**Creation and Characterization of a Cystic Fibrosis Model Cell Line**

**PROJECT DESCRIPTION**

**ABSTRACT**

Recent reports and preliminary results from our laboratory, suggest that the cystic fibrosis transmembrane conductance regulator (CFTR) could be involved in pH regulation. Acid-base balance may be disrupted in cystic fibrosis (CF) resulting in the acidification of the extracellular environment. In order to test the mechanism of this acidification response, it is important to have well characterized cell culture lines that are more similar to CF epithelia. A number of CF laboratories have been using the Calu-3 cell line primarily due to its similarity to lung epithelial tissue. Calu-3 cells may be an excellent candidate as a model of the airway submucosa most affected in CF. However, as yet there is no CFTR-knockout Calu-3 to act as a model for the CF disease state. To this end, we propose to make a CFTR-downregulated Calu-3 (Calu-3/LO) and CFTR-knockout Calu-3 (Calu-3/KO) using two antisense approaches. To accomplish this both antisense oligonucleotide and antisense vector insertion techniques will be applied. Calu-3 cells will be stably transfected with a previously constructed CFTR antisense plasmid. Successful transfecants will be screened using molecular approaches to confirm successful gene insertion and expression in selected Calu-3 lines. In addition, the presence (or absence) of functional CFTR chloride channels will be assayed. Antisense vector expression may lead to complete knockout (Calu-3/KO) in some clonal cell lines and intermediate downregulation (Calu-3/LO) in others. If all properly transfected cells with the antisense CFTR vector exhibit significant, but incomplete knockout of CFTR expression, further downregulation of CFTR will be achieved using antisense oligodeoxynucleotides. Our intent is to generate Calu-3 lines with three levels of CFTR expression; normal, intermediate and knockout. We then propose to use these variably expressing CFTR Calu-3 lines to examine their role in extracellular pH acidification/alkalinization (pHo) more closely. To do this our laboratory will utilize a highly sensitive pH biosensor (microphysiometer) that allows real-time measurement of changes in extracellular pH. Studying different Calu-3 lines will enable our laboratory to demonstrate whether acidification anomalies previously seen in CFTR-deficient lines also exhibit a dose-dependence on CFTR expression. Channel blockers and other treatments can be used in an effort to determine the possible role CFTR plays in pH regulation. In addition to dissecting out the mechanism of pH regulation in CF, these assays may prove useful in the screening for, and rigorous testing of, putative corrective treatments of the disease cystic fibrosis. Yet perhaps most importantly, the successful generation and characterization of Calu-3/LO and Calu-3/KO lines will enable numerous CF research laboratories to test their findings on new, relevant cells that express CFTR at normal, low, and knockout (CF) levels.

**RESEARCH PLAN**

**A. Hypothesis and Specific Aims**

The general research aims of our laboratory are to develop simple assays that can quickly differentiate between cells expressing the cystic fibrosis (CF) disease phenotype versus cells that are ‘normal’ (or ‘corrected’), and to further determine the mechanism by which this differentiation occurs. Later, it may be possible to optimize those assays in the screening for, and rigorous testing of, putative corrective treatments of cystic fibrosis.

More specifically, the current goal of our laboratory is to test the human cystic fibrosis transmembrane conductance regulator (CFTR) for its capacity to regulate cellular pH. As a result of previous reports in the literature and preliminary findings in our laboratory (1) (see Appendix), our hypothesis is that the extracellular pH of the milieu surrounding normal CFTR expressing (CFTR+) cells is less acidic than that of CF (CFTR-) cells and that under optimum conditions a very sensitive pH probe could differentiate normal from CF cells. These findings were made feasible by a new
technology in the form of a silicon chip-biosensor, the microphysiometer (Molecular Devices Corporation, Sunnyvale CA), that can detect subtle changes in extracellular pH (2, 3).

The findings above were based on experiments conducted comparing CFTR expressing cells (2WT2, 3T3/WT) and CFTR deficient cells (NIH-3T3 and C127). These cell lines, however, may not offer the best model system for elucidation of the acidification mechanism and therefore, it is desirable to use cell lines which more closely resemble the epithelial cells most affected by cystic fibrosis.

There is good evidence that the Calu-3 cell line may be an excellent candidate as a model of the lung epithelium most affected in CF (4) however, as yet there is no CFTR-knockout Calu-3 to act as a model for the disease state. To this end, we propose to 1) make and characterize a CFTR-knockout cell culture line of Calu-3 cells using a CFTR antisense vector, 2) if necessary, further extend downregulation of CFTR expression in vector transfected lines using antisense oligonucleotides and 3) continue pH studies using the biosensor to test acidification responses and mechanisms in cell lines variably expressing levels of CFTR.

More specifically, we propose:

1. To create a CFTR-knockout Calu-3 cell line using an antisense plasmid.

Calu-3 cells will be stably transfected with a previously constructed CFTR antisense plasmid (5). Antisense vector expression may lead to complete knockout (Calu-3/KO) in some clonal cell lines and intermediate downregulation (Calu-3/LO) in others. The antisense vectors were previously assembled using a 920 bp EcoRI insert from CFTR exons 1-6 in antisense orientation into pH series plasmids. Sense versions of vector will also be used to generate control lines. Successful transfectants will be screened using PCR, RT-PCR, Western, Northern and Southern approaches to confirm successful gene insertion and/or expression in selected Calu-3 lines. In addition, the presence (or absence) of functional CFTR chloride channels will be assayed via halide efflux techniques.

2. To downregulate CFTR expression further using antisense oligonucleotides (if necessary).

Following transfection, it is possible that all properly transfected cell lines with the antisense CFTR vector will exhibit significant, but incomplete knockout of the CFTR protein expression consistent with other research findings (5, 6). If this is the case, Aim 1 will generate only low expressing lines (Calu-3/LO) which by itself would be extremely useful in testing CFTR dependent findings when contrasted to results from the normal Calu-3 line. It will also be very useful in assays that can compare dosage effects of normal, low (Calu-3/LO) and knockout (Calu-3/KO) CFTR cells. In an attempt to further downregulate the expression of the CFTR, transfected cell lines cultured in a number of antisense oligodeoxynucleotides will be screened. Downregulated Calu-3 antisense vector transfected cells treated with oligodeoxynucleotides (an antisense cocktail approach) will be screened via Western blot and halide efflux for CFTR expression and function.


These cell lines will be used to study acidification/alkalinization responses in the Cytosensor microphysiometer in variably expressing, but otherwise identical cell lines (normal, low, knockout). This affords the opportunity to demonstrate whether acidification anomalies previously seen in CFTR-deficient lines also exhibit a dose dependent response in a manner consistent with the degree of CFTR expression. Since a number of other membrane channels and exchangers could contribute to acid/base efflux, or be under the influence of CFTR, a variety of channel blockers will be used in an attempt to elucidate the mechanism responsible for the acidification/alkalinization response seen in our earlier work (1, 7).
B. Significance

Background and Relation to Present State of Knowledge

Cystic Fibrosis is an inherited (autosomal recessive) disease caused by the mutation of a gene on the long arm of chromosome 7 (8). The cystic fibrosis gene has been cloned and sequenced (9), expressed in various cell lines (10, 11) and its gene product, the cystic fibrosis transmembrane conductance regulator (CFTR), has been shown to be a small conductance cAMP-stimulated Cl\(^-\) channel (12). The mutation of CFTR, most commonly a deletion of phenylalanine 508 (ΔF508), and the concomitant loss of cAMP-stimulated Cl\(^-\) conductance can explain the pathophysiology of many organ systems altered in CF where reduced Cl\(^-\) permeability leads to dehydrated and clogged ducts (8, 12, 13, 14). However, the fatal consequences of CF have been difficult to rationalize solely as a defect in Cl\(^-\) secretion (15). The latest breakthrough in the field indicates that CF lung disease may be due to a loss of endogenous bactericidal activity as a result of changes in the salt composition of airway surface fluid (16). These findings break with the standard explanation of CF lung disease pathology (due to dry clogging mucus) and have renewed interest in alternative defects linked to CFTR malfunction. Defective CFTR also has been reported to lead to changes in other channels Na\(^+\) (17), ORCC (17, 18, 19), mucin secretions (20), SO\(_4\)\(^-\) (21), and pH (22, 23). This last property is at the center of our studies. The literature concerning whether CFTR effects cellular pH isn't yet conclusive and pH analysis may shed light on other pleiotropic effects of CFTR mutations.

Initially CFTR was associated with intracellular pH (pHi) regulation in studies of CF pancreatic cells (CFPAC) (22) and transfected fibroblasts (24). Early findings (24, 25) that suggested endosomal or lysosomal pH was altered in CF were not consistently replicated by later investigations (26, 27, 28). More recently, studies have refocused attention on cytoplasmic pH as well as the capacity of CFTR to directly conduct bicarbonate ions (23, 29, 30, 31, 32, 33) or alter their transport through other pathways (30, 34, 35, 36). However, with few exceptions (37), the above investigations examined intracellular pH (pHi), while the most important CF pathophysiology may be due to abnormalities in the airway extracellular fluid (16).

In addition to well-known extracellular pH abnormalities in pancreatic secretions (38), in 1996 Smith et al reported that the primary cause of CF lung disease may reside in the extracellular milieu, the airway surface fluid (ASF). Antimicrobial agents in the ASF maintain lung sterility (39) and Smith et al found elevated extracellular salt concentration inhibits antimicrobial function in CF. This finding highlighted the importance of characterizing changes in the composition of extracellular fluid surrounding CF epithelia cells.

To examine CFTR's effect on extracellular pH (pHo) more closely, our laboratory has applied a highly sensitive pH biosensor that allows real-time measurement of acid and base efflux rates via assaying changes in extracellular pH (2, 3). The microphysiometer, a semiconductor based instrument, detects the rate of which a cell acidifies its extracellular environment. The device can detect changes in extracellular pH as a result of transient acid/base fluxes such as those caused by short-lived transporter activity, alterations in metabolic rate and changes in intracellular pH (2).

Recently, we have demonstrated using CFTR expressing and nonexpressing cultures (2WT2, C127, NIH/3T3, 3T3WT) that CFTR-expressing cells alkalinize extracellular media in response to forskolin while CFTR-deficient cells (as is the case in the disease) acidify it. This could be the result of either increased base efflux or decreased acid efflux in CFTR-expressing cells (1, 7). The broad number of hypotheses which may explain these observations in part are generated due insufficient knowledge of alternative pathways of bicarbonate secretion and possible artifactual effects of cell immortalization and CFTR cDNA overexpression in nonepithelial cell lines. Well characterized cell lines that are physiologically and biochemically similar to lung epithelia are needed.
Background on Calu-3 cells and Antisense Technology

Moon et al (1997) suggested that Calu-3 cells might serve as a more effective model in the study of cystic fibrosis due to the high degree of biochemical and physiological similarity to submucosal cells. The polarized Calu-3 cell line has been well characterized and studied. Calu-3 cells express CFTR highly, form tight junctions (40, 41, 42), and appear to be the sole, or at least main channel for Cl\(^-\) conductance on the apical membrane (4). Epithelial cells lining the lung do not express much CFTR, and only recently were submucosal gland serous cells found to contain the majority of CFTR expressed in the airways (41, 43). The Calu-3 cell line may be the best CFTR model cell type available since it retains a well-differentiated phenotype resembling primary cultures of airway serous cells. It is also noteworthy that Calu-3 cells have been used in a number of studies that examined the role of CFTR in bicarbonate secretion (31, 32, 44, 45). Their studies, however, may have been strengthened had there been a CFTR-knockout of this cell line to serve as a CF model.

Recently, the development of antisense technology has been gaining ground and interest not only for its possible clinical applications, but also as an effective means of studying aspects of cell physiology, gene expression and protein function \textit{in vitro}. Antisense technology involves Watson and Crick binding of a small target region of RNA by its antisense complement, thus effectively blocking expression of the RNA transcript (6). While a number of newer approaches have been recently proposed, the use of oligodeoxynucleotides exogenously applied, and vector delivery of antisense compounds have been the most widely reported (6).

Because of their promising pharmacotherapeutic applications, the use of oligodeoxynucleotides has been most widely reported and studied of the two strategies. Exogenously applied oligodeoxynucleotides (and their derivatives to avoid action of nucleases) have shown some promise \textit{in vitro} and \textit{in vivo}. Although there is some way to go before the use of these compounds can be used therapeutically (46), they do show promise and have, in a number of studies, been used successfully to downregulate the expression of target genes \textit{in vitro}. However, in these cases, and cases specific to CFTR, the effects of downregulation are transient, being dose and time dependent (46, 47, 48). In addition, antisense work on CFTR has observed that the normally short half-life of native CFTR before application of the oligodeoxynucleotides may be artificially extended following treatment, thus confounding attempts to down regulate expression (47). While it is clear that further synthesis is inhibited, attempts to down regulate CFTR or other target proteins by oligodeoxynucleotides alone, even for short periods of time, may lead to experimental uncertainty with regards to the levels of expression in the cell.

The use of vectors (plasmid and viral) offers advantages over oligodeoxynucleotides, in that the expression of antisense RNA is extended over longer periods of time. Use of plasmid vectors containing small antisense DNA inserts, despite their lower probability of transfection compared to oligonucleotides, nevertheless offers stability since plasmid vector antisense technology is not transient, which make experimentation and replication troublesome. With the use of plasmid vector antisense technology alone, however, initial \textit{in vitro} attempts to down regulate CFTR have lead to partial, but not complete downregulation of the protein (5).

While both techniques have their shortcomings, both show promise in developing partial knockout variants of cell lines. To the best of our knowledge however, there have not been studies that have examined the effects of dual antisense treatment with oligodeoxynucleotides and expression vectors in an attempt to extend downregulation of a target gene product (an antisense cocktail). In this study, we propose to: 1) establish and characterize a knockout version of Calu-3, using an antisense CFTR expression vector, 2) if necessary, treat successful vector transfectants further using a number of oligodeoxynucleotides in order to extend downregulation of CFTR and to 3) test these variably CFTR expressing cell lines (normal, low and knockout) against our early pHo studies. Using the microphysiometer, we would like to determine whether these lines also show dose-dependent changes in alkalization/acidification curve in response to CFTR downregulation. In addition we will be investigating the efficacy of a two level antisense approach to downregulating CFTR in Calu-3 cells. Yet perhaps most importantly, the successful generation and characterization of Calu-3/LO and Calu-3/KO lines will enable numerous CF research laboratories to test their findings on new, relevant cell lines that express CFTR at normal, low, and knockout (CF) levels.
C. Experimental Design and Methods

Specific Aim 1: To construct a CFTR-knockout Calu-3 cell line using an antisense plasmid.

Hypothesis: Transfection with an antisense CFTR plasmid will lead to significant (yet perhaps incomplete) downregulation of CFTR expression in Calu-3 cells.

A) Transfection

The first step in achieving this goal, the construction of CFTR antisense plasmid vectors (5) has already been accomplished. Three sets of vectors were assembled by cloning a 920 bp EcoRI CFTR cDNA fragment in both sense and anti-sense configurations in pJΩ, a well characterized and effective mammalian plasmid expression vector. Ann Harris has kindly provided our lab with six plasmid vectors, three sense (s) and three antisense (a/s) expressing vectors: pJ4Ωs, pJ4Ωa/s, pJ5Ωs, pJ5Ωa/s, pJ5ΩEs and pJ4ΩEa/s (Figure 1). These constructs use the same sense and antisense inserts and differ only in the promoters used. The pJ4 vector series (49) contains the Mo-MULV promoter while the pJ5 series uses MMTV LTR as the promoter, and pJ5E containing the same promoter as the J5 series but also includes an MSV enhancer.

Wardle and Harris (1995) were able to demonstrate successful transfection and downregulation, but not complete knockout of CFTR expression in antisense vector transfected HT29 cell lines, using the pJ4Ω series plasmid. In addition, they were also able to demonstrate a reduction in Cl efflux in these same lines. Although they did not estimate the degree of downregulation from their halide efflux data, it appears that transfected lines had reduced expression of CFTR to <10%.

Calu-3 cells will be stably transfected with each of three control sense CFTR plasmid vectors, and three antisense CFTR plasmids. Since the plasmid does not contain a mammalian marker, the cells will be cotransfected in a 1:9 ratio of pSVneo and pJΩ plasmids. Following successful screening of transfectants, the cell lines will be characterized for expression of CFTR.

B) Characterization

In order to determine the downregulation of CFTR expression in vector transfected Calu-3 cells, Southern, PCR, Northern, RT-PCR, Western, and halide efflux assays will be performed on successful (G418 resistant) transfectants. Based on previous in vitro knockout studies (5, 46), it is expected that lines successfully transfected with antisense plasmid vector will exhibit downregulation, but perhaps not complete suppression of, CFTR expression. If that is the case, see Aim 2.

1) Identification of clones: Successful neomycin resistant clones will be screened for vector and CFTR cDNA. Since growth in a neomycin culture is already confirmatory for the pSV2neo plasmid, it will not be analyzed further. Proper orientation and insertion of the 920 bp CFTR insert can be assayed by PCR amplification using 20 bp primers from the vector which immediately flank the insert. In this way, we should determine the presence of the insert, and the proper size and orientation. Additional confirmation of presence and relative copy number will be obtained by Southern blot using a radioactive oligo probe for the CFTR insert. In this analysis, two separate approaches could prove useful, especially if one assay is problematic.

2) Examination of native CFTR gene and antisense plasmid expression (RNA): Expression of RNA in control and vector transfected Calu-3 cells will be examined by Northern blot using a radioactive oligo probe for the CFTR insert. In this analysis, two separate probes can be designed to bind either expressed sense or antisense (vector derived) RNA. RT-PCR will also be run on RNA extracts to generate cDNA from stably transfected and control cell lines. PCR oligo primers for sense and antisense sequences can be used to determine the presence of either of these RNA sequences. This will give some indication of the general expression of vector and native RNA expression. Once again, the capacity to perform either of two assays may be advantageous.

3) Expression of CFTR protein: Western blot will be performed to determine expression of CFTR in stably transfected and control cell lines. The presence of CFTR will be determined by Western blot analysis with the anti-CFTR antibody, α-1468 (1).
4) Chloride channel function: Halide efflux assays via I-125 or Cl\(^-\) selective electrodes, will be performed in order to detect activity of the CFTR channel. Since the only channel responsible for cAMP-activated Cl\(^-\) flux in Calu-3 cells is the CFTR (4), this test should be sensitive to the residual expression of any CFTR.

Figure 1. **Schematic of the pJ4\(\alpha\) vector series constructed by Wardle and Harris (1995)**. The pJ4 series is composed of a mammalian promoter, flanking polylinker, an intron and a termination sequence which are linked to a pBR 322 backbone. The 920 bp CFTR insert from exons 1-6 was ligated into the EcoRI site in sense and antisense orientations. In the same way vectors for the pJ5 and pJ5E series were also constructed. The pJ5 series is the same as the pJ4 series except that the pJ5 plasmids use the MMTV LTR promotor, while 5E series uses the same promoter and a MSV enhancer.

**Specific Aim 2: To downregulate CFTR expression further using a number of antisense oligonucleotides (if necessary).**

Hypothesis: Additional treatment with oligodeoxynucleotides will lead to further downregulation of vector transfected cell lines.

**A) Incubation**

Several studies using CFTR antisense oligodeoxynucleotides have been performed on cell culture lines *in vitro* and demonstrated downregulation of CFTR. We propose to replicate the most successful oligonucleotide sequences tested in these studies as well as screen several other sequences. We will incubate cells with oligonucleotides from the initiation site (46, 47), and downstream sequences 14-37 (48) and 19-36 (46). Despite the fact that many studies have been using modified sequences in order to reduce the effects of nucleases, notably phosphorothioate oligodeoxynucleotides, nucleases are less of a concern in *in vitro* studies that allow for the insurance of continual delivery of the antisense RNA as long as experimental conditions persist.
B) Characterization

Given that vector transfected cells will only exhibit low CFTR (<10%) expression, initially only the most sensitive assays will be applied to oligodeoxynucleotide treated cultures to determine CFTR expression levels. Treated cell lines will be screened for the expression of Cl- channel activity by RT-PCR and halide efflux (see above). After potential knockouts conditions (which oligonucleotide is most effective at what concentration) are identified, the exact conditions that optimize knockout will be determined. Our goal is also to elucidate the timecourse of downregulation over days of exposure to determine the optimal conditions that lead to knockout. When conditions are well understood and the knockout of CFTR can be repeatably replicated, our goal will be to complete a full characterization (e.g. Northern, Southern Western etc see above) of the Calu-3/KO and publish both a full protocol and characterization for generating these knockouts.


Hypothesis: The extracellular pH of the milieu surrounding normal (CFTR-expressing) cells would be less acidic than that of CF (CFTR-deficient) cells.

In previous microphysiometer experiments (1) we observed that forskolin-stimulation caused an extracellular *alkalinization* of CFTR-expressing cells while the same conditions elicit an *acidification* response to in CFTR-deficient cell lines (Figure 2). Our observations appear to be solely dependent on CFTR expression. Yet these experiments were not ideal. They were performed in cell lines that were different in a number of respects to lung serous cells. In addition, we did not have access to similar cell lines which express much lower levels of CFTR which would allow controlling for 'CFTR-dosage.' As a result, we propose the establishment of two CFTR downregulated Calu-3 cell lines (Calu-3/LO, Calu-3/KO) for further microphysiometer (and other) studies.

The microphysiometer utilizes a very small flow chamber (the sensor chamber) in which living cells are continually perfused by warm lightly-buffered media. Cells grown in culture conditions are moved to the flow chamber on their permeable support and remain in very similar conditions during the pH experiment. During the assay, cells continue to grow on a polycarbonate membrane, and are in diffusive contact with a silicon chip pH sensor, called a "light-addressable potentiometric sensor" (LAPS) (3, 50). The LAPS determines the pH around the cells and samples every second. It displays raw extracellular pH as millivolts that are determined in a Nernstian fashion (61mV per pH unit change at 37°C) from the sum of a continuously applied voltage and the voltage at the surface of the chip which changes relative to acidification at its surface(2). During perfusion the raw extracellular pH is clamped to the pH of the perfusion media (usually pH 7.4). Thus to assay the acid or base efflux rates, rate constants (extracellular acidification rates) are generated. During periodic interruptions of fluid flow, which causes transient acidifications of < 0.1 pH unit, the change of raw pH over time is calculated and plotted as the extracellular acidification rate. Two fluid paths are associated with each flow chamber, and both data acquisition and fluid path selection is controlled by a Power Macintosh computer.

In these studies, one microvolt per second (µV/sec) corresponds closely to an acidification rate of one milli pH unit/min. When rates of acidification are compared across different chambers in which the absolute number of cells varied, the data will be normalized to account for variation in acidification levels between chambers. The final data represented in figure 2 are extracellular acidification rates that have been normalized prior to forskolin stimulus.

Extracellular pH (pHo), specifically that of airway luminal fluid, might be controlled by the conductance of bicarbonate through CFTR's pore as well as other carriers. To examine CFTR’s effect on extracellular pH (pHo) more closely, our laboratory will apply the sensitive pH biosensor to the Calu-3, Calu-3/LO, Calu-3/KO cell lines.

A) pH characterization.

These studies will extend our findings from previous experiments with various transfected cell types to cells with native CFTR expression derived from a critical cell type in the CF lung. Based on our previous findings, stimulation with forskolin should cause the greatest extracellular alkalinization in Calu-3 cells fully expressing CFTR. Cells that express CFTR at low levels (Calu-3/LO) should
show a dose sensitive decrease in extracellular alkalinization response based on the level of CFTR expression. Knockout cell lines (Calu-3/KO) should show no alkalinization but an acidification response.

Cyclic AMP modulators (forskolin, cpt-cAMP, 8-bromo cAMP, IBMX (isobutylmethylxanthine)) and controls (e.g. dideoxyforskolin) will be used to assay and pharmacologically dissect the pathway of this forskolin response. Metabolic stimulants that do not elicit CFTR’s channel activity will also be tested. Potential regulation by PKC (which does phosphorylate CFTR but alone effects no stimulation in channel activity), and the calcium second messenger pathways will be tested (e.g. TPA/PMA, ionomycin, thapsigargin, carbachol, EGTA, BAPTA). General metabolic stimulants (A23187, insulin, metabolites/sugars, stress) will also be tested for their capacity to elicit this signature CFTR-dependent response that previously has been shown to vary reliably between CFTR and control -transfected cell lines. We will also test agents that cause significant decreases in CFTR Cl- conductance for their effect on CFTR inhibition of extracellular alkalinization. Glibenclamide, DPC (Diphenylamine-2,2'-dicarboxylic acid), NaSCN, or NaI have been reported to reduce CFTR channel conductance. These will be tested for a capacity to uncouple wild-type CFTR expression from alkalinization and other nonspecific effects.

![Graph showing normalized acidification responses of CFTR-expressing (3T3/WT) and CFTR-deficient (NIH/3T3) NIH fibroblasts.](image)

**Figure 2.** Effect of 10 uM forskolin treatment on normalized acidification responses of CFTR-expressing (3T3/WT) and CFTR-deficient (NIH/3T3) NIH fibroblasts. Fibroblasts expressing CFTR showed greatly reduced acidification rates (extracellular alkalinization) in response to forskolin treatment while fibroblasts not expressing CFTR increase extracellular acidification. After forskolin washout, the CFTR-expressing cells continue to show a reduced acidification rate when compared to controls. (* indicates significance when compared to controls at P< 0.05; error bars as S.E.M.).

**B) Characterizing alternative pathways.**
The observation that CFTR-expressing cells alkalinize their extracellular environment, while CFTR-deficient cells acidify it, is perhaps most easily explained through a mechanism of bicarbonate secretion through the CFTR. However, our previous observations do not only support this hypothesis. Bicarbonate movement is only one possibility and CFTR may only play a passive role in facilitating bicarbonate flux through traditional carriers. To test whether bicarbonate is the primary source of alkalinization, inhibition of carbonic anhydrase by acetazolamide (29) should reduce the generation of cytoplasmic bicarbonate and reduce the signal. If bicarbonate is identified as the agent, pathways other than CFTR will be examined. The anion exchanger (AE) may be responsible for bicarbonate secretion, and CFTR could possibly be playing a secondary electrogenic role to this carrier. Blocking the AE by DIDS (29) should offer some insight into the role of this channel. If CFTR-expressing cells under inhibition of DIDS continue to alkalinize their environment in relation to CFTR-deficient lines, this would be better evidence for the movement of bicarbonate through CFTR. Alternatively, CFTR may have some regulatory influence on the adjacent Na/H exchanger, resulting in suppression of its activity, thus reducing proton efflux. In the absence of functional CFTR then, proton efflux is not inhibited resulting in extracellular acidification. This will be tested by using amiloride derivatives (29) or DEPC, both blockers of this pathway. The microphysiometer allow these, and a number of other tests to be performed on cell lines expressing variable levels of CFTR in a non-invasive way. In a similar way, once the role of CFTR is more clearly understood, the microphysiometer offers the opportunity in the future to test therapeutic drugs in an attempt to ameliorate the pathophysiological state of this disease.

E. Percentage of Time on Project
The principal investigator will devote 40% of his time to the project. Since the PI has a large teaching load at MSU, both the undergraduate, Errett C. Hobbs and the postdoc John H. Wilterding will work full time on this project to assist in its progress.

F. Future Funding Sources
This project is key to the long term goals of our research laboratory. The successful generation and characterization of these CF model cell lines would lead to strong support from the CF research community and the sharing of the cell lines with other investigators would create bridges of research collaborations and publications that would greatly increase our ability to gain external funding. Here are several examples of opportunities for future funding:

Title **R01 Research Grant**: Sponsor: *National Institutes of Health* (NIH), National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) or National Heart, Lung, and Blood Institute (NHLBI). Both NIDDK and NHLBI support basic research into the mechanisms responsible for the lung pathophysiology responsible for cystic fibrosis.

Title: **CFF - NIH Funding Award**, Sponsor: *Cystic Fibrosis Foundation (CFF)*. The objective of this award is to support excellent Cystic Fibrosis (CF) related research projects that have been submitted to and approved by the National Institutes of Health (NIH), but cannot be supported by NIH funds. Support from the CFF, through various mechanisms is intended to provide for the development of sufficient preliminary data to make CF grand related application highly competitive in the NIH review process. Applications must fall within the upper 40th percentile with a priority score of 200 or better.

Title: **CF Research Grant**: Sponsor *Cystic Fibrosis Research, Inc*. A private institute has previously supported my work while I was working at Stanford University. They also would be interested in the dissemination and distribution of cystic fibrosis model cell lines. CFRI, 560 San Antonio Road, Suite 103, Palo Alto, CA 94306.

Title: **Research Award**: Sponsor: *The Whitaker Foundation*. A private institute, the Whitaker Foundation, supports innovative use and creation of technology (antisense approaches and silicon chip based microphysiometry) to solve biological problems (like cystic fibrosis).
G. Literature Cited