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Kirtishri Mishra
Case Western Reserve University

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Gastrointestinal Histology in Knock Out Mouse Models and Temporal Induction of CFTR Protein Function in Mice

ABSTRACT

Cystic Fibrosis (CF) is a fatal hereditary disease that is characterized by gastrointestinal, pancreatic, and pulmonary disorders. CF is caused by a mutation in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. An inherited condition, CF causes wide defects in epithelial tissue function in an organism. This ubiquitous affect poses a challenge for researchers studying the disorder, because it is difficult to distinguish primary and secondary effects from the result from CFTR mutation. Knock-out (KO) mouse models have provided a way for researchers to delete CFTR functions from specific tissues. In order to fully understand this disease, we created a mouse model that allows us to activate/deactivate CFTR function in cells at any given time point using a drug called tamoxifen (TM). We also created a panel of mouse models showing variable levels of CFTR function. We used these mice to study the correlation of CFTR function with growth and survival seen in mice. We also used these same animals to determine if there is any correlation between the number of mucus producing cells (goblet cells) in the intestines and CFTR activity. It was established that CFTR function is necessary in the intestines for survival but it does not affect growth. Furthermore, in general animals with CFTR function reduction or deletion lead to higher number of goblet cells.

BACKGROUND

Cystic fibrosis (CF) is a hereditary disease affecting the exocrine (mucus) glands of the lungs, liver, pancreas, and intestines, causing progressive disability due to multisystem failure. The presence of CF is directly related to the absence of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein, which is responsible for the transport of chloride ions, and water molecules across epithelial layers throughout the body (1). The presence of CFTR proteins allows individuals to maintain a thick, slippery substance called mucus that lubricates and protects the linings of the airways, digestive system, reproductive system, and other organs and tissues throughout the body. However, patients with CF, who lack functional CFTR proteins, form mucus that becomes thick and obstructs the organs that they would normally lubricate. This accumulation of thick mucus in organs, such as the lungs, can provide an area of bacterial growth, which can lead to

Kirtishri Mishra

Kiritishri Mishra is a senior Biology student at Case Western Reserve University. He aspires to study medicine after completing his undergraduate degree. Kirtishri has been an active member of the Department of Pediatrics at Case Western for four years. He is also involved in service projects at the Free Clinic of Greater Cleveland and the University Hospitals. Kirtishri is an avid sports fan, and loves to play tennis and basketball.

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chronic lung infections. Over time, mucus buildup and infections result in permanent lung damage, including the formation of scar tissue (fibrosis) and cysts in the lungs.

Most people with cystic fibrosis also have digestive problems because thick, sticky mucus interferes with the function of the pancreas. The pancreas, a gland in the digestive and endocrine system, produces insulin, a hormone that helps control blood sugar levels. The exocrine pancreas makes enzymes that help digest food. In people with cystic fibrosis, mucus blocks the ducts of the pancreas preventing these enzymes from reaching the intestines to aid digestion. Problems with digestion can lead to diarrhea, malnutrition, poor growth, and weight loss. Some babies with cystic fibrosis have meconium ileus, a blockage of the intestine that occurs around the time of birth (1).

The development of transgenic mice bearing null mutations of different genes encoding channel proteins has opened a large field of investigation, which allows a better understanding of the physiological mechanisms controlled by various proteins. This novel method allows researchers to induce mutations in proteins in desired tissues and conduct a genome wide study of the affects.

CF studies have made a prolific use of the system applying the transgenic mouse scheme to study the effect of CFTR deletion in specific tissues. Dr. Craig Hodges (Department of Pediatrics, Case Western Reserve University), in coordination with Dr. Mitchell Drumm's Lab (Case Western Reserve University), has developed mice that show varying severity of CFTR deletion in the intestines. The purpose with this mouse model is to determine the correlation of CFTR activity in the organism with the survival and growth of the mice populace being studied. We will also study the correlation between CFTR activity in the intestines and the number of goblet cells present in the epithelia of the intestines. Furthermore, Dr. Hodges has created an inducible mouse system in which the CFTR gene can be activated or inactivated by tamoxifen-induced recombination. The strategy is to activate the CFTR gene in CF mice of different ages and assess the critical times of this activation on the various disease phenotypes. Before the phenotypic changes in response to CFTR activation can be assessed, it is necessary to know how well the activation system works (2).

INTRODUCTION

In order to fully understand the tamoxifen-induced system, it is integral to introduce the mechanism behind the knock out (KO) mice that we have used for our study (Figure 1). Dr. Hodges has created these two conditional CFTR mouse models using the Cre/Lox system. The Cre/Lox system is a scheme by which small segments of DNA between two LoxP sites (34 base pairs of a specific DNA sequence) can be recombined in the presence of an enzyme called Cre-recombinase (3). Dr. Hodges created two versions of the CFTR gene with LoxP sites in it. In one case, the presence of Cre catalyzes the excision of CFTR Exon 10 ,inactivating the gene (Figure 1A). In the other case, the LoxP sites were inserted in such a way as to create an inversion of Exon 10 in the presence of Cre. The reason for the focusing on Exon 10 from the mice is because Exon 10 is known to be necessary for CFTR function in humans and mice. Consequently, the deletion of Exon 10 results in creating mice that display similar phenotypic attributes to those that have CF. The second mouse model (Figure 1B) does not cause deletion of Exon 10 but rather contains an inverted Exon 10 that prevents a normal mRNA transcription and thus is inactive. However, when these inverted LoxP sites are catalyzed using Cre-recombinase, they reverse the inversion causing CFTR to be fully functional again (1).

The initial studies of these mice have been to express Cre in specific tissues and investigate the effects of losing or gaining CFTR function in one tissue or cell type at a time. The immediate goal of the study is to determine the optimum concentration of TM and the time span of treatment that will yield the highest rate of desired recombination. In order to determine the optimum conditions a reporter system was used in which the presence of TM induces the recombination of an inhibitory sequence, allowing a ubiquitous gene called ROSA to be expressed. This gene is responsible for coding for β-Galactosidase enzyme which can be detected through X-Gal staining (4). Therefore, the efficiency of TM induced recombination can be measured by the percentage of cells that turn blue after staining due to the presence of B-gal. The optimized conditions can then be used to induce recombination and thus inactivate or activate CFTR in the mouse.

The TM inducible system was tested in an in vitro system.

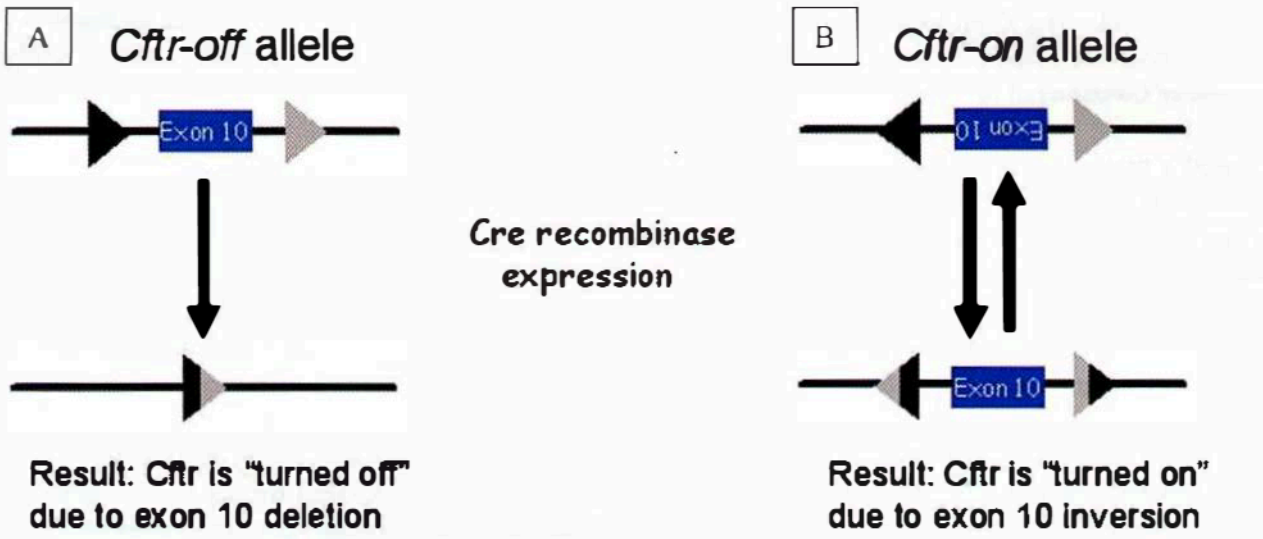


Figure 1: A schematic representation of the mechanism used to create knock out mice. Two small, 34 base pair sequences, called LoxP sites, are placed around Exon 10. A) Presence of the enzyme Cre Recombinase in similar orientation in a specific tissue causes the recombination of the floxed Exon 10, which is then degraded in the cell. B) An inverted orientation inverts Exon 10, but in the presence of Cre Recombinase the Exon 10 is re-inverted to its proper orientation, and activates CFTR function.(Courtesy of Dr. Craig Hodges.)

There are several advantages to conducting an in vitro examination at this stage of the study. First, it allows us to ensure that TM is administered uniformly to all the cells, instead of being available to a limited number of cells in an in vivo model. Second, it allows us to study CF pathogenesis at any given time point in the mouse life cycle, even prior to birth, because we are able to remove the fetus from the mother and examine the function of CFTR in undifferentiated fibroblasts. Third, it allows us to study CFTR function in a tissue while it is isolated from organismal mechanisms from other tissues. Finally, it allows us to maintain an ideal control because we can study the differences between treated and untreated cells of the same animal.

As stated earlier, individuals with CF often experience poor growth and survival, an attribute that has traditionally been attributed to malnutrition as a result of intestinal obstruction. Another common phenotype seen in CF patients is Goblet cell hyperplasia (abundance) (5). Goblet cells are mucus producing cells that are found scattered among other cells in the epithelium of many organs, especially in the intestinal and respiratory tracts. In the second part of the study we looked at a panel of conditional mouse knock-out models that display varying levels of CFTR activity to examine the relationship of CFTR function in the intestine. A total of four models was studied. The first was the control showing 100% CFTR function. The second model was the hypomorph showing approximately 5-10% CFTR function. The third model, called the FABP, did not show any mouse CFTR activity – instead a human version of the CFTR (hCFTR) was expressed in the intestine. Finally, the fourth model was a complete KO of CFTR function.

It has also been seen that patients suffering from CF show an increased production of mucus (6). The reason for this phenomenon can be attributed to the systemic response to the lack of proper amount of mucus needed to lubricate the endothelial cells of organs. However, this overproduction of mucus feeds into the vicious cycle of obstruction because the malfunction of the CFTR protein results in increased viscosity of the mucus. Therefore, another attribute that was examined was the number of goblet cells in the intestinal tissue. The abundance of goblet cells was quantified and correlated to the frequency and severity of obstruction in the knock out animals.

METHODS

Generation of Construct

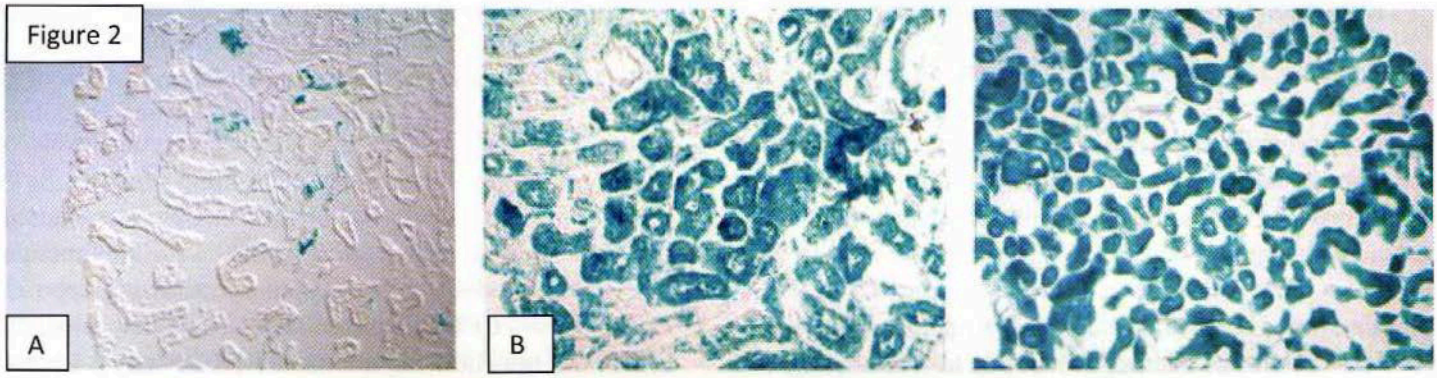
We crossed a mouse line that showed inducible Cre-recombinase expression to another mouse of interest, such as the turn-off mouse (Exon 10 flanked by LoxP sites), turn-on mouse (Exon 10 flanked by inverted LoxP sites), or the Rosa-β-Gal mouse (negative operator flanked by LoxP sites). These crosses gave us offspring that were sensitive to the presence of TM and resulted in recombination of the flanked region upon TM administration.

Fibroblast Plating, Induction, and Staining

At 12-14 days post coitum fetuses were removed from the pregnant mother. The developing brain and liver were removed from the fetuses, and the rest of the tissues were minced. The tissues were allowed to digest in trypsin for 30 minutes. Following digestion, cells were plated. Cells were split into three wells several days later and plated at a concentration of 50,000 cells in each well on a six-well plate. Two wells were treated with TM at varying concentrations, while one of the wells was maintained as a control with no treatment. After 48 hours, TM was removed from the cells, and X-gal staining was performed. Cells that underwent recombination turned blue (Figure 2). A qualitative analysis was done to determine the percentage of cells that turned blue.

Recording Growth and Survival

Growth rate and survival was recorded on each of the four strains of mice showing variable levels of CFTR activity. Data was accumulated on each mouse from birth till the 40th day of their life cycle. The new litters were weaned



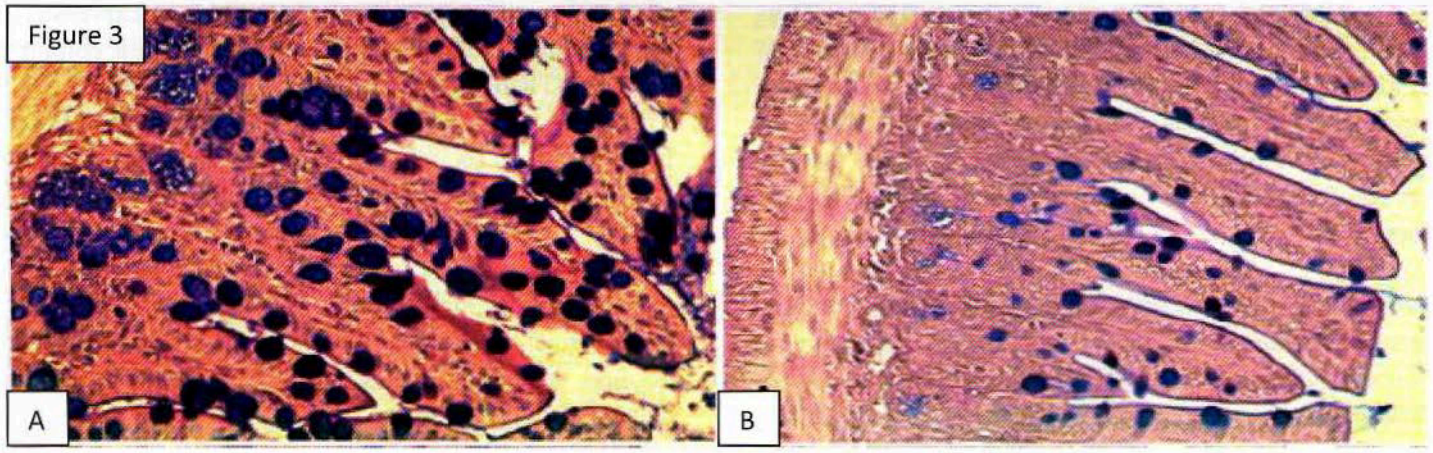


Figure 2: Renal tissue segments after X-gal staining. A) No TM treatment B) 3mg of TM treatment C) 9mg treatment of TM treatment. All treatments were performed for 48 hours (2).

Figure 3: Intestinal cross sections. A) An intestinal segment from the ileum of a CF mouse. B) An intestinal cross section of a wildtype animal. The number and size of goblet cells in wildtype animal is frequently lower. Only the cells on the periphery of the villi were counted.

from the parents' cage at day 20. Males and females were placed in separate cages. Growth was recorded every five days as a measurement of the animal's weight through this period. Survival was recorded with respect to the rate of mortality seen in the populace of a specific strain. A minimal ten animals were examined for each strain. Statistical analyses were performed to verify correlation between the observed attributes and CFTR activity levels.

Gastrointestinal Histology – Quantification of Goblet Cells

Paraffin-embedded sections (5 μm) were stained with dye for the evaluation of goblet cell hyperplasia in the mice showing variable levels of CFTR activity. A thousand cells were counted from the peripheral lining of the intestinal villi (Figure 3). Goblet cells were included in the total of thousand cells; however, the number of goblet cells was also recorded to determine the ratio of goblet cells to epithelial cells. Varying sections from two different slides were examined in order to gain a wide sampling of the tissue segments. Statistical analyses were performed to verify correlation of CFTR activity to number of goblet cells for each strain.

RESULTS

Tamoxifen Inducible System and X-Gal Assay

Much of our work with the inducible system thus far has dealt with establishing a protocol that will yield reliable results. This process has encompassed a great deal of trial and error and has given us some preliminary parameters that we hope to build upon in the future.

By varying the concentrations of TM treatment we have established that the fibroblasts are unable to survive at TM concentrations above 1μM. 48 hours for recombination also seems to be an optimum time for treatment duration. Time points below this treatment period show significantly reduced recombination rates, and treatments longer than 48 hours have shown no significant increase in recombination. Longer durations of treatment can also lead to significant cell mortality.

We have also had to make significant modifications to our X-gal protocol, adjusting the time of the perfusion step and adjusting the pH of the X-gal solution. The protocol has been inconsistent to this point; however, our modifications to the starting protocol have shown improvement in the results.

Growth and Survival

We examined the growth and survival of the strains of mice that showed variable quantity of CFTR activity. Figure 4 presents the data for growth seen in the four strains of mice being studied. It was seen that the wildtype mice, showing 100% CFTR activity, grew the most (measured in grams). The complete KO mice grew the least consistently showing half the weight of the wildtype animals. The R117H and the FABP mice showed similar growth and survival patterns (Figure 5). They showed a significant ($t<.05$) decrease in growth from the wildtype; however, survival was almost equivalent in these animals to the control group. A slight increase in mortality was seen around day 21 in all strains; however, in the complete KO survival decreased drastically around day 23. The survival rate decreased from approximately 85% on day 20 to 20% on day 26. This is compared to 93% survival in R117H, and 95% survival in both FABP and wildtype control on day 26.

Goblet Cell Number

Cells from two slides were counted in order to quantify the percentage of overall cells that were goblet cells. For each animal, 1,000 cells were counted. T-tests were performed on the group data. A significant increase was seen in the number of goblet cells in the hypomorph as compared to the control group. The hypomorph showed an average of 222 goblet cells/thousand cells, whereas the control group showed an average of 157 goblet cells/thousand cells. The complete KO also showed goblet cell hyperplasia; however, no statistical significance was reached. Finally, the FABP showed a decreased level of goblet cell number compared to the control. Many of these groups do not have a large enough sample size at this point.

DISCUSSION

Tamoxifen Inducible System

It has long been established that Cystic Fibrosis is caused

by a mutation in the CFTR gene. Despite rigorous studies, researchers are uncertain of the primary and secondary manifestations that result due to direct loss of CFTR function. In order to distinguish some of these manifestations, Dr. Hodges has created mouse KO models in which we can delete CFTR function on a the function of CFTR in specific tissues by observing how these deletions affect the overall phenotype of the animal.

Along with studying the manifestations of the disease phenotype, it is also important to determine the pathogenesis of the condition. In order to accomplish this we have created a inducible KO system in which we can control the times in the mouse life cycle when CFTR function is either deleted or activated – referred to as turn-off and turn-on systems respectively. We have also created a reporter model that allows us to determine the concentration and duration of treatment that will give us the highest percentage of desired recombination in cells.

Thus far, we have used the reporter system to determine that the optimum concentration for TM treatment is somewhere between 500nM and 1μM. Concentrations below the lower limit do not seem to induce significant amounts of recombination. On the other hand, concentrations of TM breeching the upper limit become toxic to the cells. The reason for this toxicity can be attributed to the oncogenic qualities of TM. At high enough concentrations, TM can interfere with normal metabolic processes in cells, thereby, resulting in cell mortality.

We have also established that the optimum duration of treatment for the cells is 48 hours. Similar to concentrations, we must establish a time period that will give the cells an ample opportunity to respond to TM treatment; however, we do not cells to become over exposed to TM leading to death.

The optimization of this protocol would allow conditional activation of the CFTR gene. This approach will allow researchers to circumvent problems associated with germline ablation and early embryonic lethality caused by a standard knockout or over expression approaches, thereby allowing a more complete analysis of gene function. In future experiments, we aim to conduct in vitro studies of primary cells, which would allow us to examine the affect on gene expression with the conditional knock out of CFTR function, thereby allowing us to gauge the effects of the gene in genome wide expression studies.

Growth and Survival

One of the most common phenotypes characterized in CF patients is poor growth and malnutrition. Traditionally this phenotype has been solely attributed to the lack of CFTR function in the intestinal epithelia. Lack of CFTR function in the intestines causes the buildup of viscous mucus and feces resulting in obstruction and the inability of the intestines to absorb proper nutrients. In order to validate, this idea we created a panel of mouse KO models that showed varying degrees of CFTR activity in the intestine. The purpose of this study was to determine the correlation between CFTR activity in the intestine, and growth and survival in mice.

As seen in Figure 4, there is a strong correlation between CFTR activity and growth for the mice that have full CFTR function throughout the body as opposed to no CFTR function in the complete KO. However, the hypomorph (R117H) does not clearly establish a correlation. The hypomorph is a strain that expresses 5-10% CFTR function in the organism, however, the strain does not show a close similarity to the wildtype growth as would be expected if the intestines were the sole cause of malnutrition. Therefore, attributing the whole growth phenotype to the intestines is not validated with our model. Our last model, the FABP, is essentially a whole KO, but it contains a human version of the CFTR gene in the intestines. The FABP showed a very similar growth phenotype to the hypomorph. It was interesting to see that restoration of CFTR function in the intestines still gave us a growth phenotype that was more similar to the complete KO than the wildtype. This data reaffirms our conclusion that growth phenotype is associated to other tissues besides the intestines.

Survival patterns showed some interesting results. As expected the control showed a very high survival rate, whereas the complete KO showed a drastic decrease in survival over time. By the time the animals reach day 30 of their life cycles, only 20% of the complete KO animals survive, as compared to ≈95% survival in wildtype. This decline can be attributed a significant point in the mice life cycle in our study, because at day 20 the mice are weaned

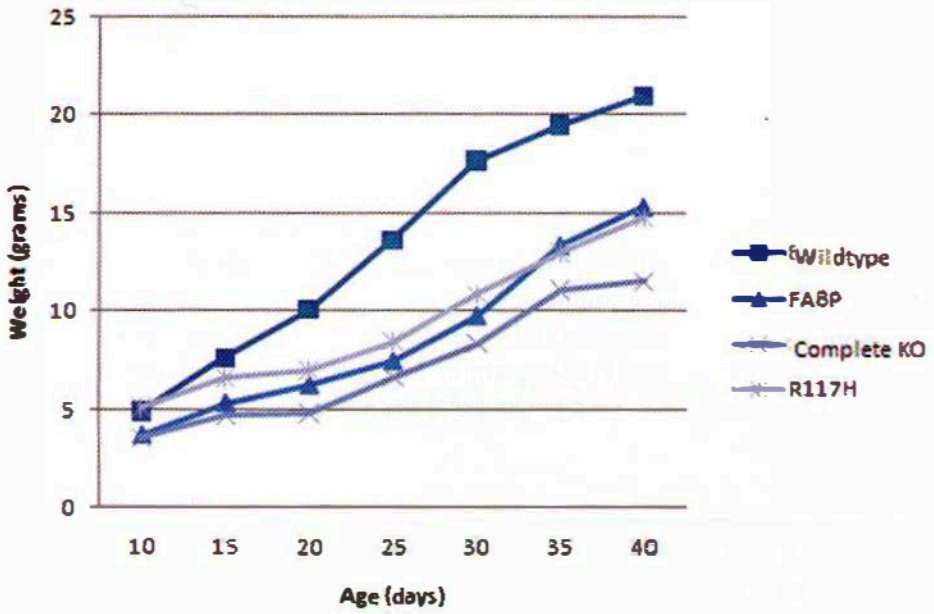


Figure 4: Weight of four different strains of mice showing variable quantities of CFTR activity. The four mice represented are the wildtype, the complete KO, the hypomorph, and the intestinal CFTR restoration. The wildtype animals with 100% CFTR activity show the greatest growth, whereas the complete KO shows the least amount of growth. The FABP and R117H strains show similar growth attributes to each other, and they are closer to the CF phenotype than the wildtype phenotype (Courtesy of Dr. Craig Hodges).

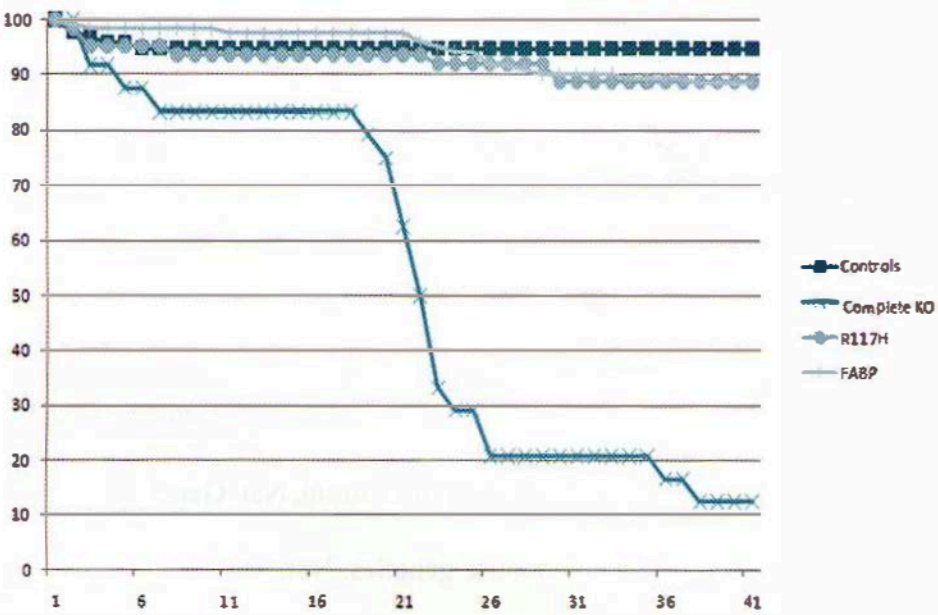


Figure 5: Survival of the four strains being studied. Survival is similar in the wildtype, the gur corrected FABP, and the hypomorph (R117H); however, there is a great deal of mortality in the complete KO around day 21 (Courtesy of Dr. Craig Hodges).

from the parent cages and placed in new cages separated by sex. We believe the sudden decline in survival is a direct result of the change in the type of food the mice consume. When the mice are with their mothers, they are able to be nurtured by the lactating female; however, in the new cage they must consume the solid food. This could exacerbate the obstructive condition in their intestines and lead to an increased death due to obstruction.

We also see that the R117H and the FABP model show a very high rate of survival – very similar to the wildtype. Therefore, we can conclude that there is a very strong correlation between CFTR function in the intestines and survival. This phenomenon could occur due to sufficient restoration of CFTR function that could lead to prevention of obstruction; however, it does not address the issue of malnutrition.

Goblet Cell Number

Goblet cells are mucus producing cells present in the epithelial lining of various organs. A common phenomenon seen in CF patients is an increase in goblet cell number, a condition known as Goblet Cell Hyperplasia. The pur-

pose of this part of the study was to determine if there is a correlation between CFTR activity and goblet cell number. The same strains of mice that were used to study growth and survival were used in this study.

In our model it was seen that there is a general trend of goblet cell hyperplasia in animals that had complete or partial CFTR activity deletion. However, aside from this general trend there is no definite relationship between the gradations of CFTR activity and goblet cell number. For example: it was seen that the complete KO showed a higher number of goblet cell number in the intestines than the wildtype. However, the R117H model, which has 5-10% CFTR activity showed a significant increase in goblet cell number, even more than the complete KO. Finally, it was seen that the strain with hCFTR restoration in the intestines showed a lower number of golblet cells in the intestines.

At this point we are uncertain of the statistical significance of the data because most of the group data is composed of approximately three to four animals, amongst which the FABP only has one animal data. More animal intestines need to be quantified in order to verify the trends.

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