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Src kinase inhibition reduces inflammatory and cytoskeletal changes in F508 human cholangiocytes and improves CFTR correctors efficacy

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Abstract

CFTR, the channel mutated in cystic fibrosis (CF), is expressed by the biliary epithelium (i.e. cholangiocytes) of the liver. Progressive clinical liver disease (CFLD) occurs in about 10% of CF patients and represents the third leading cause of death. Impaired secretion and inflammation contribute to CFLD, however the lack of human-derived experimental models has hampered the understanding of CFLD pathophysiology and the search for a cure. We have investigated the cellular mechanisms altered in human CF cholangiocytes using induced pluripotent stem cells (iPSC) derived from healthy controls and a F508 CFTR patient. We have devised a novel protocol for the differentiation of human iPSC into polarized monolayers of cholangiocytes. Our results show that iPSC-cholangiocytes reproduced the polarity and the secretory function of the biliary epithelium. PKA/c-AMP mediated fluid secretion was impaired in F508 cholangiocytes and negligibly improved by VX-770 and VX-809, two small molecule drugs used to correct and potentiate F508 CFTR. Moreover, F508 cholangiocytes showed increased phosphorylation of Src kinase and TLR4 and pro-inflammatory changes including increased NF- κ B activation, secretion of pro-inflammatory chemokines (i.e MCP-1 and IL8), as well as alterations of the F-actin cytoskeleton. Treatment with Src inhibitor (PP2) decreased the inflammatory changes and improved cytoskeletal defects. Inhibition of Src along with administration of VX-770 and VX-809 successfully restored fluid secretion to normal levels. **Conclusions:** Our findings have strong translational potential and indicate that targeting Src kinase and decreasing inflammation may increase the efficacy of pharmacological therapies aimed at correcting the basic F508 defect in CF liver patients. These studies also demonstrate the promise of applying iPSC technology in modeling human cholangiopathies.

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Keywords

Cystic fibrosis liver disease; induced pluripotent stem cells; cytokines; F-actin; secretion

Cystic fibrosis (CF) is an autosomal recessive multisystemic disease caused by mutations in the gene coding for the CF transmembrane conductance regulator (CFTR), a cAMP-dependent Cl^- channel expressed at the apical membrane of secretory epithelia, including the biliary epithelium (1–3). Around 30% of CF patients present liver abnormalities (CFLD) and in about 10% a chronic cholangiopathy progresses into sclerosing cholangitis and eventually focal biliary cirrhosis and liver transplantation (4, 5). Improved care of lung disease has significantly increased the life expectancy in CF patients, and now CFLD represents the third cause of death in CF (6).

In the biliary epithelium, CFTR promotes the transmembrane efflux of chloride and bicarbonate and bile secretion in response to cAMP/PKA signaling(7). The pathogenesis of CF-associated liver disease (CFLD) is still unclear and there is no specific genotype/phenotype correlation. In general, CFLD patients harbor severe mutations that are mostly associated with absent or decreased CFTR at the membrane and decreased secretion of HCO_3^- (8, 9). About two third of CF patients carry a deletion of a phenylalanine in position 508 (F508), that affects CFTR function by preventing its trafficking to the plasma membrane, as well as by causing a gating defect and decreased CFTR half-life when rescued (10). F508 patients have a higher risk of developing CFLD (11).

A cure for CFLD is not available and patients are routinely treated with ursodeoxycholic acid (UDCA) in the hope to increase bile flow, known to be reduced in CF and to decrease bile acid toxicity (12). Novel agents acting on the F508 mutation and able to increase CFTR transport to the membrane (correctors) and to increase the activity of the rescued CFTR (potentiators) are being used to treat human lung disease, but there is no data regarding their use in CFLD (13).

The prospects for curative treatments are hampered by a lack of clear understanding of the pathogenesis of CFLD. The classic hypothesis that reduced CFTR causes ductal cholestasis and therefore biliary damage, has been challenged by recent studies in CFTR knock-out mice (CFTR-KO), showing that, in addition to its secretory functions, CFTR is critical to control the innate immune properties of biliary cells and the structural polarity of the epithelium (14, 15). At the apical membrane of the cell, CFTR assembles a protein complex able to maintain Src tyrosine kinase in an inactive state. In CFTR-KO cells this protein complex does not assemble, Src self-activates and phosphorylates toll-like receptor TLR4. Phosphorylation enhances the activity of TLR4 in response to endogenous or exogenous ligands (i.e LPS). As a result, NF- κ B activity increases, instructing the epithelium to secrete a number of proinflammatory cytokines able to attract inflammatory cells and damage the bile ducts. Furthermore, the increased inflammatory status of CFTR-KO cholangiocytes affects the organization of F-actin cytoskeleton and the cell junctional complexes impairing the barrier function of the epithelium.

Inhibition of Src or NF- κ B and the use of PPAR γ agonists *in vivo* ameliorates liver damage in CFTR-KO mice, indicating that this is indeed a disease-relevant mechanism (15). Whether this mechanism is relevant for the human disease it is unknown. Therefore, studies in a human cellular model are necessary to validate the findings and to exploit their therapeutic implications.

In this study, we took advantage of iPSC technology to generate a well-differentiated human cholangiocytes model bearing the F508 defect suitable to study the pathophysiologic mechanisms responsible for cholangiocyte dysfunction that might be therapeutically targeted and translated into clinical applications. We have used this model to address the role of CFTR in innate immunity and fluid secretion and to study how novel therapeutic molecules affect epithelial functions in CF. The results strongly advocate for the value of multi-target combination treatments in CF.

Materials and Methods

Reagents and additional methods are detailed in the supplemental materials and methods.

Human iPSC (hiPSC) generation and culture

Control iPSC were obtained from the Coriell cell repository and from Dr. Flora Vaccarino, Yale University. These lines were generated using the classical retroviral approach through the ectopic expression of the canonical transcription factors Oct4, Klf4, Sox2 and cMyc (16, 17).

F508-iPSC were a gift of Dr. Laura E. Niklason, Yale University and were generated in the laboratory of Dr. Darrel Kotton, Boston University by a viral free reprogramming method using a single excisable polycistronic lentiviral Stem Cell Cassette (STEMCCA) encoding the Yamanaka factors (18).

All hiPSC lines were maintained in defined culture conditions on Matrigel (Corning Inc., Corning, NY) coated plates in mTEsR media (STEMCELL Technologies Inc., Vancouver, Canada).

Differentiation of cholangiocytes from iPSC and expansion

A detailed description of the protocol is reported in the Supplementary Material and Methods section.

Polarization of iPSC-cholangiocytes and measurement of trans-epithelial resistance (TER)

To obtain a polarized monolayer either hepatoblasts or iPSC-cholangiocytes in expansion were seeded on top of collagen coated transwell inserts with a 0.4 μ m pore semipermeable membrane (Corning Inc.) and the same media was applied apical and basolateral. In this condition cells grow as a polarized monolayer accessible from both the apical and basolateral domain distinctly. Establishment of a confluent monolayer was routinely checked, measuring transepithelial resistance (Millicell ERS System; EMD Millipore, Billerica, MA). Cells were confluent and ready for further experiments when transepithelial resistance was $>1000 \Omega \cdot \text{cm}^2$. Where indicated cells were treated with VX-770 and VX-809

(10uM) (Selleck Chemicals LLC, Houston, TX) for 24 hours and PP2 (1uM) (EMD Millipore) for 2 hours.

Animals

All procedures involving animals in this study were approved by the Yale University Institutional Animal Care and Use Committee according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). F508-CFTR (B6-129-Cfr^{tm1Kth}) (19) mice with targeted mutation corresponding to the F508 mutation in human, were used for the isolation of primary cholangiocytes cell lines. Animals were bred in our facility and were maintained as previously described (14, 20, 21).

Immunofluorescence and confocal microscopy

Mouse and human cholangiocytes were fixed with either a) 1:1 methanol/acetone solution and washed with PBS; b) or with 4% PFA, washed with PBS/10mM glycine, and permeabilized with PBS/TritonX-100 0.2%. Unspecific binding sites were blocked with Protein Block Serum-Free solution (Dako, Agilent Technologies, Santa Clara, CA) for 1 hour at RT. Cells were then incubated overnight at 4°C with specific primary antibody (see supplementary table 1) and with the proper secondary antibody for 1 hour at RT. Cells were then mounted using a Vectashield Kit (Vector Laboratories, Burlingame, CA) with 4',6-diamidino-2-phenylindole (DAPI). Confocal analysis was performed using a Zeiss LSM 710 Duo confocal microscope or a Zeiss LSM510 Meta.

Determination of cytokine secretion

Polarized iPSC-derived cholangiocytes were untreated or treated with PP2 (1 μM), LPS (10 ng/ml) (Sigma, St Louis, MO) or their combination for 24 h. At the end of the treatment, apical media were collected and processed for MCP-1 and IL8 quantification analysis by using the Milliplex[®] MAP Human Cytokine/Chemokine Magnetic Bead Panel (EMD Millipore) coupled with BioPlex Luminex platform (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions. Data were normalized for the cell protein content as previously described (14).

Measurement of Apical Surface Liquid (ASL)—Fluid level at the apical membrane of confluent human and mouse cholangiocyte monolayers was quantified according to a modified technique previously described (22). Briefly, confluent monolayers of cholangiocytes were washed with PBS and labeled with CellMask[™] Orange Plasma membrane Stain (Invitrogen, Life Technologies, Carlsbad, CA) (1:1000) for 10–15 minutes at 37°C. To label the ASL, 20ul KRB containing 0.5 mg/ml FITC-dextran (40KDa) was added to the apical surface of the monolayer and 100 ul Fluorinert FC-770 (Sigma) was layered on the top to prevent evaporation. Transwells were then placed on the surface of a glass-bottomed MatTek 35mm dish (MatTek Corp, Ashland, MA) over a serosal reservoir of 80 ul (KRB) and left in the incubator for 30 minutes. A mix of forskolin (10uM) and IBMX (100 uM, Sigma) were dissolved in the serosal reservoir and in the dextrans mixture where indicated. Transwells were then mounted in an incubator that maintains 37°C and 5% CO₂

on the stage of a Zeiss LSM 710 Duo confocal microscope and images acquired with the Zeiss AIM software. Samples were imaged using a 40× water immersion lens. XZ high-resolution images (512×96 pixels) were acquired from different points of each transwell using the same electronic gain, offset, and laser powers. The surface area covered by dextrans (square pixels) was measured by ImageJ software by applying a fluorescence threshold to obtain the average ASL in different conditions.

Statistical analysis—Data are shown as means $\pm$ SD and compared using Student's t test, or ANOVA with repeated measures when appropriate. All tests were two-sided. Statistical analyses were carried out using GraphPad Prism. A P value of less than 0.05 was considered statistically significant.

Results

Successful differentiation of human iPSC into mature, polarized cholangiocytes optimized for pathophysiological and pharmacological applications

Recent studies have shown that it is possible to differentiate human iPSC cells into cholangiocyte-like cells (23–26). To use this technology as a *bona fide* disease model and a platform to conduct cell biology studies we have spent significant effort in optimizing several methodological aspects, to generate a competent epithelium, improve its functional polarity and produce sufficient amounts of cells to study (see material and methods).

Human iPSC lines were differentiated into biliary epithelial cells (cholangiocytes) by modifying the protocol published by Dianat et al. (24)(see methods). Briefly, iPSC were sequentially exposed to signals involved in liver development and “accompanied” through the following sequential steps: definitive endoderm (DE), hepatic endoderm (HE) and hepatoblasts (HB), i.e. the common progenitors of hepatocytes and cholangiocytes. A schematic representation of the protocol is shown in Fig. 1A. Using this protocol, we were able to reproducibly differentiate iPSC into cholangiocytes with high efficiency. By immunohistochemistry (Fig. 1B), iPSC-derived cholangiocytes expressed specific biliary markers such as CK19, CK7, HNF1 β , SOX9 and the NOTCH2 intracellular domain (NICD) and were also positive for the sodium-dependent bile acid transporter (ASBT) and gamma-glutamyltransferase (GGT). Gene expression of functional cholangiocyte markers, such as CFTR and the membrane-bound bile acid receptor TGR5 was also present, whereas markers typical of hepatoblast such as HNF4 α and AFP were down-regulated (Fig. 1C).

The apico-basal polarity is a key feature of a mature biliary epithelium that deeply influences its function; therefore we optimized culture conditions to enable the establishment of polarized monolayers. To achieve this goal iPSC at the stage of hepatoblast differentiation were plated on top of collagen coated transwell inserts before inducing cholangiocytes specification. Cholangiocyte monolayers obtained under these conditions acquired the characteristics of a tight epithelium with a competent trans-epithelial resistance (TER) $> 1000 \Omega\cdot\text{cm}^2$ (Supplementary Fig.1). The localization of the tight junction marker ZO-1 at the apical cell junction, the presence of primary cilia on the apical membrane (Fig. 2A) and the basolateral localization of functional secretin receptors (Fig. 2B)(see below)

confirmed the differentiation of iPSC-derived liver cells into mature cholangiocytes (iPSC-cholangiocytes) that acquire apico-basal polarity when cultured as a monolayer.

Cyclic AMP (c-AMP) signaling in response to the hormone secretin that binds its receptor at the basolateral membrane represents the main pathway regulating Cl^- and HCO_3^- secretion in biliary cells(27, 28). To assess the functional maturation of polarized iPSC-cholangiocytes we evaluated their intracellular cAMP production in response to secretin exposure; forskolin (10uM) was used as a positive control (Fig. 2B). Consistent with basolateral expression of functional secretin receptor, stimulation of cholangiocytes with secretin (50nM and 100nM) lead to a dose-dependent significant increase in intracellular cAMP levels when administered from the basolateral but not from the apical side.

The use of iPSC-cholangiocytes for cell biology studies requires the availability of large number of cells. Starting a new differentiation from iPSC for every experiment would be time consuming, expensive and subject to significant variability. To solve these issues we devised a method that allowed us to subculture and expand iPSC-cholangiocytes for up to ten passages (see methods).

At the end of the differentiation period cholangiocytes were harvested and plated on collagen-coated flasks and supplemented with an enriched media (see material and methods for details). As shown by immunofluorescence and real-time qPCR these cells maintained specific biliary markers expression at the same or higher level compared to the iPSC at the end of the differentiation (Fig. 3A,B).

Having optimized the protocol and culture conditions in healthy controls, we then differentiated iPSC-cholangiocytes obtained from a patient carrying the most common CFTR mutation F508 in homozygosis (Supplementary Fig. 2). As expected, both control and mutated cholangiocytes expressed CFTR at the mRNA level but in the case of F508 cholangiocytes, the glycosylated mature form of the protein was not detected by western blot (Fig. 4A). Functional and morphological characterization of F508 cholangiocytes was similar to the one above described for normal cholangiocytes as also shown by similar gene and protein levels of expression of SCRT and AE2 (Fig. 4A).

cAMP-stimulated secretion is defective in human cholangiocytes derived from F508-iPSCs

We quantified the cAMP-stimulated secretory function in normal and mutated cells by apical surface liquid measurement (ASL) (see methods section for further details). To define the apical surface of the epithelium, human cholangiocytes cultured as polarized monolayers were visualized with the membrane dye CellMask™, while fluorescent dextrans (40KDa) were applied on top of the monolayers. Following stimulation with forskolin (10 uM) in the presence of the phosphatase inhibitor IBMX (100 uM) for 30 minutes, cells were live-imaged by confocal microscopy and serial z-stack images were acquired. The threshold area of the fluorescent dextrans on top of the epithelium was used as a parameter to quantify fluid secretion (Fig. 4B). As shown in Fig. 4C, treatment with forskolin significantly increased apical secretion of fluid in control iPSC-cholangiocytes, but not in cholangiocytes from the F508 iPSC, consistent with the expected secretory defect.

Pro-inflammatory changes in F508-mutated human cholangiocytes

Using a full knock-out rodent model for CFTR (CFTR^{tm1UNC}, CFTR-KO), we have recently showed that CF cholangiocytes respond to LPS with increased activation of TLR4/NF- κ B pathway and excess production of pro-inflammatory cytokines as result of Src tyrosine kinase activation (14, 15). However, it is at present unclear if these findings can be translated to humans and to patients with the F508 mutation.

Here we show that in human F508 cholangiocytes these changes are already quite evident at baseline, before challenging cells with LPS. In fact, NF- κ B activity measured using a reporter gene assay (14), was significantly higher in F508 cholangiocytes, with respect to control cells, (Fig. 5A). In addition, secretion of MCP-1 and IL-8, chemoattractants for pro-inflammatory monocytes and neutrophils were also significantly increased at baseline with respect to control cholangiocytes (Fig. 5B). Having shown increased NF- κ B activity and cytokine secretion in human F508 cholangiocytes we focused on the events upstream. Western blot analysis showed that TLR4 phosphorylation at the tyrosine 674 is significantly increased in F508 cholangiocytes, as compared to normal cholangiocytes (Fig. 5C). Furthermore, Src activity, measured as phosphorylation at the tyrosine 419 residue of the catalytic domain is also increased in human cholangiocytes bearing the F508 mutation, with respect to cholangiocytes normally expressing CFTR at the plasma membrane (Fig. 5D). Next, we exposed control and F508 cholangiocytes to LPS (10ng/ml) and we measured NF- κ B activation 6 hours later and MCP-1 and IL-8 secretion 24 hours later. As reported in Fig. 5A,B, LPS treatment further increased significantly NF- κ B activation and cytokines secretion in F508 cholangiocytes.

To determine if Src activation was upstream to the pro-inflammatory changes identified in F508 human cholangiocytes we repeated the experiments in the presence of the Src inhibitor PP2 (1 μ M). The significant decrease in NF- κ B activation and in cytokine secretion after treatment with PP2 confirmed the pathophysiological role of Src in the inflammatory phenotype displayed by F508 human cholangiocytes (Fig. 5A,B).

The distribution of actin cytoskeleton is altered in F508 cholangiocytes

Changes in cytoskeleton have been reported in a number of cells and tissues in CF (15, 29, 30). Working with CFTR-KO mice that completely lack CFTR, we suggested that the destabilization of F-actin fibers was a consequence of the increased activity of the Src kinase/TLR4/NF- κ B pathway (15). To study the integrity of the F-actin cytoskeleton in human F508 cholangiocytes and controls we analyzed phalloidin staining in polarized monolayers after fixation. Immunofluorescence revealed significant differences in F-actin distribution between control and F508 cholangiocytes. In control cells F-actin was distributed at the area of apical membrane and cell-cell junctions, where normally the subcortical actin ring co-localizes closely with cell-cell junctions. On the contrary, in F508 cells a large amount of F-actin was de-localized and diffused in the cytoplasm significantly distorting the polygonal shape of the cells in the monolayer (Fig. 6A). Interestingly, short-time treatment with the Src inhibitor PP2 (1 μ M) was able to recover the altered distribution of F-actin in human F508 cells, indicating that changes in actin are a consequence of activation of Src kinase/TLR4/NF- κ B pathway (Fig. 6B).

The same defect was present also in primary cholangiocytes isolated from F508-CFTR mice (B6-129-Cftr^{tm1Kth}) that bear a targeted mutation corresponding to the F508 mutation in human (Supplementary Fig. 3).

Src inhibition increases the efficacy of VX-770 and VX-809 in rescuing the F508 defect in cholangiocytes

The F508 mutation causes multiple structural defects in CFTR. As a consequence, not only the majority of the protein is unable to reach the membrane, but also the stability of the

F508-CFTR that it is able to arrive at the cell surface is decreased (31). The search for a pharmacological therapy able to correct the F508 mutation has produced two small molecules (VX-770 and VX-809) recently approved by FDA (32, 33).

VX-770 is a “potentiator” of the channel activity, while VX-809 works as a “corrector” allowing the F508 CFTR to bypass proteomic degradation and increasing the trafficking of the protein to the plasma membrane. Unfortunately, *in vitro* studies (34) and recent clinical data (35, 36) show that the efficacy of these drugs may be decreased in long-term treatments, because the half-life of the rescued CFTR at the plasma membrane is lower than that of wild-type CFTR.

Thus, we tested the ability of VX-770 and VX-809 to recover the secretory function in iPSC-cholangiocytes bearing the F508 mutation by measuring apical fluid secretion in polarized monolayers as described above. As shown in Fig. 7B,C there was a moderate increase in forskolin-stimulated fluid secretion after treatment with the correctors in F508 human cholangiocytes, however secretion remained 80% less than that of normal human cholangiocytes. We also found a similar low response in F508 mouse cholangiocytes (Supplementary Fig. 3).

By western blot analysis we confirmed that the mature CFTR form was expressed in F508 human cholangiocytes after treatment with VX-770 and VX-890 (see Fig. 7A) suggesting that the small effect of treatment on fluid secretion is likely related to a predicted instability of F508 CFTR at the membrane. The integrity and organization of the F-actin cytoskeleton is known to be necessary for CFTR retention at the plasma membrane and its association in multiprotein membrane complexes (37). Therefore, we reasoned that the normalization of F-actin distribution could have a “stabilizing” effect on the retention of the rescued F508 CFTR at the membrane and would possibly improve the efficacy of the VX-770 and VX-809.

To this aim we tested the effect of Src inhibition in combination with VX-770 and VX-890 in F508 cholangiocytes on actin microfilaments distribution and fluid secretion. We treated polarized cholangiocytes with VX770 and VX-809 (10 uM) for 24h with or without PP2 (1uM) that was administered two hours before analysis. As shown by phalloidin staining in Fig. 6A, F-actin organization partially improved after treatment with VX-770 and VX-809 (probably as an effect of more CFTR reaching the apical membrane), but did not fully normalize, unless the Src inhibitor PP2 was administered in combination with VX-770 and VX-809. We demonstrated the same effect also in cholangiocytes isolated from mice carrying the mutation F508 (Supplementary Fig. 3). As we predicted, in cells treated with

the combination of correctors and PP2, secretion was significantly improved reaching levels comparable to normal cells (Fig. 4C and 7B,C). Fluid secretion did not improve after treatment with PP2 alone (Fig. 7C), suggesting that PP2 does not affect the residual activity of F508 CFTR that escaping the ER checkpoint eventually arrives at the membrane. The treatment with the combination of correctors and PP2 in F508 cholangiocytes significantly increases the amount of the glycosylated mature form of F508-CFTR. In addition, PP2 alone has a synergistic effect on forskolin stimulated fluid secretion in control cells (Supplementary Fig.4). Altogether these data suggest that normalizing F-actin by inhibition of Src kinase improves and potentiates the efficiency of the small molecules, most likely by increasing the stability of the rescued F508 at the membrane or by decreasing the recycling of CFTR from the membrane.

Discussion

The recently discovered induced pluripotent stem cells (iPSCs) technology provides a source of cells derived from patients that can be differentiated in the somatic cell of interest. iPSC-derived cells retain the genetic background of the donor, making them a suitable tool for disease modeling (38, 39). Recent studies have shown that it is possible to differentiate patient-derived iPSC into liver epithelial cells and that these cells reproduce phenotypic features of the genetic disease of origin (25, 26, 40). This is of great importance in CFLD, because the lack of a valuable human model has hampered the research in the field. We have generated a modified protocol for the differentiation of iPSC into cholangiocytes able to overcome some of the barriers to the routine use of this technology for cell biology studies, and applied this system to model CFLD *in vitro*.

The pathogenesis of CFLD is unclear and although it has been associated with the lack of CFTR function and of biliary bicarbonate secretion leading to ductal cholestasis, several open questions remain (41). Our recent studies using a CFTR-KO mouse model have shown that altered TLR/NF- κ B-dependent innate immune mechanisms and abnormalities of the cytoskeletal biliary cell structure could contribute to the pathogenesis of CFLD and suggest that an attempt to restore only the secretory functions of the biliary epithelium may not be curative in itself (14, 15). However, CF rodent models do not fully reproduce the human disease, thus we decided to use cholangiocytes generated from iPSC derived from a CF patient to model the cystic fibrosis, to study if our previous findings in rodents apply also to humans and to expand their translational implications.

To this aim we devised an improved step-wise protocol that recapitulates the different stages of biliary development and allows us to culture and expand the iPSC-derived cholangiocytes. Our protocol presents two important improvements, compared to previously published studies. Notably, we were able to generate a polarized and functionally competent monolayer of cholangiocytes as confirmed by the specific expression of markers, the receptor-mediated response to secretin and the acquisition of a trans-epithelial resistance and polarized secretion. Culture in 2D polarized conditions has the advantage to preserve the functional polarity of the epithelium while overcomes the difficult experimental accessibility of the apical domain in 3D organoid cultures. In addition, we were able to sub-culture and

expand our differentiated cholangiocytes, thus reducing the experimental cost and the variability of multiple differentiations.

We have used our protocol to model *in vitro* human CFLD and to validate in human cholangiocytes bearing the most prevalent pathogenetic mutation (F508) the changes in innate immunity described in a CFTR-KO mouse model. There is still no correlation between the liver complications and specific CFTR mutations, but it is recognized that liver disease progresses in patients with more severe mutations mainly associated with a loss of CFTR at the membrane (8). Thus, we used iPSC derived from a CF patient homozygous for the CFTR class II mutation F508, the most common mutation in CF. This mutation is caused by the deletion of three nucleotides in position 508 of the CFTR gene on chromosome 7, and produces an abnormal CFTR protein that is not properly folded and is unable to leave the endoplasmic reticulum for further processing. Therefore the F508 mutation prevents CFTR from reaching the apical membrane. A small percentage of the protein is still able to escape the ER checkpoint but is not functional because of a gating defect and decreased half-life at the membrane (10).

We first confirmed that the mature form of CFTR was undetectable in human iPSC-derived F508 cholangiocytes and that fluid secretion in response to increased intracellular cAMP was absent. Then, we tested the response to currently used therapeutic compounds. The FDA has recently approved a treatment to correct the F508 defect in CF patients, based on the combination of a “potentiator” of the CFTR gating function (VX-770) and a “corrector” that allows CFTR to bypass proteomic degradation, thereby increasing its trafficking to the plasma membrane (VX-809) (33). Since no clinical data are available for CF liver patients with F508, we tested their ability to correct the F508 defect and rescue the impaired fluid secretion in iPSC-derived F508-cholangiocytes. Although the treatment with combined VX-770 and VX-809 increased significantly the expression of the mature CFTR protein as expected based on their pharmacological properties, their effect on c-AMP-stimulated CFTR-dependent fluid secretion was small and fluid secretion improved only by 20%, as compared to values in control cells.

However, total amount of cellular CFTR protein does not predict the actual level of protein present at the membrane. Indeed, our results on fluid secretion in human F508 cholangiocytes are consistent with recent clinical reports on lung function from F508 patients treated with the small molecules. Despite the encouraging results during phase III of the study, the actual efficacy of the new molecules in clinic was not impressive because of the instability and reduced half-life of the rescued CFTR at the plasma membrane. The reason for the low efficacy has been attributed to the inflammatory status of the patients or to a negative interference of the drugs combination in long-term treatments (34, 42).

Interestingly, we found that human F508 cholangiocytes are characterized by an increased basal NF- κ B activation and actively secrete pro-inflammatory chemokines known to attract neutrophils (i.e MCP-1 and IL-8). Similar to our previous findings in CFTR-KO mice, human F508 cholangiocytes show increased responsiveness to endotoxins compared to cholangiocytes control, as well as an increased Src tyrosine kinase activation and TLR4 phosphorylation. These data imply that in CF liver disease there is a cell autonomous “auto

inflammatory” component as result of an aberrant activation of the innate immune system. We have also shown that direct inhibition of Src or NF- κ B (through PPAR γ agonists) improves liver damage in a rodent model of CFLD (15, 43).

In addition to increased secretion of pro-inflammatory cytokines, we have observed the presence of structural alterations of the subcortical F-actin distribution in iPSC-derived cholangiocytes bearing the F508 mutation as well as in F508 mouse cholangiocytes. In polarized epithelia, actin filaments at the apical domain form an organized circumferential belt that connects the cell junctions. Actin distribution improved after Src inhibition in human F508 cholangiocytes, suggesting that the defect is secondary to Src activation as a consequence of the inflammation. Indeed, several studies have documented that inflammatory mediators can induce a disassembly of the F-actin belt and cause the relocation of junction proteins into the cytosol, therefore affecting the epithelial barrier function. Intriguingly, a similar phenotype has been reported also in a human airway immortalized CF cell line expressing F508-CFTR (29). In physiologic conditions, the integrity of the actin cytoskeleton is critical for CFTR tethering at the plasma membrane (44, 45). Thus, we hypothesized that the altered distribution of subcortical cytoskeleton caused by the inflammatory responses of the biliary epithelium might decrease the stability of the rescued CFTR at the membrane and limit the efficacy of VX-770 and VX-809.

Because inhibition of Src tyrosine kinase reduces NF- κ B activation and cytokine secretion and rescues F-actin distribution, if our hypothesis is correct, targeting Src should improve the efficacy of VX-770 and VX-809. Indeed, our study shows that treatment with a combination of CFTR potentiators/correctors and the Src inhibitor results in the restoration of the F-actin architecture and in a parallel drastic increase in fluid secretion in F508 cholangiocytes, both of human and mouse origin. Interestingly, Src inhibition also enhanced PKA stimulated fluid secretion in control cells. Further studies will clarify whether Src kinase plays a role in the mechanism of recycling of the channel at the membrane

In summary, we have developed an improved protocol for the generation of iPSC-derived human cholangiocytes in polarized monolayers. Our culture conditions allow cholangiocytes to reach high level of functional maturation while expanding their experimental applicability for modeling of biliary diseases and drug screening compared to 3D cultures. We have derived human cholangiocytes from a CF patient with the common homozygous mutation

F508 CFTR and used them to model the liver disease. Using iPSC-derived cholangiocytes, we have unveiled the pathophysiological mechanism that underlies cholangiocyte dysfunction in CF and showed that CFTR-defective biliary cells present inflammatory and cytoskeletal changes affecting the ability to respond to current treatments. Normalization of these changes by Src inhibition strongly improves the efficiency of the small molecules active with the F508 mutation. Our study has further proved that given the multiple functions of CFTR, its pharmacological therapy requires a combination of therapeutic approaches. Most likely these findings can be applied to other organs affected by CF, such as the airway epithelium and the pancreas.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of abbreviations

iPSC	induced pluripotent stem cells
CFTR	CF transmembrane conductance regulator
CF	cystic fibrosis
CFLD	CF-associated liver disease
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
TLR	toll-like receptor
Src	Rous sarcoma oncogene cellular homolog
TER	trans-epithelial resistance
WT	wild type
PP2	4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine
IBMX	3-isobutyl-1-methylxanthine
c-AMP	cyclic adenosine monophosphate
KRB	Krebs-Ringer Bicarbonate Buffer
LPS	Lipopolysaccharide
FITC	fluorescein isothiocyanate
MCP-1	Monocyte chemotactic protein 1
IL8	Interleukin 8
ZO-1	zona occludens-1

CK19	cytokeratin 19
CK7	cytokeratin 7
HNF1β	hepatocyte nuclear factor 1 homeobox B
SOX9	SRY-box 9
HNF4α	Hepatocyte nuclear factor 4 alpha
AFP	alpha fetoprotein
EGF	epidermal growth factor
HGF	hepatocyte growth factor
RA	retinoic acid
FGF4	fibroblast growth factor 4
HGH	human growth hormone
IL6	inteinterleukin-6
SCRT	Secretin receptor
AE2	Anion exchange protein 2

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Figure 1. Differentiation of cholangiocytes from human iPSC

(A) Schematic overview of the protocol used to differentiate cholangiocytes from human iPSC; bright field images of the sequential differentiation steps showing the changes in cell morphology. DE, definitive endoderm; HE, hepatic endoderm; HB, hepatoblasts; Chol, cholangiocytes. SR, KO-Serum replacement; EGF, epidermal growth factor; HGF, hepatocyte growth factor; RA, retinoic acid; FGF, fibroblast growth factor; IL6, interleukin-6. (B) Characterization by immunofluorescence shows the expression of biliary specification markers and the enzymatic activity for gamma-glutamyl transferase (GGT) at the end of the differentiation process (day 21). (C) Gene expression profile shows that iPSC-derived

cholangiocytes (day 21) express high levels of biliary specification markers compared to the hepatoblasts (day 12) while the hepatocyte marker HNF4 α and the progenitor marker AFP are significantly decreased. Bars represent means $\pm$ SD of n=3 independent differentiation experiments. *p<0.05, **p<0.01.

A

B

Figure 2. iPSC-cholangiocytes growing in monolayers develop functional apical/basal polarity (A) iPSC-derived cholangiocytes were cultured on a transwell insert from the stage of hepatoblasts and throughout the cholangiocyte specification (day 21). Immunofluorescence and confocal analysis show the presence of apical primary cilia (acetylated alpha-tubulin) in green and the expression of the protein ZO-1 (in red) restricted at the apical cell junctions confirming the acquisition of apical/basal polarity. (B) Z-stack section shows the basolateral expression of SCRT. Polarized cholangiocytes on transwells at the end of the differentiation (day 21) were left untreated or stimulated with secretin (50nM and 100nM) from the basolateral side or with secretin (50nM) from the apical side or with forskolin (10μM). At

the end of the treatment cells were lysed and intracellular cAMP levels were quantified by ELISA. A significant dose-dependent increase in cAMP after treatment with secretin only from the basolateral side is consistent with a functional receptor at the basolateral membrane. Forskolin, a broad activator of adenylyl cyclases was used as a positive control. Bars represent means $\pm$ SD of n=3–4 independent differentiation experiments. *p<0.05, **p<0.01 vs CTRL. Scale bar=10 μ m.

Figure 3. iPSC-derived cholangiocytes retain biliary epithelial marker when propagated in culture for several passages

iPSC-derived cholangiocytes at the end of the differentiation (day 21) were expanded and propagated in culture for up to 10 passages. (A) Bright field images show the epithelial morphology of cells at different passages up to 10 and the characterization by immunofluorescence confirms that sub-cultured iPSC-cholangiocytes preserve specific biliary and epithelial markers. (B) Gene expression profiling shows that specific markers of mature cholangiocytes are expressed at the same or higher level compared to the cholangiocytes at the end of the differentiation. * <0.05 , ** <0.01 vs iPSC-Chol at the end of the differentiation.

Figure 4. iPSC-derived cholangiocytes from a patient bearing the $\Delta F508$ CFTR mutation reproduce key features of cystic fibrosis

Cholangiocytes were differentiated from iPSC of a CF patient homozygous for the $\Delta F508$ CFTR mutation ($\Delta F508$ -chol) and of a healthy control (Control-chol). (A) Protein and gene expression levels for CFTR, SCRT and AE2 were quantified by western blot and RT-PCR in $\Delta F508$ -chol and Control-chol. Gene expressions were comparable between $\Delta F508$ -chol and Control-chol; as expected, Western blot analysis shows that the mature CFTR glycoform (band C) is not expressed in $\Delta F508$ -chol, while SCRT and AE2 protein are expressed at similar levels. Bars represent means $\pm$ SD of RT-PCR data from n=3 independent differentiation experiments. (B) Representative confocal z-stack section of human Control

and F508 iPSC-chol for ASL quantification showing in green the FITC dextrans on the top of the epithelial monolayer and in red the epithelial cells labeled with the cell membrane dye CellMask Orange. Fluorinert FC-770 was layered on the top of the dextrans to prevent evaporation during the experiment. ASL was quantified as threshold areas in pixels of the fluorescent dextrans. (C) Bar graph shows ASL measurements in Control and F508-chol. Fluid secretion is significantly increased in Control-chol after stimulation with forskolin (10 μ M) but not in F508-chol. Bars represent means $\pm$ SD of n=7–9 different monolayers from 3 independent experiments.

Figure 5. Src-kinase dependent pro-inflammatory changes are present in human F508-chol
(A) NF- κ B activity was measured by Luciferin/Luciferase reporter gene assay in human iPSC cholangiocytes in control conditions and after stimulation with LPS (10ng/ml) for 6 hrs. Basal NF- κ B activity was significantly elevated in F508-chol as compared to controls and further increased after stimulation with LPS. NF- κ B activity in F508-chol was significantly decreased by treatment with a Src kinase inhibitor (PP2, 1 μ M). Bar graph represents mean $\pm$ SD of the relative Luciferase activity in n=3 different experiments repeated in triplicate; *p<0.05, **p<0.01. **(B)** MCP-1 and IL-8 concentration was determined by Luminex analysis in the apical medium of polarized human iPSC

cholangiocytes. Values were normalized for total protein content. Both MCP-1 and IL-8 were highly secreted by F508-chol compared to controls. Secretion was significantly increased by treatment with LPS for 24 hrs. Treatment with PP2 (1 μ M) significantly decreased their concentration in F508-chol. Bar graphs represent mean $\pm$ SD of n=3 different experiments repeated in triplicate; **p<0.01 vs Ctrl-chol; Ctrl, ##p<0.01 vs F508-chol Ctrl; p<0.05 vs Ctrl-chol LPS, ^p<0.01 vs F508-chol LPS. (C) TLR4 tyrosine (Y674) phosphorylation was determined by Western blot. Bar graph represents the ratio between p-TLR4 (pY674) and total TLR4. Tyrosine phosphorylation of TLR4 is significantly increased in F508-chol. *p<0.05. (D) Src activity was determined by Western blot using an antibody for Src (pY418). Bar graph represents the optical density ratio of p-Src (pY418)/ total Src. F508-chol show an increased amount of p-Src compared to Control-chol. *p<0.05.

Figure 6. Effect of PP2 and F508 CFTR correcting drugs on the altered F-actin organization in F508 human cholangiocytes

Distribution of F-actin cytoskeleton in polarized human iPSC cholangiocytes was analyzed by phalloidin staining followed by quantification of the fluorescence intensity along a 30 μm line traced across the cell (see inset in Control-chol). Fluorescence intensity was measured in 10 cells per field and in 3 fields per sample using ImageJ software, NIH. (A) Confocal imaging of phalloidin-labeled actin shows an altered distribution of F-actin filaments in F508-chol with an intense positive staining in the cytoplasm. Quantification of the phalloidin fluorescence intensity shows the presence of sharper peaks corresponding to membranes staining in Control-chol while similar levels of fluorescence were detected along

membrane and cytoplasm in F508-chol. **(B)** Treatment with F508 CFTR correcting drugs (VX-770 and VX-809, 10 μ M) partially redistributed F-actin in the submembrane compartment as also shown by the two small membrane peaks in the quantification graph. Treatment with PP2 (1 μ M) alone or in combination with VX-770 and VX-809 completely recovered the actin distribution in F508-chol similar to Control-chol. Phalloidin fluorescence intensity confirms the presence of membrane sharper peaks that reach intensity values comparable to Control-chol.

Figure 7. Src kinase inhibition increases the efficacy of VX-770 and VX-809 to rescue the secretory defect in human F508-chol

(A) Western blot shows the expression of the glycosylated mature form of F508-CFTR (band C) in iPSC-derived F508-chol after treatment with VX-770 and VX-809 (10 μ M), confirming their pharmacological property. (B) Representative confocal z-stack sections of human F508-chol for ASL quantification showing in green the FITC dextran on the top of the epithelial monolayer and in red the epithelial cells labeled with the cell membrane dye. Cells were untreated or treated with VX-770 and VX-809 alone or in combination with PP2 and exposed to forskolin (10 μ M) to measure their c-AMP mediated secretory function. Dextran level was unchanged in untreated cells and slightly increases after treatment with

VX-770 and VX-809 alone but visibly increased by the combined treatment with PP2. (C) ASL was quantified as intensity of the dextran fluorescence area in pixels. Bars represent means $\pm$ SD of n=7–9 different monolayers from 3 independent experiments; *p<0.05, **p<0.01 vs basal.