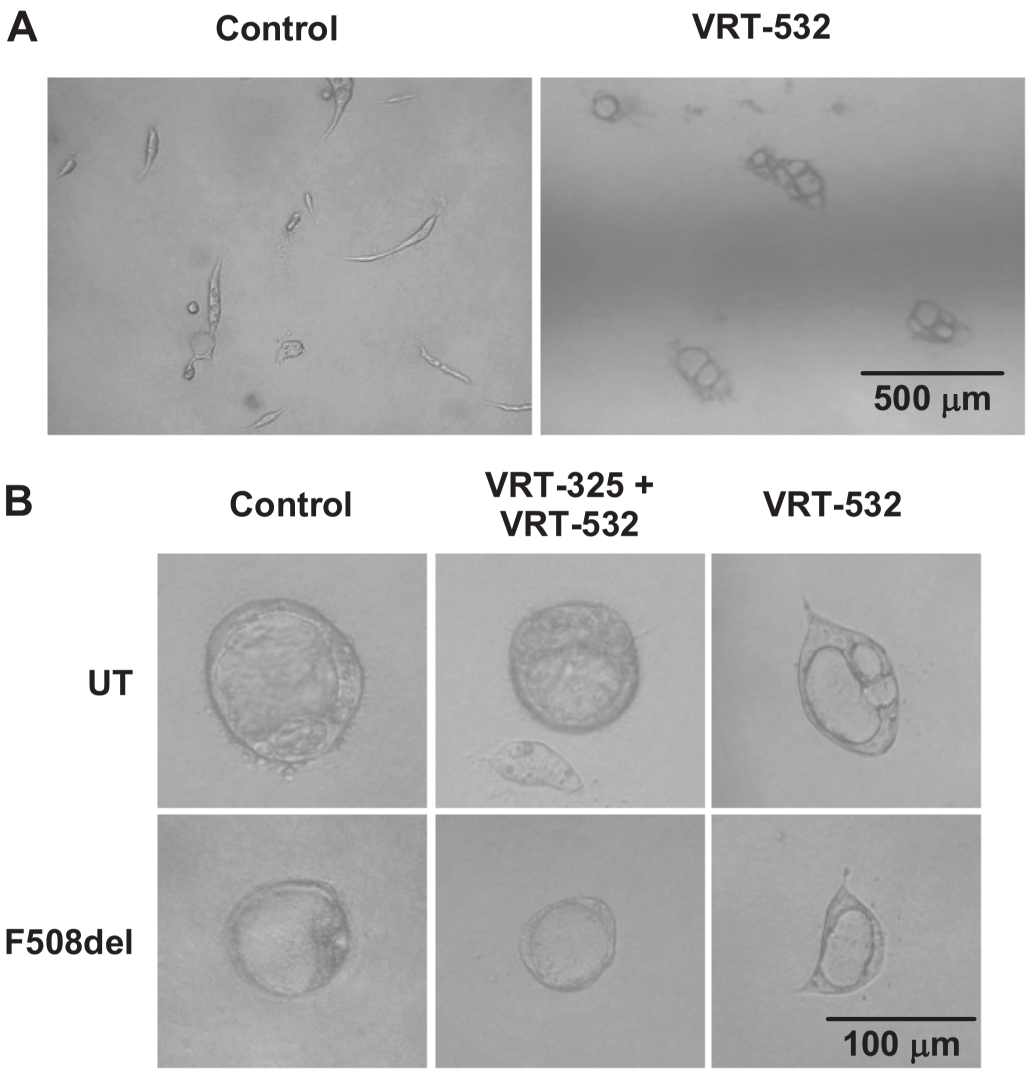

B
Untransfected
WT-CFTR
WT-CFTR + C172 from Day 0
WT-CFTR + C172 from Day 6
F508del-CFTR
Cyst Volume (mm3)
0
1
2
3
4
5
6
8
10
12
Time (Day)
**
##
##
**

Fig. 3


A
Control
VRT-532
500 μm
B
Control
VRT-325 + VRT-532
VRT-532
UT
F508del
100 μm

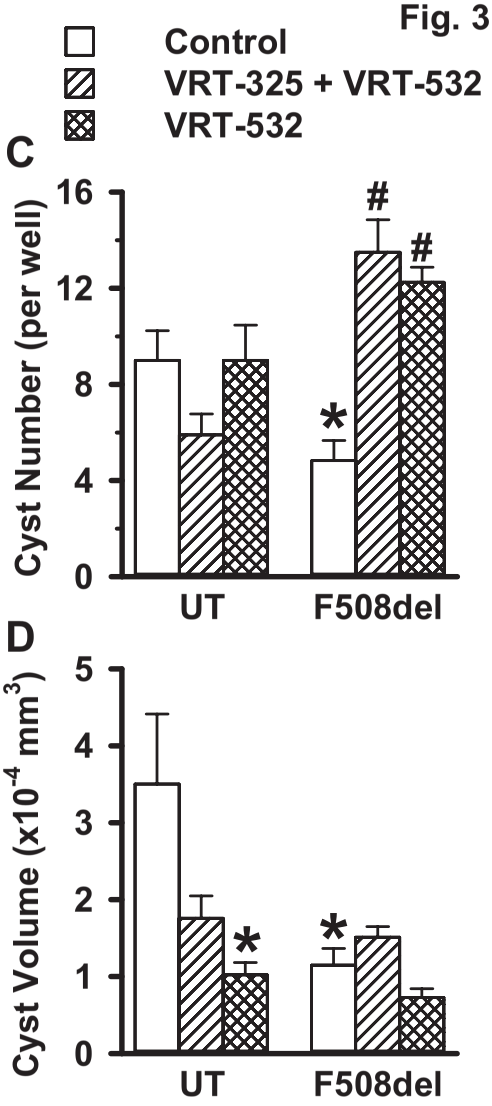
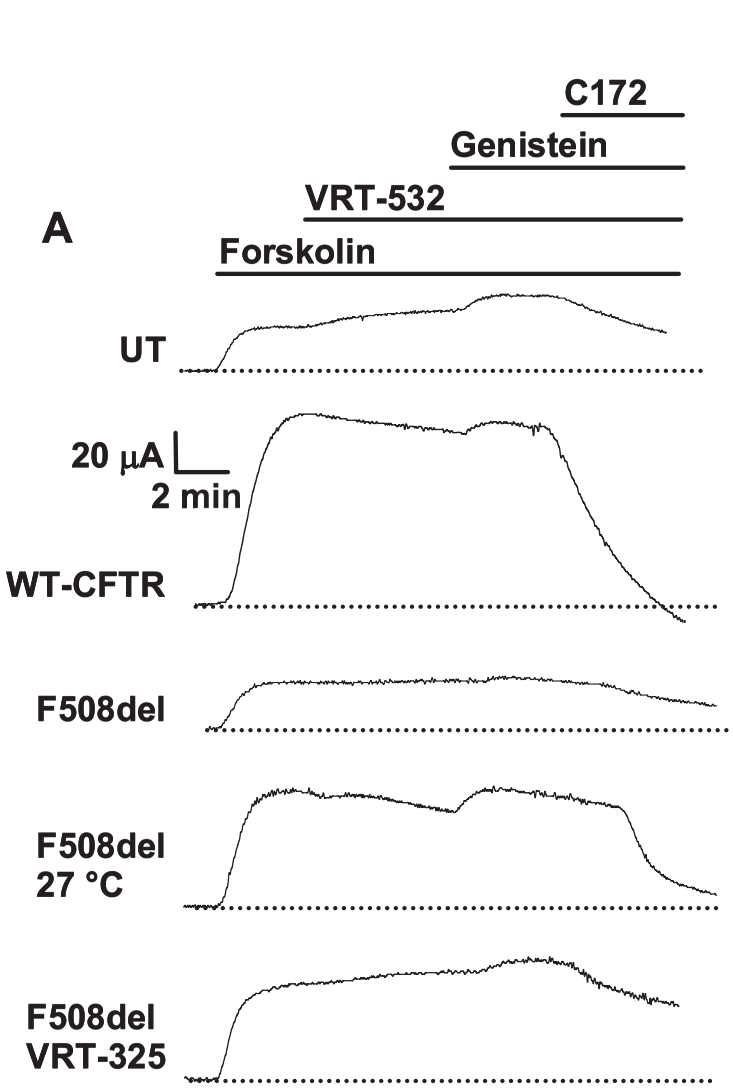

Control
VRT-325 + VRT-532
VRT-532
C
Cyst Number (per well)
16
12
8
4
0
#
#
*
UT
F508del
D
Cyst Volume (x10^{-4} mm^3)
5
4
3
2
1
0
*
*
UT
F508del

Figure 4


A
C172
Genistein
VRT-532
Forskolin
UT
20 μA
2 min
WT-CFTR
F508del
F508del
27 °C
F508del
VRT-325

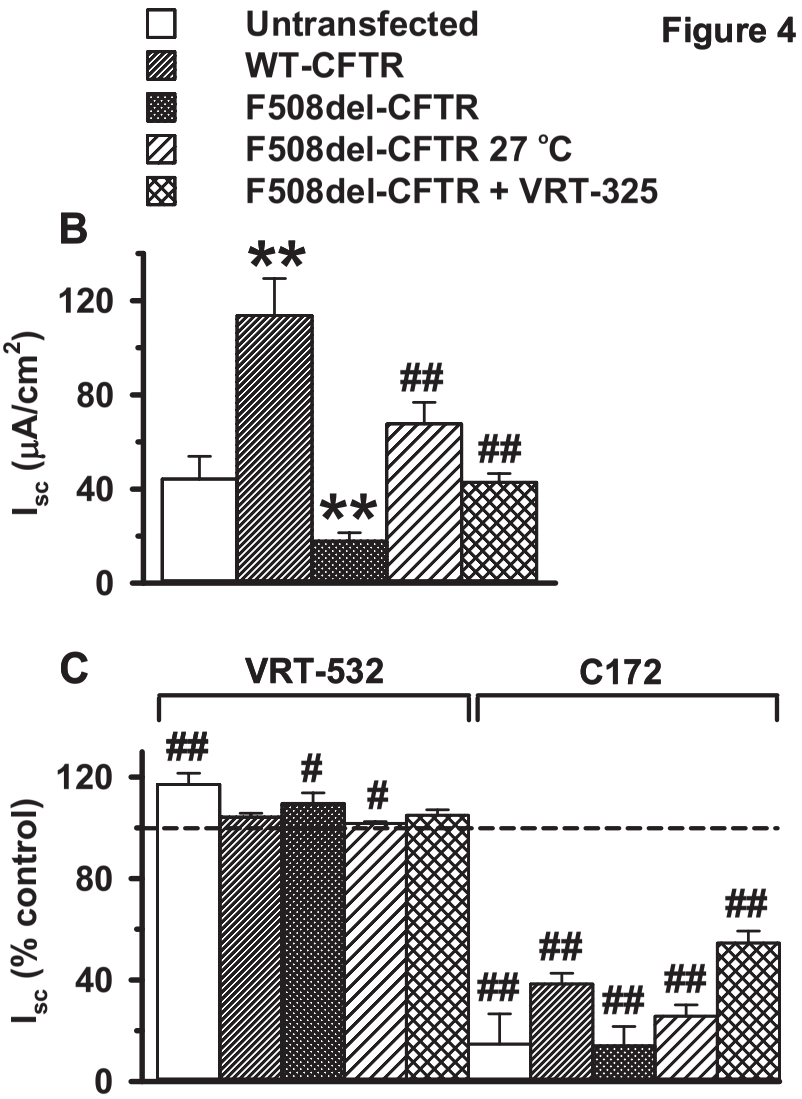
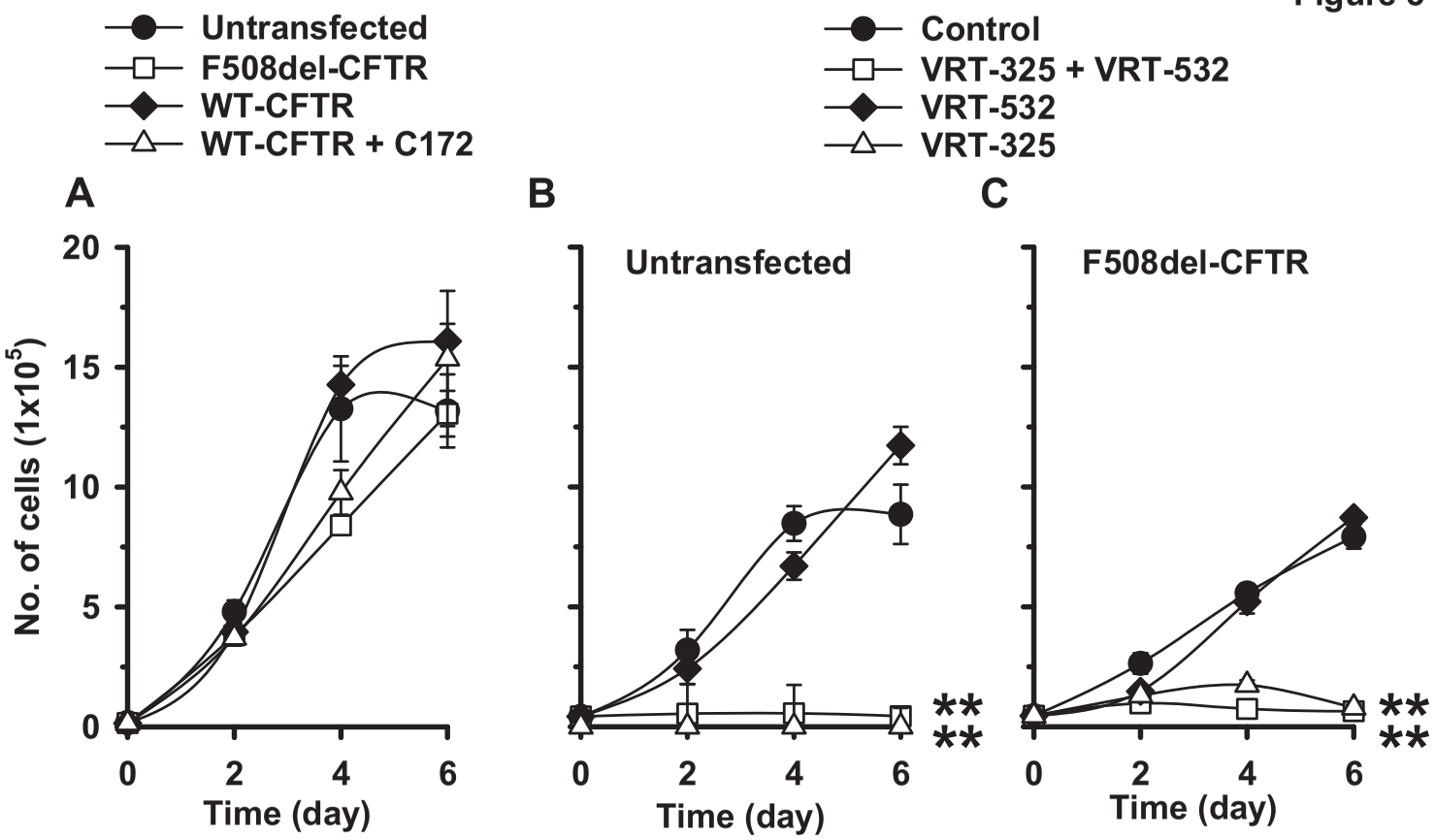

Untransfected
WT-CFTR
F508del-CFTR
F508del-CFTR 27 °C
F508del-CFTR + VRT-325
B
I_{sc} (μA/cm^2)
120
80
40
0
**
**
##
##
C
VRT-532
C172
I_{sc} (% control)
120
80
40
0
##
#
#
##
##
##
##
##

Figure 5


Untransfected
F508del-CFTR
WT-CFTR
WT-CFTR + C172
Control
VRT-325 + VRT-532
VRT-532
VRT-325
A
B
C
No. of cells (1x10^5)
20
15
10
5
0
0
2
4
6
Time (day)
Untransfected
F508del-CFTR
**
**