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Induction of CFTR gene expression by 1,25(OH)₂ vitamin D₃, 25OH vitamin D₃, and vitamin D₃ in cultured human airway epithelial cells and in mouse airways

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Abstract

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which often leads to protein misfolding and no CFTR surface localization. This then leads to chronic airway infections, inflammation, and tissue damage. Although vitamin D has been explored as a therapy to treat CF due to its antimicrobial-inducing and anti-inflammatory properties, the effect of 1,25-dihydroxyvitamin D₃ (1 α ,25(OH)₂D₃) on CFTR directly has not been studied. We treated cultured healthy and diseased bronchial epithelial cells (BEC) with 10 nM 1 α ,25(OH)₂D₃ for 6 and 24 hours and found that 1 α ,25(OH)₂D₃ increases both mRNA and protein CFTR levels using RT-qPCR, flow cytometry and fluorescence immunohistochemistry. Treatment of CF cells with 10 nM 1 α ,25(OH)₂D₃ led to an increase in both total and surface CFTR expression, suggesting 1 α ,25(OH)₂D₃ could be used to increase properly localized CFTR in airway cells. To determine if BEC could convert the more clinically relevant cholecalciferol to 25OHD₃, cultured non-CF and CF BECs were treated with a range of cholecalciferol concentrations, and 25OHD₃ levels were quantified by ELISA. We found that 25OHD₃ levels increased in a concentration-dependent manner. Treatment of BEC with 10 μ M cholecalciferol led to increases in both CYP24A1 and CFTR mRNA levels, even when added to the apical surface of cells grown in an air-liquid interface, suggesting that topical administration of vitamin D could be used therapeutically. To demonstrate this *in vivo*, we intranasally delivered 1 μ M 1 α ,25(OH)₂D₃ into mice. After 6 hours, we observed induction of both Cyp24A1 and CFTR expression in the tracheas of treated mice. The major findings of this study are that vitamin D can be converted to the active form when topically administered to the airway, and this could be used to increase CFTR levels in patients with CF. This could potentially be useful as an adjunctive therapy, together with newly developed CF treatments.

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Keywords

cystic fibrosis; 25-hydroxylase; 1-alpha-hydroxylase; airway epithelium

1. Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encodes a chloride ion channel. CFTR is expressed in many organs, including the lungs, pancreas, salivary glands, kidneys, liver, sweat glands, and reproductive tract[1]. There are over 2,000 CFTR mutations that, depending on the specific mutation, affect either trafficking, synthesis, or function of CFTR[2]. The most common mutation in Caucasians is a phenylalanine deletion at position 508 (F508del). The loss of this amino acid causes misfolding of the protein in the endoplasmic reticulum (ER), where it is then subsequently degraded prior to reaching the plasma membrane[3–5].

Improper chloride transport from defective CFTR leads to thickened mucous secretions, persistent bacterial infections, lung tissue damage and malnutrition due to poor vitamin absorption[6–10]. The lungs are the organ most commonly studied in CF because of the severity of the effects at that site. Itself, the thickened mucus in the lungs provides an environment favorable for microbial growth[11]. The most common bacterial species found in CF is *Pseudomonas aeruginosa*, with 80% of adult CF patients colonized by the bacteria [12]. Chronic bacterial infection leads to immune system activation and release of neutrophilic proteases. However, instead of clearing the bacteria, tissue damage ensues and a lung transplant becomes necessary. In fact, 80% of CF patients' deaths are due to lung infection[1].

Besides the lungs, other organs are affected in CF due to the widespread expression of CFTR. For example, malnutrition is common because mucus clogs the pancreas and digestive enzymes can't be released, resulting in poor vitamin absorption, especially fat-soluble vitamins A, D, E, and K[8, 13, 14]. Vitamin D is best known to be a principal factor that maintains calcium homeostasis and is required for bone development and maintenance[15]. Early vitamin D deficiency could lead to rickets while adult deficiency leads to osteoporosis and increases in fractures in the elderly (reviewed in [16]). Additionally, vitamin D deficiency is also associated with a number of non-bone related disorders including multiple sclerosis, diabetes and Crohn's disease, as well as a variety of cancers[17–21]. Vitamin D is of particular interest for CF patients because of its diverse functions and ability to act on a wide variety of tissues. Up to 90% of CF patients are deficient in vitamin D, even with supplementation, and this deficiency is associated with increased pulmonary exacerbations, increased bacterial infection and inflammation[22, 23].

Vitamin D undergoes a multistep, multiorgan conversion process that ultimately leads to its final hydroxylation and activation to 1,25-dihydroxyvitamin D₃ (1 α ,25(OH)₂D₃) (reviewed in [24]). Initially, vitamin D₃ is either taken as an oral supplement or converted by the skin after sunlight exposure and subsequently hydroxylated in the liver to 25OHD₃. Multiple 25-hydroxylases are reported to convert vitamin D₃ to 25OHD₃, with the best-studied being

Cyp27A1 and Cyp2R1[25, 26]. 25OHD₃ is then transported to the kidneys where it undergoes a second hydroxylation step by the 1- α -hydroxylase Cyp27B1 to be converted to the active 1 α ,25(OH)₂D₃[24, 27]. Recently, the conversion of 25OHD₃ to 1 α ,25(OH)₂D₃ has been reported in extrarenal tissues including macrophages, colon, keratinocytes, osteoclasts and lung[28–30]. The actions of 1 α ,25(OH)₂D₃ are mediated by binding to the vitamin D receptor (VDR), where it heterodimerizes with retinoid X receptor and interacts with a specific consensus sequence called a vitamin D response element (VDRE) [31, 32]. This then leads to the activation and transcription of target genes. While the VDR/1 α , 25(OH)₂D₃ transcription complex can take several hours to result in an observable change in cellular activities, 1 α ,25(OH)₂D₃ has also been reported to rapidly affect intracellular processes by binding membrane VDR or 1,25D₃-membrane-associated, rapid response steroid-binding protein (1,25D₃-MARRS) receptor to turn on a number of kinases, such as protein kinases A and C[33–41].

Currently, the greatest appeal of vitamin D lies in its antimicrobial and anti-inflammatory properties (reviewed in [9]). Vitamin D is known to downregulate the expression of inflammatory cytokines IL-6 and IL-8, which have been reported to be upregulated in CF patients [42, 43]. Ideally, these anti-inflammatory properties would prevent recruitment of neutrophils, which contribute to the lung tissue damage. This was observed in an *in vivo* hamster model where LPS inhalation followed by 1 α ,25(OH)₂D₃ administration decreased neutrophil recruitment[44]. 1 α ,25(OH)₂D₃ treatment was also found to upregulate gene expression of the antimicrobial peptides LL-37 and β -defensins, as well as other innate immune mediators in CF and normal airway epithelial cells, which would be beneficial to combat opportunistic infections[45–47]. *P. aeruginosa* viability was decreased when exposed to supernatants of bronchial epithelial cells treated with 1 α ,25(OH)₂D₃ [48].

While examining the effect of vitamin D treatment on airway epithelial cells, we observed an unexpected induction of CFTR mRNA, suggesting a potential therapeutic role for vitamin D in CF treatment. Currently, a major obstacle for using oral vitamin D to treat cystic fibrosis is that vitamin D₃ must undergo two hydroxylation steps in the liver and kidney, resulting in 25(OH)₂D₃ and 1 α ,25(OH)₂D₃, respectively. This multiorgan activation combined with poor vitamin D absorption in CF patients would most likely lead to insufficient levels of activated vitamin D reaching the lungs and ultimately no change in CFTR. While treating patients with either 25OHD₃ or 1 α ,25(OH)₂D₃ would avoid the need for multiple hydroxylations, treating with either metabolite is not feasible because of their short half-lives of several hours when ingested and the dangerous side effect of hypercalcemia[49–53]. As of now, these two forms are only used to raise calcium levels in patients on long-term renal dialysis. In this study, we investigated the effect of 1 α , 25(OH)₂D₃ on CFTR both *in vitro* and *in vivo*, the conversion of vitamin D₃ to active 1 α , 25(OH)₂D₃ in lung epithelial cells, and the feasibility of topical administration of vitamin D to the airways.

2. Methods and materials

2.1 Cell Culture

Human bronchial epithelial (HBE) cells were from two sources. Normal HBE cells (NHBE) were purchased from Lonza (Walkersville, MD) and grown in bronchial epithelial growth medium (BEGM) from the same company. CF HBE cells were obtained from lungs explanted during transplantation under IRB-approved protocols and were cultures at an air liquid interface (ALI) until well differentiated using well described protocols ([54]). UCN3T (non-CF) and UNCCF1T (CF) cells were a gift from Scott Randell (University of North Carolina), and have been described previously[55]. BEAS-2B cells were obtained from the American Type Culture Collections (Rockville, MD) and grown in BEGM medium. HEK293 cells were a generous gift from Dr. Edward Chan (University of Florida) and were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and penicillin/streptomycin (Thermo Scientific, Rockford, IL). All cells were grown at 37°C in 5% CO₂. Submerged cells were grown in 12-well plates and cells grown at an ALI were cultured in Transwell® inserts (Fisher Scientific, Pittsburgh, PA).

2.2 RNA isolation, RT-PCR, and PCR

After cells were treated with 1 α ,25(OH)₂D₃ (Sigma-Aldrich, St. Louis, MO) or vitamin D₃, total RNA was isolated using RNeasy Plus Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, and was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) using the T100 ThermoCycler (Bio-Rad). RT-PCR was performed using SoAdvanced Universal SYBR Green Supermix (Bio-Rad) using the CFX96 Real Time PCR Detection System (Bio-Rad). Data was analyzed with CFX Manager Software (Bio-Rad). For PCR, cDNA was amplified using iProof HF Master (Bio-Rad) and fragments were visualized on a 2% agarose gel. Bands were cut out and sent to UF Interdisciplinary Center for Biotechnology Research for Sanger sequencing to confirm identity of the bands. β -2 – macroglobulin (B2M) was used as the reference gene and primers for both RT-PCR and PCR are listed in Table 1 and were designed and purchased from Integrated DNA Technologies (Coralville, IA).

2.3 Western blot

Cells were lysed using RIPA buffer. Samples were mixed with LDS sample buffer (Thermo Scientific) and run on a 10% NuPAGE Tris-Acetate gel (Thermo Scientific). Proteins were transferred using the iBlot (Thermo Fisher) transfer apparatus to nitrocellulose membranes. Membranes were blocked in 5% milk in Tris-buffered saline with Tween 20 (TBS-T) and incubated with either anti-Cyp2R1 (Abcam Inc., Boston, MA), anti-Cyp27A1 EPR7529 (Abcam), anti-Cyp27B1 H-90 (Santa Cruz Biotechnology) or anti- β -actin BA3R (Thermo Fisher) overnight and bands were visualized by chemiluminescence. Densitometric analysis of the bands was performed using ImageJ software.

2.4 Confocal Microscopy and Flow Cytometry

Cells were treated with 1 α ,25(OH)₂D₃ (Sigma-Aldrich, St. Louis MO) and fixed with 2% paraformaldehyde (Santa Cruz Biotechnology, Santa Cruz, CA). For surface CFTR staining,

cells were subsequently blocked in 1% BSA and incubated with anti-CFTR H-182 (Santa Cruz Biotechnology) overnight. Cells were washed with phosphate-buffered saline and incubated with CruzFluor 488 (Santa Cruz Biotechnology) for 1 hour at room temperature. Fluorescence was visualized using the Leica TCS-SP5 at the University of Florida Interdisciplinary Center for Biotechnology Research.

For intracellular staining of CFTR, cells were permeabilized after fixation with 0.5% TWEEN 20 (Sigma) in PBS. Cells were then blocked in 1% BSA and the same steps as above were followed. Fluorescence was detected by either confocal microscopy using the Leica TCS-SP5 or flow cytometry using the FACS Calibur cytometer (BD Biosciences, San Jose, CA). Confocal data was analyzed using LAS AF Lite Software (Leica, Buffalo Grove, IL) and flow cytometry data was analyzed with FCS Express 4 (De Novo Software, Glendale, CA).

2.5 25OHD₃ ELISA

Bronchial epithelial cells (n=3) from non-CF controls or CF-bronchial epithelial cells (n=3) (Lonza) cultured on collagen coated-6 well plates were treated with vitamin D₃ (Sigma Aldrich) for 24 hours. All experiments were conducted in the absence of fetal bovine serum. Ethanol matched to the highest vitamin D₃ dose was used as a vehicle control. Supernatants were collected and 25OHD₃ measured by enzyme immunoassay (EIA) (Immunodiagnostic Systems, Fountain Hills, AZ) according to the manufacturer's instructions. Media with vitamin D₃ alone was also assessed for 25OHD₃ and these values were subtracted from the total 25OHD₃ levels reported. The assay detection limit for the 25OHD₃ EIA is 5–152 ng/mL

2.6 Animal experiments

All animal experiments were conducted in accordance with University of Florida Animal Care and Use Committee. C57BL/6J mice were obtained directly through University of Florida ACS Breeding. Mice were sedated with an injection of 40 mg/kg ketamine and 5 mg/kg xylazine (Sigma-Aldrich) and were intranasally administered 50 μ L (25 μ L/nares) 1 μ M 1 α ,25(OH)₂D₃ (Sigma-Aldrich) or EtOH vehicle control (Sigma-Aldrich) for 6 hours with n=5 per condition. After 6 hours, nasal epithelia, trachea, and lungs were obtained and RNA was isolated as described above and RT-PCR was performed.

2.7 Statistical Analysis

The results obtained from three independent experiments were expressed as the fold change relative to EtOH-treated cells $\pm$ standard error of the mean (SEM). Each independent experiment was also run in technical triplicates. All differences were analyzed by a two-tailed unpaired Student's t-test. Statistics for the ELISA were performed with one-way ANOVA with post-hoc multiple comparisons versus the control group. A p-value of p<0.05 was considered statistically significant.

3. Results

3.1 Effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on CFTR expression in bronchial epithelial cells

To quantify the effect of vitamin D on CFTR gene expression, we treated cultured commercially available cell lines (NHBE and BEAS-2B), immortalized primary cells (UNCN3T and UNCCF1T) and primary differentiated cell lines with 10 nM $1\alpha,25(\text{OH})_2\text{D}_3$ for either 6 or 24 hours and quantified the change in CFTR mRNA levels. All primary patient and UNCCF1T cells are positive for the F508del mutation. RNA was harvested, and RT-qPCR was performed. Significant increases in CFTR mRNA levels were observed in all cultures at 6 hr. (figure 1). In five of the six samples, maximum upregulation was achieved by 6 hours. In the other two samples, CFTR expression was greatest after 24 hours.

To quantify the effect of vitamin D on CFTR protein expression, UNCCF1T cells were treated with 10 nM $1,25(\text{OH})_2\text{D}_3$ for 24 hours, fixed, permeabilized, and stained for total CFTR protein. Flow cytometry revealed a second population of cells with a greater fluorescence compared to the EtOH control, which suggests that there was an overall increase in total protein with $1,25(\text{OH})_2\text{D}_3$ treatment (figure 2).

3.2 The effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on surface expression of CFTR in CF cells

The F508del mutation leads to an improperly folded CFTR, and subsequent degradation of the protein prior to reaching the membrane. Therefore, we evaluated surface expression of CFTR by confocal microscopy. We treated UNCCF1 cells with $1,25(\text{OH})_2\text{D}_3$ for 4 and 24 hours, permeabilized the cells and stained for both extracellular and intracellular CFTR protein using immunofluorescence. Intracellular protein was detected in EtOH treated cells, which is expected because F508del mutants are capable of CFTR protein translation, and the protein reaches the cell surface. After both 4 and 24 hours, there is an increase in total fluorescence and therefore total protein, which is consistent with the flow cytometry data (figure 3). In addition, there is increased fluorescence at the perimeter of these cells, suggesting that $1,25(\text{OH})_2\text{D}_3$ increases surface CFTR expression. To detect surface CFTR, UNCCF1 cells were fixed, but not permeabilized, and stained for CFTR. As expected, no surface CFTR was detected in the EtOH treated conditions. However, after both 4 and 24 hours of $1,25(\text{OH})_2\text{D}_3$ treatment, CFTR was detected on the surface, suggesting that $1,25(\text{OH})_2\text{D}_3$ can not only increase CFTR mRNA and protein, but also bring CFTR to the surface of the cell.

3.3 The conversion of vitamin D_3 to 25OHD_3 in bronchial epithelial cells

Bronchial epithelial cells are known to convert 25OHD_3 to $1,25(\text{OH})_2\text{D}_3$ due to the expression of the 1-alpha hydroxylase Cyp27B1 in these cells [30]. However, both 25OHD_3 and $1,25(\text{OH})_2\text{D}_3$ are not useful clinically due to their dietary short half-life and negative effects on the kidneys, respectively [49–52]. Therefore, we chose to determine if bronchial epithelial cells could convert the dietary vitamin D_3 to 25OHD_3 and ultimately $1,25(\text{OH})_2\text{D}_3$. First, we determined the presence of the two most significant 25-hydroxylases, Cyp27A1 and Cyp2R1, in primary non-CF donors and cystic fibrosis patients (figure 4A). In all samples, Cyp2R1 and Cyp27A1 expression was detected by PCR and confirmed by DNA sequencing. We also observed a similar pattern of expression in

BEAS-2B cells (not shown). Expression of Cyp2R1 and Cyp27A1 protein was also confirmed by western blot of BEAS-2B whole cell lysate. Alongside BEAS-2B cells, kidney HEK293 cell lysates were also probed for both 25-hydroxylases as well as Cyp27B1. However, very low to undetectable levels of Cyp27A1 and Cyp2R1 were found in the HEK cell line, especially compared to the BEAS-2B cell line. Cyp27B1 was expressed in both HEK293 and BEAS-2B cells, although substantially higher in the kidney cells, which was expected due to the kidney's previously established major role in vitamin D metabolism (figure 4B). To confirm that this activation occurs, NHBE cells were treated with vitamin D at increasing concentrations, and 25OHD₃ levels were quantified in the supernatant by ELISA. The result shown in Figure 4C demonstrates a dose-dependent increase in 25OHD₃ levels, indicating that the vitamin D₃ was converted to 25OHD₃ by the cultured cells. In addition to NHBEs from healthy donors, we also examined if diseased, CF-derived epithelial cells were capable of converting vitamin D₃ to 25OHD₃. We observed a nearly identical conversion compared to NHBE's and confirmed that disease does not impact bronchial epithelial cells ability to metabolize vitamin D locally.

After confirming bronchial epithelial cells express both Cyp27A1 and Cyp2R1, we treated three non-CF cultured lung epithelial cell lines with 10 μ M D₃ for 6 and 24 hours to quantify expression change in the 1,25(OH)₂D₃ dependent gene Cyp24A1 (figure 5A). Cyp24A1 was upregulated 31 fold after 6 hours and 6 fold at 24 hours in NHBE cells. Cyp24A1 was upregulated 62 fold at 6 hours and increased to 132 fold relative to the control in UCN3 cells. In BEAS-2B cells, Cyp24A1 was upregulated only 12.5 fold and was the least amount of the three cell lines at 6 hours, but by 24 hours, Cyp24A1 expression was the greatest of the cell lines at 300 fold. In addition, we also quantified the relative expression of LL-37, which is known to be upregulated after 1 α ,25(OH)₂D₃ treatment in bronchial epithelial cells. Like Cyp24A1, LL-37 was upregulated in all three cell lines, with BEAS-2B cells expressing the greatest levels after 24 hours of vitamin D₃ treatment (data not shown). Because these genes can only be upregulated in the presence of 1 α ,25(OH)₂D₃, the upregulation observed with vitamin D₃ treatment strongly implies vitamin D₃ is converted to the active 1 α ,25(OH)₂D₃, which is then responsible for the changes in gene expression.

3.4 Topical administration of vitamin D *in vitro* and *in vivo*

While oral supplementation may be considered as a way to increase overall 1,25(OH)₂D₃ levels in the airway to induce CFTR in CF patients, we hypothesized that direct topical application may be more efficient. Therefore, we examined the effect of delivery of vitamin D to the apical surface of BEAS-2B cells grown both submerged and at the air liquid interface (ALI) with vitamin D₃ for 6 hours and observed an increase in CFTR expression (figure 5B). In this experiment we added vitamin D₃ at 10nM in the submerged culture, as we have in the experiments above, to approximate physiological levels found in the circulation. On the apical surface the vitamin D₃ was applied in a small volume (100 μ l) at 10 μ M in order to demonstrate the ability to deliver high concentrations to the airway surface that could be rapidly converted to the active form.

To demonstrate the feasibility of topical delivery of vitamin D *in vivo*, mice were intranasally treated with EtOH, or 1 μ M 1 α ,25(OH)₂D₃ for 6 hours, sacrificed, and the nasal

epithelia, trachea, and lungs were harvested. RT-qPCR confirmed Cyp24A1 mRNA upregulation in both the nasal epithelia (data not shown) and trachea in $1\alpha,25(\text{OH})_2\text{D}_3$ - treated mice. CFTR mRNA expression was also upregulated in the $1\alpha,25(\text{OH})_2\text{D}_3$ treated mice (figure 6).

4. Discussion

Our results show that vitamin D and its metabolites are able to increase CFTR mRNA and protein levels, as well as surface expression of the protein in both normal and F508del CF airway epithelial cells. In this most prevalent CF mutation, CFTR protein is synthesized, but does not fold correctly. This misfolding results in degraded protein, no surface expression of CFTR, and ultimately no chloride transport. Thus, treatment of BEC with vitamin D could provide a benefit to individuals with this and other mutations where CFTR surface localization is defective.

With $1\alpha,25(\text{OH})_2\text{D}_3$ treatment, CFTR mRNA, protein, and surface expression were upregulated significantly in bronchial epithelial cells. These changes were detected through qPCR, flow cytometry and confocal microscopy and were observed through a wide variety of cultured and primary lung epithelial cells from both CF and non-CF donors. As we were able to observe an increase in CFTR protein levels as early as 6 hours, we speculate that vitamin D may simultaneously be acting on multiple pathways: the standard transcriptional pathway, and one that regulates CFTR transport. While all cultures demonstrated induction of CFTR mRNA, there was variability in both the extent of induction and the timing. This can be seen both in cell lines and patient samples. This may be due to variability in other aspects of gene regulation between the cells, which are known to exist in vitamin D-mediated gene regulation [56].

The standard mechanism of transcriptional activation would involve the entry of $1\alpha,25(\text{OH})_2\text{D}_3$ into the cell, followed by the VDR-mediated activation of the transcription of CFTR, which would lead to an overall increase in CFTR protein expression. When analyzing the promoter region for transcription factor binding sites using the JASPAR database, several weak putative VDREs were detected. Considering that the CFTR mRNA increased approximately two fold in most cell types tested, a weak VDRE may account for these low levels. However, it would likely not account for the increase in surface expression seen so early.

Vitamin D is also known to have numerous intracellular nongenomic roles in addition to transcriptional activation. $1\alpha,25(\text{OH})_2\text{D}_3$ has been reported to activate intracellular signaling molecules such as protein kinases A and C, phosphatidylinositol 3-kinase, phospholipase C as well as open calcium channels[36, 57–59]. Therefore, vitamin D could also be affecting CFTR transport post-transcriptionally. There also exist proteins that are common to both CFTR and the vitamin D pathway. For example, vitamin D has been reported to interact with the heat shock proteins and other intracellular chaperones, such as Bag-1, which are vital to correct CFTR folding and surface expression[60]. A second protein common to both pathways is ERp57, which has been found to be complexed with F508del CFTR but also

serves as the vitamin D receptor found in the plasma membrane[38, 40, 61–63]. Therefore, vitamin D may be binding to these molecules to allow for correct CFTR folding.

These findings demonstrate that $1\alpha,25(\text{OH})_2\text{D}_3$ upregulates CFTR, and could be potentially useful therapeutically. $1\alpha,25(\text{OH})_2\text{D}_3$, while effective *in vitro*, has its limitations as a therapy. It only has a half-life of several hours and has the undesirable side effect of hypercalcemia [49–52]. Currently, it is only approved to raise calcium levels in patients undergoing long-term dialysis. The precursor, 25OHD_3 has a longer half-life in the serum, but like $1\alpha,25(\text{OH})_2\text{D}_3$, is only approved for increasing serum calcium due to the same negative side effects. Vitamin D_3 is the form most commonly taken as an oral supplement and is the most stable, but it must be converted to 25OHD_3 in the liver and $1\alpha,25(\text{OH})_2\text{D}_3$ in the kidney to be activated. It is known that BEC can convert 25OHD_3 to $1\alpha,25(\text{OH})_2\text{D}_3$ [30]. We demonstrate here for the first time that these epithelial cells not only express the hydroxylases to convert vitamin D_3 to 25OHD_3 , but treatment with vitamin D_3 also activates $1\alpha,25(\text{OH})_2\text{D}_3$ dependent genes. More importantly, vitamin D_3 treatment also exhibited the same increase in CFTR mRNA as the $1\alpha,25(\text{OH})_2\text{D}_3$ treatment. HEK cells were unable to activate these genes after vitamin D_3 treatment nor was any substantial protein expression of 25-hydroxylases detected. This was not surprising due to the fact that HEK cells are kidney cells and are primarily focused on the second conversion.

The ability of lung cells to metabolize $1\alpha,25(\text{OH})_2\text{D}_3$ from vitamin D_3 has several important implications. First, this opens the possibility of other cell types outside the liver possessing the ability to undergo both conversion steps which would greatly redefine what is understood about vitamin D metabolism. In addition, the new data we present here of vitamin D_3 conversion to 25OHD_3 coupled with the existing evidence that bronchial epithelial cells can convert 25OHD_3 to $1\alpha,25(\text{OH})_2\text{D}_3$ also introduces the prospect of a new method to inexpensively maximize $1\alpha,25(\text{OH})_2\text{D}_3$ exposure to the lungs[30].

One important hurdle that must be considered is how to deliver vitamin D to the lungs. CF patients may be chronically vitamin D deficient because they are unable to absorb fat-soluble vitamins[8, 14]. Whatever is successfully absorbed must undergo two hydroxylation steps in two different organs before the activated form can be delivered to the lung. Even with supplementation, it is unlikely that sufficient levels of $1\alpha,25(\text{OH})_2\text{D}_3$ will reach the airway to induce CFTR expression. Topical administration of vitamin D_3 , which would be converted locally, would therefore have the maximum effect on CFTR as well as the additional benefit of the activation of antimicrobial and anti-inflammatory genes.

Since *in vivo* data for topical administration of vitamin D to the lung is absent in the literature, we chose to initially examine a mouse model, to determine whether this could be a potential method. $1\alpha,25(\text{OH})_2\text{D}_3$ intranasally administered to mice for 6 hours upregulated both Cyp24A1 and CFTR in the nasal epithelia and trachea (figure 6) but no effect was observed in the lungs. This is most likely due to insufficient concentrations reaching the lungs and emphasizes the necessity to deliver vitamin D through aerosolization. Moderate Cyp24A1 upregulation was observed after treatment when all samples were obtained from the proximal lung, which also confirms that intranasal administration does not reach all regions of the lung (data not shown). However, together, these data serve as proof of concept

that $1\alpha,25(\text{OH})_2\text{D}_3$ can be topically administered and CFTR expression can be affected by that method of delivery.

This is the first instance of topical administration of vitamin D by a method other than to the skin and represents a novel approach to treat disorders involving genes expressed in the airway. Here we demonstrate that vitamin D can induce the expression of CFTR in the airway cells, which is a first step to correcting the defect in many of the mutations of this disease. In order to demonstrate full correction, further experiments are required to quantify the effect on CFTR function, including an increase in chloride conductance of fully differentiated Ussing chamber cultures, and ultimately an increase in the airway surface liquid height. While the vitamin D-mediated induction of CFTR shown here may only lead to a small effect on CFTR function, this may be sufficient to be used together with the recently developed CFTR modifiers. To date, only two drugs are approved by the FDA to target CFTR in cystic fibrosis but each must be taken orally and both have displayed limitations clinically. Ivacaftor corrects faulty chloride ion transport and only can treat patients who have a CFTR mutation that allows CFTR to reach the surface, which amounts to just 4% of the CF population [64]. Lumacaftor is designed to treat patients with the more prevalent F508del mutation by correcting faulty CFTR processing, but only produces small effects on lung function and lower exacerbation rates [65–67]. While determination of whether vitamin D can directly correct the defective ion transport will require further experimentation, both *in vitro* and *in vivo*, because we demonstrate that vitamin D results in increased CFTR surface localization in CF bronchial epithelial cells, we speculate that vitamin D can be utilized as an adjunctive therapy to improve the efficacy of the approved drugs.

Currently, vitamin D is an appealing therapeutic agent for the treatment of CF due to its anti-inflammatory properties and ability to induce antimicrobial activity, but the results in this study suggest another, more direct role for vitamin D by increasing CFTR levels. Like the other drugs, vitamin D targets CFTR directly and is FDA approved, but is also inexpensive. Vitamin D is well-tolerated at high levels, which would minimize the concern of overdosing patients. Not only are the results presented promising on their own, but future studies involving synergy of vitamin D and the FDA approved drugs holds great potential.

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References

1. O'Sullivan BP, Freedman SD. Cystic fibrosis. *Lancet*. 2009; 373:1891–1904. [PubMed: 19403164]
2. C.F.M.D.A.a. Cystic Fibrosis Mutation Database. <http://www.genet.sickkids.on.20ca/cftr/> Available at: <http://www.genet.sickkids.on.20ca/cftr/>
3. Lukacs GL, Verkman AS. CFTR: folding, misfolding and correcting the F508 conformational defect. *Trends Mol Med*. 2012; 18:81–91. [PubMed: 22138491]

4. Farinha CM, Matos P, Amaral MD. Control of cystic fibrosis transmembrane conductance regulator membrane trafficking: not just from the endoplasmic reticulum to the Golgi. *FEBS J.* 2013; 280:4396–4406. [PubMed: 23773658]
5. Ward CL, Omura S, Kopito RR. Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell.* 1995; 83:121–127. [PubMed: 7553863]
6. Tirouvanziam R, de Bentzmann S, Hubeau C, Hinnrasky J, Jacquot J, Péault B, Puchelle E. Inflammation and infection in naive human cystic fibrosis airway grafts. *Am J Respir Cell Mol Biol.* 2000; 23:121–127. [PubMed: 10919974]
7. Valenza G, Tappe D, Turnwald D, Frosch M, König C, Hebestreit H, Abele-Horn M. Prevalence and antimicrobial susceptibility of microorganisms isolated from sputa of patients with cystic fibrosis. *J Cyst Fibros.* 2008; 7:123–127. [PubMed: 17693140]
8. Rayner RJ. Fat-soluble vitamins in cystic fibrosis. *Proc Nutr Soc.* 1992; 51:245–250. [PubMed: 1438333]
9. Herscovitch K, Dauletbaev N, Lands LC. Vitamin D as an anti-microbial and anti-inflammatory therapy for Cystic Fibrosis. *Paediatr Respir Rev.* 2014; 15:154–162. [PubMed: 24332502]
10. Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science.* 1989; 245:1066–1073. [PubMed: 2475911]
11. Boucher RC. Airway surface dehydration in cystic fibrosis: pathogenesis and therapy. *Annu Rev Med.* 2007; 58:157–170. [PubMed: 17217330]
12. Davies JC. *Pseudomonas aeruginosa* in cystic fibrosis: pathogenesis and persistence. *Paediatr Respir Rev.* 2002; 3:128–134. [PubMed: 12297059]
13. Bronstein MN, Sokol RJ, Abman SH, Chatfield BA, Hammond KB, Hambidge KM, Stall CD, Accurso FJ. Pancreatic insufficiency, growth and nutrition in infants identified by newborn screening as having cystic fibrosis. *J Pediatr.* 1992; 120:533–540. [PubMed: 1552390]
14. Maqbool A, Stallings VA. Update on fat-soluble vitamins in cystic fibrosis. *Curr Opin Pulm Med.* 2008; 14:574–581. [PubMed: 18812835]
15. Bikle, JSADD., Christakos, S. C.J.R. and Disorders of Mineral Metabolism. Vitamin, m. D: production, mechanism of action and clinical requirements., i.P.o.t.M.B. Diseases. Wiley, B; 2013. p. 235-248.
16. Holick MF. Vitamin D: importance in the prevention of cancers, type 1 diabetes, heart disease, and osteoporosis. *Am J Clin Nutr.* 2004; 79:362–371. [PubMed: 14985208]
17. Raghuwanshi A, Joshi SS, Christakos S. Vitamin D and multiple sclerosis. *J Cell Biochem.* 2008; 105:338–343. [PubMed: 18655192]
18. Kamen DL, Tangpricha V. Vitamin D and molecular actions on the immune system: modulation of innate and autoimmunity. *J Mol Med (Berl).* 2010; 88:441–450. [PubMed: 20119827]
19. Aguirre M, Manzano N, Salas Y, Angel M, Díaz-Couselo FA, Zylberman M. Vitamin D deficiency in patients admitted to the general ward with breast, lung, and colorectal cancer in Buenos Aires. Argentina. *Arch Osteoporos.* 2016; 11:4. [PubMed: 26732091]
20. Simard A, Vobecky J, Vobecky JS. Vitamin D deficiency and cancer of the breast: an unprovocative ecological hypothesis. *Can J Public Health.* 1991; 82:300–303. [PubMed: 1768986]
21. Trump DL, Chadha MK, Sunga AY, Fakh MG, Ashraf U, Silliman CG, Hollis BW, Nesline MK, Tian L, Tan W, Johnson CS. Vitamin D deficiency and insufficiency among patients with prostate cancer. *BJU Int.* 2009; 104:909–914. [PubMed: 19426195]
22. Chesdachai S, Tangpricha V. Treatment of vitamin D deficiency in cystic fibrosis. *J Steroid Biochem Mol Biol.* 2015
23. Sexauer WP, Hadeh A, Ohman-Strickland PA, Zanni RL, Varlotta L, Holsclaw D, Fiel S, Graff GR, Atlas A, Bisberg D, Hadjiladis D, Michel SH, Mintz D, Chakraborty R, Marra B, Lomas P, Ward T, Sassman M, Imbesi GC, Kitch DM, Mallowe AM. Vitamin D deficiency is associated with pulmonary dysfunction in cystic fibrosis. *J Cyst Fibros.* 2015; 14:497–506. [PubMed: 25577127]
24. Bikle DD. Vitamin D metabolism, mechanism of action, and clinical applications. *Chem Biol.* 2014; 21:319–329. [PubMed: 24529992]
25. Sakaki T, Kagawa N, Yamamoto K, Inouye K. Metabolism of vitamin D3 by cytochromes P450. *Front Biosci.* 2005; 10:119–134. [PubMed: 15574355]

26. Zhu JG, Ochalek JT, Kaufmann M, Jones G, Deluca HF. CYP2R1 is a major, but not exclusive, contributor to 25-hydroxyvitamin D production in vivo. *Proc Natl Acad Sci U S A*. 2013; 110:15650–15655. [PubMed: 24019477]
27. Christakos S, Dhawan P, Verstuyf A, Verlinden L, Carmeliet G. Vitamin D: Metabolism, Molecular Mechanism of Action, and Pleiotropic Effects. *Physiol Rev*. 2016; 96:365–408. [PubMed: 26681795]
28. Adams JS, Rafison B, Witzel S, Reyes RE, Shieh A, Chun R, Zavala K, Hewison M, Liu PT. Regulation of the extrarenal CYP27B1-hydroxylase. *J Steroid Biochem Mol Biol*. 2014; 144(Pt A):22–27. [PubMed: 24388948]
29. Seifert M, Tilgen W, Reichrath J. Expression of 25-hydroxyvitamin D-1 α -hydroxylase (1 α OHase, CYP27B1) splice variants in HaCaT keratinocytes and other skin cells: modulation by culture conditions and UV-B treatment in vitro. *Anticancer Res*. 2009; 29:3659–3667. [PubMed: 19667162]
30. Hansdottir S, Monick MM, Hinde SL, Lovan N, Look DC, Hunninghake GW. Respiratory epithelial cells convert inactive vitamin D to its active form: potential effects on host defense. *J Immunol*. 2008; 181:7090–7099. [PubMed: 18981129]
31. Pike JW, Meyer MB. The vitamin D receptor: new paradigms for the regulation of gene expression by 1,25-dihydroxyvitamin D(3). *Endocrinol Metab Clin North Am*. 2010; 39:255–269. table of contents. [PubMed: 20511050]
32. Carlberg C, Bendik I, Wyss A, Meier E, Sturzenbecker LJ, Grippo JF, Hunziker W. Two nuclear signalling pathways for vitamin D. *Nature*. 1993; 361:657–660. [PubMed: 8382345]
33. Lösel R, Wehling M. Nongenomic actions of steroid hormones. *Nat Rev Mol Cell Biol*. 2003; 4:46–56. [PubMed: 12511868]
34. Deeb KK, Trump DL, Johnson CS. Vitamin D signalling pathways in cancer: potential for anticancer therapeutics. *Nat Rev Cancer*. 2007; 7:684–700. [PubMed: 17721433]
35. Wali RK, Baum CL, Sitrin MD, Brasitus TA. 1,25(OH)₂ vitamin D₃ stimulates membrane phosphoinositide turnover, activates protein kinase C, and increases cytosolic calcium in rat colonic epithelium. *J Clin Invest*. 1990; 85:1296–1303. [PubMed: 2156898]
36. Zanatta L, Zamonier A, Zanatta AP, Bouraïma-Lelong H, Delalande C, Bois C, Carreau S, Silva FR. Nongenomic and genomic effects of 1 α ,25(OH)₂ vitamin D₃ in rat testis. *Life Sci*. 2011; 89:515–523. [PubMed: 21565203]
37. Boyan BD, Chen J, Schwartz Z. Mechanism of Pdia3-dependent 1 α ,25-dihydroxy vitamin D₃ signaling in musculoskeletal cells. *Steroids*. 2012; 77:892–896. [PubMed: 22569272]
38. Khanal RC, Nemere I. The ERp57/GRp58/1,25D₃-MARRS receptor: multiple functional roles in diverse cell systems. *Curr Med Chem*. 2007; 14:1087–1093. [PubMed: 17456022]
39. Nemere I, Farach-Carson MC, Rohe B, Sterling TM, Norman AW, Boyan BD, Safford SE. Ribozyme knockdown functionally links a 1,25(OH)₂D₃ membrane binding protein (1,25D₃-MARRS) and phosphate uptake in intestinal cells. *Proc Natl Acad Sci U S A*. 2004; 101:7392–7397. [PubMed: 15123837]
40. Nemere I, Garbi N, Hammerling G, Hintze KJ. Role of the 1,25D₃-MARRS receptor in the 1,25(OH)₂D₃-stimulated uptake of calcium and phosphate in intestinal cells. *Steroids*. 2012; 77:897–902. [PubMed: 22546984]
41. Tunsophon S, Nemere I. Protein kinase C isoforms in signal transduction for the 1,25D₃-MARRS receptor (ERp57/PDIA3) in steroid hormone-stimulated phosphate uptake. *Steroids*. 2010; 75:307–313. [PubMed: 20079367]
42. Zhang Y, Leung DY, Richers BN, Liu Y, Remigio LK, Riches DW, Goleva E. Vitamin D inhibits monocyte/macrophage proinflammatory cytokine production by targeting MAPK phosphatase-1. *J Immunol*. 2012; 188:2127–2135. [PubMed: 22301548]
43. McNally P, Coughlan C, Bergsson G, Doyle M, Taggart C, Adorini L, Uskokovic MR, El-Nazir B, Murphy P, Grealley P, Greene CM, McElvaney NG. Vitamin D receptor agonists inhibit pro-inflammatory cytokine production from the respiratory epithelium in cystic fibrosis. *J Cyst Fibros*. 2011; 10:428–434. [PubMed: 21784717]
44. Takano Y, Mitsuhashi H, Ueno K. 1 α ,25-Dihydroxyvitamin D₃ inhibits neutrophil recruitment in hamster model of acute lung injury. *Steroids*. 2011; 76:1305–1309. [PubMed: 21745487]

45. Yim S, Dhawan P, Ragunath C, Christakos S, Diamond G. Induction of cathelicidin in normal and CF bronchial epithelial cells by 1,25-dihydroxyvitamin D₃. *J Cyst Fibros*. 2007; 6:403–410. [PubMed: 17467345]
46. Hollox EJ, Armour JA, Barber JC. Extensive normal copy number variation of a beta-defensin antimicrobial-gene cluster. *Am J Hum Genet*. 2003; 73:591–600. [PubMed: 12916016]
47. Rigo I, McMahon L, Dhawan P, Christakos S, Yim S, Ryan LK, Diamond G. Induction of triggering receptor expressed on myeloid cells (TREM-1) in airway epithelial cells by 1,25(OH)₂ vitamin D₃. *Innate Immun*. 2012; 18:250–257. [PubMed: 21690199]
48. Wang TT, Nestel FP, Bourdeau V, Nagai Y, Wang Q, Liao J, Tavera-Mendoza L, Lin R, Hanrahan JW, Mader S, White JH, Hanrahan JH. Cutting edge: 1,25-dihydroxyvitamin D₃ is a direct inducer of antimicrobial peptide gene expression. *J Immunol*. 2004; 173:2909–2912. [PubMed: 15322146]
49. Bailie GR, Johnson CA. Comparative review of the pharmacokinetics of vitamin D analogues. *Semin Dial*. 2002; 15:352–357. [PubMed: 12358640]
50. Baeke F, Takiishi T, Korf H, Gysemans C, Mathieu C. Vitamin D: modulator of the immune system. *Curr Opin Pharmacol*. 2010; 10:482–496. [PubMed: 20427238]
51. Peppone LJ, Hebl S, Purnell JQ, Reid ME, Rosier RN, Mustian KM, Palesh OG, Huston AJ, Ling MN, Morrow GR. The efficacy of calcitriol therapy in the management of bone loss and fractures: a qualitative review. *Osteoporos Int*. 2010; 21:1133–1149. [PubMed: 19960185]
52. Osborn JL, Schwartz GG, Smith DC, Bahnson R, Day R, Trump DL. Phase II trial of oral 1,25-dihydroxyvitamin D (calcitriol) in hormone refractory prostate cancer. *Urol Oncol*. 1995; 1:195–198. [PubMed: 21224117]
53. Bischoff-Ferrari HA, Dawson-Hughes B, Stöcklin E, Sidelnikov E, Willett WC, Edel JO, Stähelin HB, Wolfram S, Jetter A, Schwager J, Henschkowski J, von Eckardstein A, Egli A. Oral supplementation with 25(OH)D₃ versus vitamin D₃: effects on 25(OH)D levels, lower extremity function, blood pressure, and markers of innate immunity. *J Bone Miner Res*. 2012; 27:160–169. [PubMed: 22028071]
54. Fulcher ML, Randell SH. Human nasal and tracheo-bronchial respiratory epithelial cell culture. *Methods Mol Biol*. 2013; 945:109–121. [PubMed: 23097104]
55. Fulcher ML, Gabriel SE, Olsen JC, Tatreau JR, Gentzsch M, Livanos E, Saavedra MT, Salmon P, Randell SH. Novel human bronchial epithelial cell lines for cystic fibrosis research. *Am J Physiol Lung Cell Mol Physiol*. 2009; 296:L82–91. [PubMed: 18978040]
56. Pike JW, Meyer MB. Fundamentals of vitamin D hormone-regulated gene expression. *J Steroid Biochem Mol Biol*. 2014; 144(Pt A):5–11. [PubMed: 24239506]
57. Civitelli R, Kim YS, Gunsten SL, Fujimori A, Huskey M, Avioli LV, Hruska KA. Nongenomic activation of the calcium message system by vitamin D metabolites in osteoblast-like cells. *Endocrinology*. 1990; 127:2253–2262. [PubMed: 2226314]
58. Wali RK, Kong J, Sitrin MD, Bissonnette M, Li YC. Vitamin D receptor is not required for the rapid actions of 1,25-dihydroxyvitamin D₃ to increase intracellular calcium and activate protein kinase C in mouse osteoblasts. *J Cell Biochem*. 2003; 88:794–801. [PubMed: 12577313]
59. Haussler MR, Jurutka PW, Mizwicki M, Norman AW. Vitamin D receptor (VDR)-mediated actions of 1 α ,25(OH)₂vitamin D₃: genomic and non-genomic mechanisms. *Best Pract Res Clin Endocrinol Metab*. 2011; 25:543–559. [PubMed: 21872797]
60. Chun RF, Gacad M, Nguyen L, Hewison M, Adams JS. Co-chaperone potentiation of vitamin D receptor-mediated transactivation: a role for Bcl2-associated athanogene-1 as an intracellular-binding protein for 1,25-dihydroxyvitamin D₃. *J Mol Endocrinol*. 2007; 39:81–89. [PubMed: 17693608]
61. Oliver JD, van der Wal FJ, Bulleid NJ, High S. Interaction of the thiol-dependent reductase ERp57 with nascent glycoproteins. *Science*. 1997; 275:86–88. [PubMed: 8974399]
62. Jessop CE, Tavender TJ, Watkins RH, Chambers JE, Bulleid NJ. Substrate specificity of the oxidoreductase ERp57 is determined primarily by its interaction with calnexin and calreticulin. *J Biol Chem*. 2009; 284:2194–2202. [PubMed: 19054761]
63. Okiyonedo T, Harada K, Takeya M, Yamahira K, Wada I, Shuto T, Suico MA, Hashimoto Y, Kai H. Delta F508 CFTR pool in the endoplasmic reticulum is increased by calnexin overexpression. *Mol Biol Cell*. 2004; 15:563–574. [PubMed: 14595111]

64. Jones AM, Helm JM. Emerging treatments in cystic fibrosis. *Drugs*. 2009; 69:1903–1910. [PubMed: 19747007]
65. Wainwright CE, Elborn JS, Ramsey BW. Lumacaftor-Ivacaftor in Patients with Cystic Fibrosis Homozygous for Phe508del CFTR. *N Engl J Med*. 2015; 373:1783–1784.
66. Flume PA, Liou TG, Borowitz DS, Li H, Yen K, Ordoñez CL, Geller DE. V.-.-S. Group. Ivacaftor in subjects with cystic fibrosis who are homozygous for the F508del-CFTR mutation. *Chest*. 2012; 142:718–724. [PubMed: 22383668]
67. Clancy JP, Rowe SM, Accurso FJ, Aitken ML, Amin RS, Ashlock MA, Ballmann M, Boyle MP, Bronsveld I, Campbell PW, De Boeck K, Donaldson SH, Dorkin HL, Dunitz JM, Durie PR, Jain M, Leonard A, McCoy KS, Moss RB, Pilewski JM, Rosenbluth DB, Rubenstein RC, Schechter MS, Botfield M, Ordoñez CL, Spencer-Green GT, Vernillet L, Wisseh S, Yen K, Konstan MW. Results of a phase IIa study of VX-809, an investigational CFTR corrector compound, in subjects with cystic fibrosis homozygous for the F508del-CFTR mutation. *Thorax*. 2012; 67:12–18. [PubMed: 21825083]

Highlights

- $1\alpha,25(\text{OH})_2$ vitamin D_3 induces CFTR mRNA and protein levels in cultured airway epithelial cells.
- $1\alpha,25(\text{OH})_2$ vitamin D_3 induces CFTR protein to localize to the membrane in cultured cells from CF patients.
- Vitamin D_3 and 25OH vitamin D_3 also induce CFTR mRNA levels.
- Airway epithelial cells express 25 hydroxylases and can convert vitamin D_3 to 25OH vitamin D_3
- Intranasally delivered $1\alpha,25(\text{OH})_2$ vitamin D_3 can induce CYP24A1 and CFTR in mouse tracheas

Figure 1. Induction of CFTR mRNA in cultured HBE cells

Primary cultures of normal human bronchial epithelial cells (NHBE), or immortalized HBE cells (BEAS-2B, UNC-CF1), or well-differentiated cultures of primary cells from a non-CF donor or CF patients (all in triplicate) were treated with either ethanol control, or 10nM 1,25(OH)₂D₃ for 6 hours. Total mRNA was isolated and CFTR was quantified by QRT-PCR relative to β 2-microglobulin. Data are shown as mean $\pm$ SEM. Increase in CFTR was significant (a, $p < 0.001$; b, $p < 0.03$; c, $p < 0.05$).

Figure 2. Induction of CFTR protein in UNCCF1T cells

Cells were cultured on plastic and induced with 10nM $1,25(\text{OH})_2\text{D}_3$ or 0.1% ethanol for 24 hours. Shown is flow cytometry of permeabilized cells stained with an anti-CFTR antibody followed by a fluorescent secondary antibody. Black: unstained cells; Red: Ethanol; Blue: vitamin D-treated cells.

Figure 3. Vitamin D upregulates total protein and surface expression of CFTR

Confocal microscopy of cells grown on coverslips, treated with ethanol or 1,25(OH)₂D₃. Cultures were either permeabilized with 0.5% Tween or not permeabilized, fixed and stained for CFTR (green) and DAPI (blue) and visualized by confocal fluorescence microscopy. Magnification = 200X.

Figure 4. Activation of vitamin D in airway epithelial cells

A. mRNA expression. Total mRNA was isolated from cultured BEC from a non-CF donor and a CF patient, and subjected to RT-PCR using primers specific for CYP27B1, CYP27A1 and CYP2R1. **B. Protein expression.** BEAS2B and HEK293 cells were lysed and subjected to western blot analysis using antibodies to the hydroxylases, with β-actin as a loading control. **C. Conversion of Vitamin D₃ to 25OHD₃.** BECs obtained from three healthy patients and three CF patients were treated with indicated doses of vitamin D₃ or vehicle control for 24 hours, after which time 25OHD₃ levels were measured in supernatant.

*p<0.05 vs. control

Figure 5. Activation of vitamin D in HBE

A. Activation of vitamin D mediated gene expression in HBE by inactive vitamin D.

To demonstrate that the inactive, cholecalciferol, form can be activated by HBE and lead to induction of gene expression, cultures of three airway cell lines (NHBE, UNCN3T and BEAS-2B) were treated with 10nM vitamin D (cholecalciferol). Total mRNA was isolated after 6 and 24 hours, and CYP24A1 gene expression was quantified by QRT-PCR. **B.**

Expression of CFTR in apically stimulated cultures. Cells were stimulated either in submerged cultures (final concentration, 10nM vitamin D) or with 100μl 10μM vitamin D on the apical surface of ALI cultures, for 6 hr. CFTR was quantified as above relative to 0.1% ethanol treated cultures. Induction in all cases was significant at $p < 0.001$.

Figure 6. Induction of Cyp24A1 and CFTR gene expression in mouse tracheas after intranasal administration

C57Bl/6 mice (n=5) were inoculated with 25µl/nares of 10^{-6} M $1,25(\text{OH})_2\text{D}_3$ in 0.1% ethanol, or vehicle control. After 6 hours, mice were sacrificed and tracheas removed. Shown, mean values ($\pm$ SEM) CFTR (blue) and CYP24A1 (red) mRNA levels normalized to β -actin. Increases in both Cyp24A1 and CFTR mRNA levels are significant, $p < 0.006$ by t-test.

Table 1

PCR primer sequences

Gene	Forward	Reverse
hB2M	CTCCGTGGCCTTAGCTGTG	TTTGGAGTACGCTGGATAGCCT
hCFTR	GCTTCCTATGACCCGATAAC	GGAGCAGTGCCTCACAATAA
hCyp24A1	TTGGCTCTTTGTTGGATTGTCCGC	TGAAGATGGTGCTGACACAGGTGA
hLL-37	GGGACCATGAAGACCCAAAG	CGAAGCACAGCTTCCTTGTA
mB2M	GGTCTTTCTGGTGCTTGTCT	TATGTTCCGGCTTCCCATTCTC
mCFTR	GGGAATCAGCGATGGAGAAA	GAAGAAGGCAGAGCTAGTGAAG
mCyp24A1	ACCACCGCCTAGAGAGATATT	GACAGCAGCGTACAGTTCTT
Cyp2R1 PCR	AGAGACCCAGAAGTGTTCAT	GTCTTTCAGCACAGATGAGGTA
Cyp27A1 PCR	CACAAACTCCCGATCATAGAA	CAAGGACAGCAATGCGATAAAG