

Therapeutical studies of the disrupted CFTR gene in affected sheep and rabbit models produced by genome editor CRISPR/Cas9

Cystic Fibrosis (CF) is a life-threatening, autosomal recessive disorder caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. Patients with CF have a decreased lifespan due to complications with lung infections/disease, decreased airway function, and persistent cough with phlegm. Over the past few decades, scientists have contributed to CF research by using animal models to understand the pathology of the disease. The models selected have similar pathogenesis to humans, so they are key insights for understanding the effects of CFTR gene malfunctions. More recently, animal models, such as CRISPR generated sheep and rabbits used in the two current studies, have been successful in providing scientists with insights for promising therapeutical advances that could be used in humans with CF. In the first study, sheep have been generated using CRISPR technology and somatic cell nuclear transfer techniques. The models used for the study had a disruption in the CFTR gene to produce CFTR heterozygous and homozygous offspring. Newborn homozygous sheep developed phenotypes consistent with CF pathology seen in humans. The second study used the same CRISPR technology technique to generate CF rabbits with a disrupted CFTR gene. CF rabbits receiving an oral osmotic laxative survived an average of 40 days and 100% died due to gastrointestinal disease. The rabbits also mimicked phenotypes shown in humans with CF such as decreased respiratory function and gastrointestinal obstruction. Here, we explain how the use of sheep and rabbit models help to test the effects of a mutated CFTR gene and provide further therapeutical advances that could be helpful for individuals living with Cystic Fibrosis. These two models have been successful in understanding CF pathology and suggest that there is hope for finding a cure for Cystic Fibrosis in the near future.

Thesis Mentor: Sarah Cooper

Overview:

Cystic Fibrosis (CF) is a hereditary disease that adversely affects an individual's lungs and digestive system. CF is a recessive disorder, meaning both copies of the gene must be inherited for the individual to express symptoms. Each individual has a Cystic Fibrosis transmembrane regulator (CFTR) gene that is responsible for regulating the proper flow of chloride and sodium ions in and out of membranes in the lungs and other organs throughout the body. Chloride and sodium levels are important for maintaining the proper amount of fluid inside and outside of cells. When the CFTR gene does not function correctly, there is an imbalance in both the chloride and sodium levels. Chloride becomes trapped in the cells and causes severe dehydration on the cell surfaces. Once the cells are dehydrated for an extended period of time, thick mucus builds up on the surfaces and causes complications for the individual. These complications include difficulty breathing, excessive coughing with thick phlegm, and even gastrointestinal issues. All of these symptoms are associated with the life-threatening, non-curable disease, Cystic Fibrosis (Boyd et al. 2020).

The two studies included in this thesis use genetically modified animal models to mimic CF symptoms seen in patients with this disorder. The two models are important to CFTR gene research because they have a longer survival rate and similar organ function to humans. The symptoms observed include lung disease, decreased airway function, and most commonly, gastrointestinal obstruction. Previous studies have not been able to provide useful insights for how a disrupted CFTR gene functions because the models do not survive long enough for the entire study. Also, not enough research has been done on this specific disease until the past two decades with the advancements in technology and medicine. Sheep and rabbit models used in the two current studies have allowed researchers to examine the CFTR gene more closely and use these findings to develop useful therapeutics to lessen the symptoms this disease imposes.

The first study, *A Sheep Model of Cystic Fibrosis Generated by CRISPR/Cas9 Disruption of the CFTR Gene*, (Fan et al. 2018) uses two groups of genetically modified sheep. The first group includes sheep expressing a disrupted CFTR gene causing CF disorder, and the other is a control group of sheep that do not have CF. The jejunum and colon in the gastrointestinal tract are the most recognized complications observed in this study. From the study, sheep with CF developed GI issues ultimately resulting in obstruction of the gastrointestinal tract. Liver and gallbladder disease were also detected in the models due to complications that arise with the overproduction of thick mucus. The findings from the study are consistent with complications seen in humans since intestinal obstruction is a common trait seen in CF patients due to the rapid mucus secretions in this area.

The second study *Phenotypes of CF Rabbits Generated by CRISPR/Cas9-Mediated Disruption of the CFTR Gene*, (Xu et al. 2021) uses genetically modified rabbits to create a disruption in the CFTR gene. Three groups of rabbits were generated, with each group expressing a different mutation sequence in the CFTR gene. The groups were raised at three different college campuses in the United States. Each college also had a control group of rabbits for comparison to the CF rabbits. At the University of North Carolina location, rabbits received an oral laxative for the entirety of their lives. The rabbits who received the laxative were able to surpass the lifespan of the other two groups of rabbits. This study primarily focused on airway function, which was

observed to have been improved in the rabbits who received the laxative. The airway functions observed in rabbits not receiving the laxative were limited, and lung disease began to develop in models surviving longer than five days. Decreased airway function is commonly seen in CF patients due to the presence of spotty mucus plugs in the lining of the lungs. These mucus plugs act as primary mucus producers which cause severe complications in the lungs due to the overproduction. This causes the patient to have heavier breathing and increased difficulty when taking deep breaths.

To date, there is no cure for CF, but the past few decades have provided promising insights for how a malfunctioning CFTR gene causes CF. These insights have allowed scientists to create therapeutics that mediate the effects of CF and help the patient live a less painful life. The most recent therapeutic, Trikafta, is a combination of three CF therapies approved for patients 12 years and older with at least one specific mutation in the CFTR gene. This specific mutation is found in nearly 90% of patients all patients with Cystic Fibrosis. Trikafta targets the defective CFTR protein by helping it to function more effectively. The drug does this by binding to different places on CFTR proteins to help more of these proteins reach the cell surface. Another function of the drug is to help the CFTR proteins stay open longer at the cell surface (Liessi et al. 2020). Both of the chosen studies use genetically modified animal models to observe what happens in the body when the CFTR gene is not functioning properly and how these observations are used to create promising drugs to help fix these issues. Ongoing research using these methods has allowed for promising results and could possibly lead to the first cure for this frightening disease.

Introduction

Cystic Fibrosis Transmembrane Conductance Regulator

Cystic Fibrosis (CF) is an autosomal recessive disease that results in the dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) channel due to mutations in the CFTR gene (Boyd et al. 2020). In 1989 John Riordan discovered the gene responsible for CF is located on chromosome seven and provides instructions for how the CFTR protein functions (Semaniakou et al. 2019). The CFTR protein functions as a

cyclic AMP-activated chloride channel that regulates water and ion balance across the membrane of cells that produce mucus, sweat, saliva, tears, and digestive enzymes (Boyd et al. 2020). When the protein does not function correctly, chloride becomes trapped within cells, leading to dehydration on membrane surfaces. Dehydration within the cells causes irregular thick and sticky mucus to build up in the membranes causing a chain reaction of complications throughout different organs of the body. These complications include tissue and organ damage due to infections that the mucus buildup causes (Maule et al. 2020). An individual with CF experiences a breakdown of neutrophils in the lungs leading to large amounts of extracellular DNA. The extracellular DNA increases the viscosity of sputum causing difficulty in breathing and overactive lung function.

Cystic Fibrosis was first discovered in 1938 by American pathologist Dr. Dorothy Andersen. She first called the disease “cystic fibrosis of the pancreas” based on her findings from autopsies of children who had died from malnutrition causes. The disease is more common in the Caucasian population with 1,000 new cases diagnosed each year. Cystic Fibrosis affects one in 2,500 to 3,000 newborns each year worldwide, although these numbers are beginning to decline due to less cases being diagnosed in children and more diagnoses in adulthood. To date, there have been over 2,000 CFTR mutations reported. Individuals with CF have a shorter life expectancy due to the severity of complications the CFTR gene imposes. Patients experience intermittent blockages within the lungs, airways, and ducts in the pancreas due to abnormal mucus accumulation. When the blockages occur, digestive enzymes coming from the pancreas are unable to reach the small intestine causing impaired absorption of fats. Therefore, vitamin deficiency and malnutrition are commonly found in patients living with CF (Semaniakou et al. 2019). Cystic Fibrosis is considered a multiorgan disease because once one organ system is affected, another system begins to experience complications since the organs all work together to function efficiently. Unfortunately, there is no cure to this life-threatening disease, but there are medications patients with CF could take to alleviate the symptoms they experience which helps to lengthen their lifespan. To understand the pathology of CF, animal models are used for scientific research to study the effects CF has on different organ systems in the body.

Use of Animal Models for CF Research

Scientific researchers have been using different types of animals over the past few decades for understanding and investigating the Cystic Fibrosis pathophysiology. Although each animal has a different pathology, researchers are careful when selecting models that closely resemble human CF manifestations in order to receive the most accurate results. In 1992, three years after the CFTR gene was identified, the first genetically modified CF mouse model was studied (Guilbault et al. 2006). Access to patient tissue for research is greatly limited, but genetically modified mice are abundant due to their ability to produce multiple litters each year. They have been proven to be successful over the years in CF research. Based on previous studies, mice have shown recurrent bacterial lung infections, intestinal diseases, kidney complications, liver disease and decreased nasal airway function which are all similar to human CF phenotypes (Wilke et al. 2011). Other animal models that have been used as scientific research models for CF include rats, ferrets, pigs, and in the current study, genetically modified sheep and rabbits with Cystic Fibrosis.

From previous studies, rats have shown to develop intestinal obstruction, but histology of the pancreas, liver, and lung appeared within normal limits (Dreano et al. 2019). These findings contradict the phenotypes expressed in humans because individuals with CF experience pancreas, liver, and lung complications. Both ferrets and pigs with a genetically modified CFTR gene have been shown to develop the lung disease phenotype, as well as pancreatic, gallbladder, and intestinal disease (Yan et al. 2014). Although these animals have been successful in expanding CF research, they have certain limitations. Some limitations include failure to reproduce pulmonary phenotypes observed in CF patients due to their smaller airways, and the presence of abnormal airway epithelium in the pathogenesis of CF lung disease (Xu et al. 2021).

Submucosal glands (SMGs) are also important to consider when conducting CF research. SMGs refer to exocrine glands that secrete mucus to facilitate the movement of particles along the body's various tubes such as the throat and intestines. Exocrine glands normally function to produce thin, slippery secretions such as mucus, sweat, and tears. In contrast, the exocrine glands in a patient with CF produce thick, sticky secretions

which are harmful to the airways because they cause difficulty breathing. SMGs in humans are found in the airways, sinuses, and the trachea of the bronchial tubes (Wine and Joo 2003). SMGs are studied in CF research with animal models, but mice and rats have SMGs limited to only the trachea. Ferrets and pigs have SMGs throughout their airways which is useful for CF research because it shows the greatest similarity to human phenotypes. Evidently, rabbits completely lack SMGs, so this model does not completely represent human SMG findings related to CF (Rosen et al. 2018). Using the appropriate animals as research models has allowed scientists to develop therapeutics that could be useful for patients living with CF. Although CF is incurable, these therapeutics may help to alleviate any discomfort the patient experiences, allowing for a more enjoyable lifestyle.

Examples of Human CF Therapeutics

Currently, there are advancing therapeutical treatments available for patients living with Cystic Fibrosis focusing on lung function, airway clearance, and airway surface rehydration. Although these therapeutics are costly (some up to \$300,000/year), they have helped to lengthen the average lifespan of an individual living with Cystic Fibrosis (Bilton et al. 2011). One drug, known as Dornase alfa, is a recombinant human deoxyribonuclease (DNase) known to reduce the number of lung infections and improve overall lung function in a CF patient (Edmondson & Davies, 2016). This drug works by cleaving any extracellular DNA which breaks down the thick mucus build up. Once the extracellular DNA is cleaved, the patient experiences better air flow. Dornase alfa has led to significant reduction in inflammation and in DNA concentrations, suggesting the treatment should begin in the earlier stages of the disease rather than when the lung has already been compromised at an older age (Edmondson & Davies, 2016).

Individuals experiencing CF suffer from dehydrated airways which causes hoarseness while breathing. When chloride ions become trapped within the cells of the membrane, there is no way for the cells to receive the hydration they need to properly function. Mannitol and Denufosol are two therapeutical drugs used in CF patients to restore hydration to the trapped chloride cells. Mannitol is a nonabsorbable sugar alcohol that works

as an osmotic gradient on airway surfaces to provide rehydration by increasing volume surface liquid for the cells and the clearance of mucus (Edmondson & Davies, 2016). A study by Bilton et al. (2011) has shown Mannitol produces a sustained and clinically meaningful benefit, but patients receiving this therapeutic drug have experienced haemoptysis and an intense cough (Bilton et al. 2011). Denufosal is an inhaled P2Y2 agonist drug that acts by bypassing the defective CFTR chloride channel and activates an alternative chloride channel. The alternative channel increases airway surface hydration and ciliary beat frequency for mucus clearance (Kellerman et al. 2008).

Ivacaftor is an oral agent that increases ion function of activated cell-surface CFTR. Studies using bronchial epithelial cells from the lungs of patients with CF have been shown to increase in air-surface fluid levels and ciliary beat frequency similar to the drug Denufosal (Condren & Bradshaw 2013). The most recent advancement in therapeutics for CF is Trikafta. This is the first triple combination therapy (elexacaftor/tezacaftor/ivacaftor) available to treat patients 12 years and older with CF. The combination of drugs works together by helping the CFTR protein fold correctly and function more effectively. Elexacaftor binds to different locations on CFTR proteins, Tezacaftor helps more proteins reach the cell surface, and Ivacaftor helps CFTR proteins stay open longer at the cell surface. Trikafta is approved for patients 12 years and older with Cystic Fibrosis who have at least one F508del mutation in the CFTR gene, which represents 90% of the CF population. Clinical trials show that children tolerated the drug well and showed improvements in sweat chloride. (Liessi et al. 2020).

Connecting What is Known and Recent Studies Using Animal Models

Cystic Fibrosis is a complex disease because it affects more than one organ system concurrently, but the severity of the disease differs from one individual to another. CF is usually discovered earlier in an individual's life, but in the past few years it is no longer considered a pediatric disease. Patients who are diagnosed with CF as adults show different symptoms when compared to patients who are diagnosed with CF as children. Adults

with CF express a milder case, long-term prognosis, better lung function, fewer complications, and a longer life expectancy than patients who were diagnosed as children (Santos et al. 2017).

Many patients seek lung transplantations with intentions to live a longer life, but such procedures become high-risk due to infections that could develop due to the compromised immune system CF presents. There are not enough donors available for the high demand, but if a patient is eligible to receive a lung transplant, the median survival post transplantation is only an additional 6.4 years (Adler et al. 2009). Transplantations only serve as a temporary solution to the problems CF imposes. Although there is no cure to this disease, clinical research in the past few years has led to therapeutic progress increasing the survival rate from childhood to adulthood with patients living an average of 40 to 53 years (Boeck et al. 2020).

Research using human tissue is extremely limited due to the complications that could arise. Individuals with CF have delicate tissues, so taking samples from these individuals for research could be deadly. As previously stated, other animal models have been successful in providing researchers insights for how the CFTR protein is affected in individuals with this disease. Although the models have provided insights, they have not been able to show the effects of every organ affected in humans. In the two current studies, sheep and rabbits have been used as models for furthering research on the effects CF has on the body. Both animals have the greatest similarity to the human CFTR gene and are representative for research because they have longer life spans and larger organs which provides researchers with comparison to the size of human organs (Xu et al. 2021). In this thesis, I will explain how the use of sheep and rabbit models help to test the effects of a mutated Cystic Fibrosis transmembrane conductance regulator and provide further therapeutical advances that could be helpful for individuals living with Cystic Fibrosis.

In an article titled *A Sheep Model of Cystic Fibrosis Generated by CRISPR/Cas9 Disruption of the CFTR Gene*, (Fan et al. 2018) described the generation of sheep models for Cystic Fibrosis using genome editor CRISPR and somatic cell nuclear transfer to suggest that the large animal model will be useful in advancing potential CF therapeutical studies intended to be used in humans. The authors proposed a hypothesis based on

similar pathology to humans in order to determine if the CF transmembrane conductance regulator protein (CFTR) could be altered to provide insights in developing advanced therapeutics capable in humans experiencing Cystic Fibrosis.

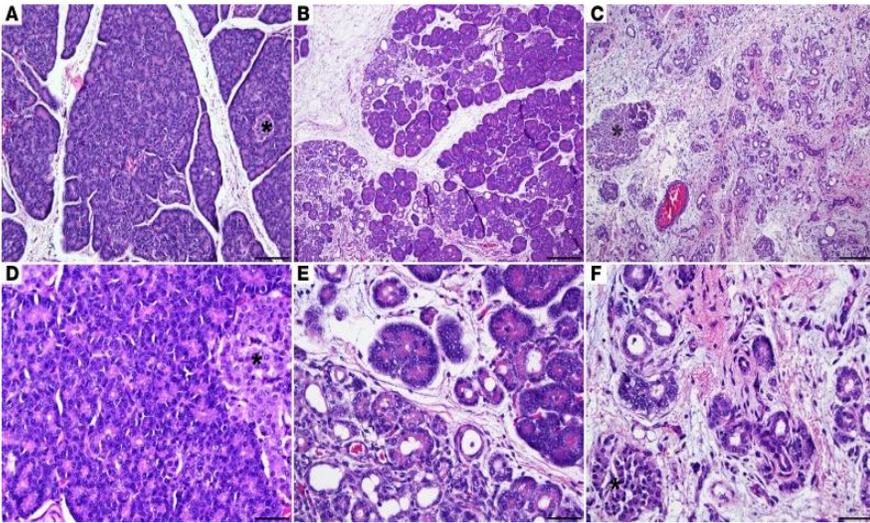
CRISPR technology was used to introduce mutations in the CFTR locus of fetal sheep fibroblasts (SFF), followed by somatic cell nuclear transfer (SCNT). Primary SFFs were used from domestic sheep (*Ovis aries*) and isolated from 45-day old fetuses. The SFFs were cultured in 15% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin at 38.5°C in an atmosphere of 5% CO₂ in air. Polymerase chain reaction (PCR) primers were designed according to the sheep CFTR genome sequence and used to amplify exons 2, 11, and partial intron sequences. The choice of these exons was determined by future plans to generate human-specific disease-associated mutations in these exons. CRISPR targeting sites were designed based on exon 2 and 11 sequences, and enzyme restriction sites were introduced to target loci in order to facilitate mutation detection of the CFTR gene. A pair of oligonucleotides for each targeting site was synthesized and ligated to the pX330 vector. Single-celled derived CFTR-mutated fibroblast colonies were isolated by limiting dilution and screened by PCR assays. Corresponding DNA oligos for each of the targeting sites were synthesized using Integrated DNA Technologies (Fan et al. 2018).

Five micrograms of circular sgRNA/Cas 9 vectors were transfected into SFFs using Amaxa 4D-Nucleofector, and three days after transfection, cells were harvested for genomic DNA isolation using a QIAGEN blood and tissue kit. Each of the targeted genomic loci was PCR amplified from the genomic DNA isolated from SFFs, and after digestion with the chosen enzyme, the PCR products were fixated on 1% agarose gel. The gel was then stained with SYBR green dye for better detection. Based on whether the PCR products were fully or partially resistant to digestion by a chosen restriction enzyme, indels were detected. Three days after targeting vector transfection, cells were subjected to single cell cloning in 96 well-plates. PCR was used to identify CFTR-mutated colonies. Fetal fibroblasts both homozygous and heterozygous for CFTR mutations were used as donor cells for SCNT. SCNT was performed as normal, with minor modifications such

as oocyte recovery instead of a slicing technique, and SOF embryo culture instead of G1 medium. Homozygous and heterozygous fetal fibroblasts were grown to 80%-90% confluence and used as nuclear donor cells for SCNT. A total of 1,029 CFTR^{-/-} and CFTR^{+/-} cloned embryos were cultured in SOF medium for 10-12 hours then transferred to 73 estrus-synchronized sheep surrogates ages ranging from 2-5 years old. PCR was then used to identify homozygous from heterozygous lambs (Fan et al. 2018).

The generation of CFTR^{-/-} and CFTR^{+/-} fetal fibroblasts using CRISPR was successful because CFTR disruption was achieved in both male and female colonies for exons 2 and 11. Sequence analysis of the PCR results indicated small insertions and deletions were introduced at each of the targeted CFTR loci in the colonies. The SCNT process produced males and females from each group, and PCR indicated all cloned fetuses carried the same mutations as the donor cells they originated from. There was no difference between the homozygous and heterozygous groups in terms of pregnancy development rates and bodyweights. Late-term abortions and still-births were recorded with 5/17 being in the homozygous group and 3/16 in the heterozygous group. Live-born CFTR^{-/-} lambs were not considered viable due to severe intestinal obstructions, so were euthanized within 48 hours after their birth. 2/13 CFTR^{+/-} lambs were euthanized due to broken ribs, and another developed septic arthritis caused by an umbilical bacterial infection. The remaining CFTR^{+/-} sheep remained healthy and developed to adulthood (Fan et al. 2018). A detailed histological evaluation was performed on 15 CFTR^{-/-} newborn lambs at 0-3 days old, and 5 CFTR^{+/-} lambs (4 at 0-3 days old, and 1 at 57 days) focusing on the lungs, intestinal tract, pancreas, and liver.

The most revealing phenotype associated with loss of CFTR in the sheep was intestinal obstruction evident in 100% of CFTR^{-/-} newborn lambs shown in Figure 1 reproduced by Fan et al. A distinction was evident between a distended oral intestine and smaller diameter aboral small intestine or colon (Figure 1B). Obstruction was found in the distal jejunum or in the proximal, mid, or distal spiral colon. The intestine oral to the obstruction site was distended with large amounts of meconium with measurements of 1-2.5 cm in diameter (Figure 1D). The intestine and/or the colon aboral to the obstruction site measured 3-5mm in



diameter and contained thick mucoid contents (Figure 1, F and H) compared to the normal limits (Figure 1, E and G). There were no intestinal lesions observed in the heterozygous and control lambs (Fan et al. 2018).

Figure 1. Reproduced from Fan *et al.* 2018 Intestinal histology of homozygous newborn sheep. (A) Intestinal tract of control sheep at <1-day old. (B) Meconium ileus shown in intestinal tract of <1-day old homozygous sheep. (C) Small intestine histology of control lamb. (D) Histology of small intestine enlarged and filled with meconium in homozygous lamb. (E) Histology of colon in control lamb. (F) Homozygous lamb colon filled with mucous. (G) Histology of colon in control lamb. (H) Homozygous lamb colon with colonic glands enlarged and filled with mucous.

The pancreas revealed hypoplasia or aplasia in both homozygous (11/15) and heterozygous lambs (3/5). A pancreas was not grossly visible in 7/15 homozygous lambs and 3/5 heterozygous lambs and was about 1/5-2/3 of expected size in 4/15 homozygous lambs. Pancreatic tissues where the pancreases should be located were collected for closer observation. No pancreatic tissue was detected from observation in 6 lambs that had no grossly visible pancreas (3/7 homozygous and 3/4 heterozygous). Pancreatic fibrosis was evident in 40% of homozygotes and none in heterozygotes as shown in Figure 2 reproduced from Fan, et al. Among the 7 homozygous lambs without a visible pancreas, some residual pancreatic tissue was evident based on histological study. Three showed mild multifocal to severe diffuse fibrosis with acinar and ductal dilation. A fourth subject was not histologically examined due to autolysis. One out of four homozygous lambs with grossly normal pancreases showed mild multifocal intestinal fibrosis. No fibrotic lesions were observed in the heterozygote or control lambs (Fan et al. 2018).

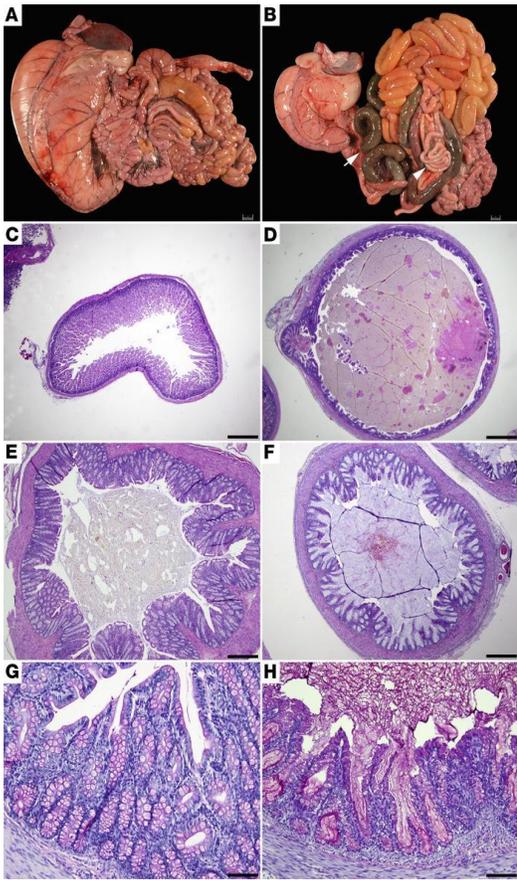


Figure 2. Reproduced from Fan *et al.*, 2018 The pancreatic pathology of Cystic Fibrosis in newborn CFTR $-/-$ sheep. (A) Tissues of the pancreas in control sheep. The endocrine cells are tightly packed together. The asterisk indicates irregularly shaped patches of endocrine tissue within the pancreas. (B) Tissues of homozygous sheep shows mild underdevelopment of the pancreas. There is a decrease in density of the endocrine cells. (C) Tissues of the homozygous sheep shows severe underdevelopment of the pancreas. Only the pancreatic ducts separated by adipose tissue are visible. (D) Magnified pancreas in the control sheep in (A). (E) Magnified pancreas in homozygous sheep in (B). (F) Magnified pancreas in homozygous sheep in (C).

In an article titled *Phenotypes of CF Rabbits Generated by CRISPR/Cas9- Mediated Disruption of the CFTR Gene*, (Xu et al. 2021) described the generation of rabbit models for Cystic Fibrosis using genome editor CRISPR to suggest that rabbits could be the useful in advancing previous therapeutical studies due to their amino acid sequence exhibiting a 92% similarity to the human CFTR protein. The authors proposed a hypothesis based on previous studies performed (Fan, et al., 2018), (Tuggle KL et al. 2014), (Stolz DA et al. 2010, and (Kent, G. et al. 1997) which used mice, rats, and sheep, but in the current study using rabbits. The authors suggest this model will be the most promising because rabbits have the greatest similarity to the human CFTR protein, therefore gut and lung CF pathogenesis are similar and could be used for practical advancement of CF therapeutics (Xu et al. 2021).

Rabbits exhibiting CF were generated using an advanced CRISPR gene-editing platform which utilized Cas9 expression of plasmid JDS246 and sgRNA expression from plasmid repository company Addgene (Xu et al. 2021). SgRNA on exon 11 of the CFTR gene in the rabbit was designed using Zifit targeter software (Xu et

al. 2021). Founder rabbits were bred with wild-type rabbits to verify germline transmission. Three lines of CF rabbits were established carrying: a. CF Δ 1 (one nucleotide deletion) generating a premature stop codon after amino acid 477, b. CF +1 (one nucleotide insertion) generating a premature stop codon after amino acid 480, and c. CF Δ 9 (nine nucleotide deletion) resulting in deletion of amino acids P477, S478, and E479. 162 embryos were transferred into 6 recipients, and once sexually matured, CFTR-knockout (KO) rabbits were bred with WT counterparts to create an F1 generation. Rabbits carrying indels in 1 allele were assigned to the F1 heterozygous group, which were then interbred to produce F2 and later generations of homozygous CFTR-KO rabbits. Genomic DNA was extracted, and ear skin tissues were biopsied to determine genotypes. The genomic DNAs were used to complete a PCR in which the products were purified and sequenced for detection of indel mutations approximate to the sgRNA target sequence. Quantitative PCR was conducted on a small group of rabbits to test whether CFTR mRNA was still expressed in the F1 generation (Xu et al. 2021).

Rabbit colonies were studied in three different locations: WSU, UM, and UNC in order to test the differences in mutations between the generations. At the UNC location, rabbits aged 2 weeks were given Golytely (an osmotic laxative) to drink replacing regular water (Xu et al. 2021). Since GI obstruction is commonly evident in the previously discussed studies, an osmotic laxative was administered to test whether or not this technique would help to lessen the number of rabbits that die due to GI obstruction. Rabbits with CF consumed the osmotic laxative for their entire life. Beginning at 4 weeks, rabbits ate Rabbit Liquid diet as a supplement to their normal rabbit chow twice a day (Xu et al. 2021). Their diet also integrated vegetables 3 times per week, and fruit once a week. At the UM location, the main diet for the rabbits was Laboratory Rabbit Diet #5321 (LabDiet). All rabbits were weighed and examined for any abdominal masses at 1 week old. If there were any masses detected, the rabbit was treated with 50 mL subacute warmed saline, 0.5 mg/kg cisapride orally, 0.5 mL/kg simethicone orally, 0.5 mg/kg methoclopramide orally, and 0.5 mL/kg lactulose orally which was received until abdominal masses resolved. Six CF rabbits were raised living to \geq 1 year using this protocol (Xu et al. 2021).

CFTR Western Blot was performed using tracheal tissues from both CF and WT rabbits. Tissues were homogenized in lysis buffer, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol with protease inhibitors, then centrifuged for 10 minutes at 4°C. The total protein concentration was assessed by protein assay and 50 µg of protein was resuspended in 6 times SDS sample buffer then denatured at 42°C for 10 minutes. The samples were resolved by SDS-PAGE, transferred to PVDF membranes, and blocked at room temperature for 1 hour with 5% skim milk. Membranes were incubated with primary antibodies with a ratio of 1:5000 in TBST buffer with 10% FBS at room temperature for 1 hour, washed 4 times with TBST, and incubated with horseradish peroxidase-conjugated secondary antibodies in TBST with 10% FBS for 1 hour, followed by 5 washes with TBST (Xu et al. 2021).

Founder animals carried different mutation alleles due to the inherent nature of CRISPR gene targeting in which specific mutation types were unpredictably generated. No off-target mutations were detected in the animals tested. 28 CFΔ1 heterozygous rabbits were bred at the WSU location which produced 186 rabbits with genotype distributions of 48 CFTR^{+/+} (WT), 96 CFTR^{=/-} (Het), and 42 CFTR^{-/-} (CF) rabbits. Male CFΔ1 CF rabbits bred with heterozygous females were infertile. Two CFΔ1 CF males had complete absence of the vas deferens and epididymis (Xu et al. 2021).

New Zealand rabbits used in the study have an average lifespan of 7-10 years (Xu et al. 2021). Exemplified by the CFΔ1 line in the study, results showed that the CF rabbits spontaneously drinking the osmotic laxative starting at 2 weeks had a median survival rate of 44 days which is significantly lower than the WT rabbits. Intestinal obstruction detected in the rabbits beginning at 2 weeks old allowed for researchers to administer the combination of drugs previously stated. This drug combination had an effectiveness of 70% in eliminating palpable masses and allowed for the subjects to restore weight gain in weaned CF rabbits. Subjects treated with the drug combination had a median survival of >80 days. At birth, CF subjects presented no significant difference in body weight or appearance compared to WT rabbits, but after 30 days, surviving rabbits failed to gain weight at the same rate at their WT counterparts. Administering Golytely early in life

significantly improved, but did not fully correct, the weight gain of CF rabbits. CFTR disruption was profoundly expressed in the rabbit's GI tract, specifically the jejunum and colon. CF rabbits were observed for abnormalities in intestinal ion transport which showed forskolin-stimulated anion secretion absent in CF rabbit jejunum at both younger and older ages. An increase in NA^+ dependent glucose transport was observed in the jejunum of younger CF rabbits (Xu et al. 2021).

CF rabbits did not exhibit perinatal meconium ileus, but intestinal dysfunction was observed by obstruction of the proximal colon which developed before weaning. Cross sections of the distal colon revealed mucus stasis in the colonic crypts that selectively occurred in the subjects, even after they received Golytely drug treatment. The CF rabbits also appeared to have submucosal infiltration of inflammatory cells. In younger rabbits <2 months old, the appendix appeared normal, but became dilated with atrophic epithelium and inflammatory cells in older rabbits >4 months old compared to WT or heterozygous controls as shown in Figure 3: A-J reproduced by Xu et al. 2021.

Routine analysis of blood counts was performed on 4-5 month old WT and CF rabbits. White blood cell counts, and hemoglobin levels were similar in CF versus WT rabbits (Figure 3: K and L). However, WBC counts revealed increased percent heterophils and decreased percent lymphocytes in CF rabbits (Figure 3M). Metabolic paneling measuring hepatic, pancreatic, intestinal, and renal function were obtained for stable rabbits age 2-3 months. The measured values for all observed areas fell within normal ranges for New Zealand rabbits. Amniotransferase levels were slightly higher in the CF rabbits than WT subjects, but liver abnormalities were undetected in either CF or WT rabbits. Upper and lower airways in WT and CF rabbits were probed to observe CFTR expression and function in airways. Distribution of rabbit CFTR mRNA in the nasal respiratory epithelium of the WT rabbit was widespread in the superficial epithelium, differing from CF rabbits who had highly clustered areas in isolated cells in the olfactory epithelium. CF rabbits surviving more than 9 months experienced degeneration of the olfactory epithelium compared to their WT littermates. Lastly, 5 rabbits

involved in the study showed evidence of lung disease at time of sacrifice, which included parenchymal consolidation and presence of intraluminal airway mucus when dissected (Xu et al. 2021).

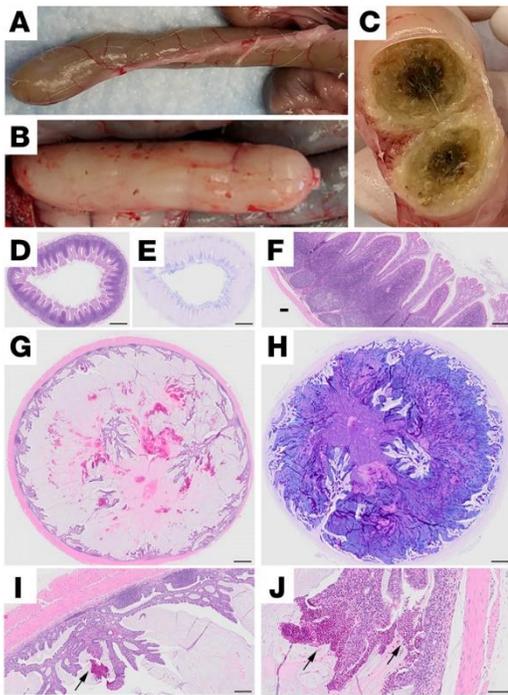
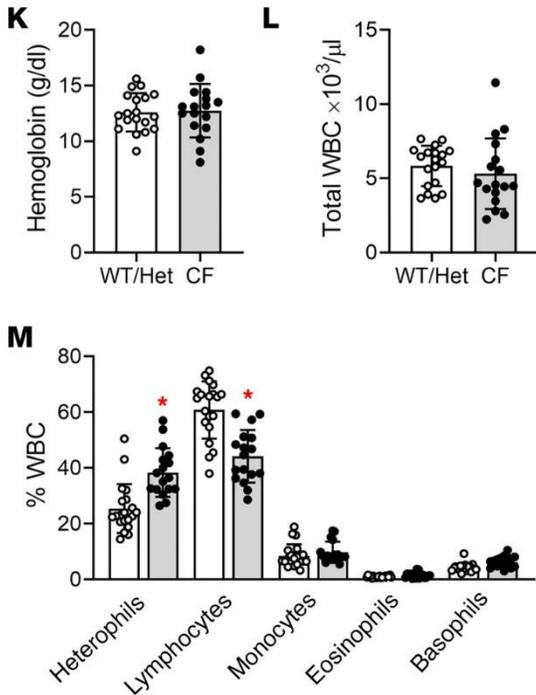


Figure 3: Reproduced from Xu et al. 2021 Histology of inflammation in CF subjects. (A-C) Appearance of appendix dissected from 1 year old WT (A) and CF (B) rabbits. CF appendix is swollen and discolored when compared to WT. In cross section (C), CF appendix lumen revealed solid caseous material and cecal contents. (D and E) Histologic image of H&E-stained cross-section (D) and PAS-stained cross-section (E) appendix dissected from 1 year old WT rabbit. (F) Higher magnification of H&E-stained appendix mucosa in WT rabbits. (G and H) Histologic image of H&E stained (G) and PAS-stained (H) appendix dissected from 1 year old CF rabbit. (I and J) Higher magnification of H&E-stained CF appendix exhibiting simplified epithelial structures compared to WT with barely detectable lymphoid tissue and granulocyte accumulation indicated by arrows. (K-M) Hematologic values for WT and CF subjects about 4-5 months. (K) represents total hemoglobin, (L) represents WBC total, and (M) represents differential cell count. n= 19 WT and 17 CF rabbits.



Discussion:

Several studies have contributed to the notion that animals share similar pathology to humans and are ideal candidates for furthering the research of Cystic Fibrosis (CF) therapeutics. CF research has been ongoing for decades, but previously studied animal models such as mice, rats, and ferrets have been inefficient for understanding the human CF pathology. By using rabbits and sheep in the current studies, scientists have been able to closely examine the effects of CFTR gene malfunctions and develop therapeutic strategies to alleviate these effects on the body (Fam et al. 2018; Xu et al. 2021).

Generation of large farm animals by genetic cloning has become more popular within the past two decades. These cloned animals have provided insights into understanding the etiology of disease by modeling human genetic diseases and advancing new therapeutics. Cystic Fibrosis is most common in individuals of European descent, and although new therapies have been effective in treating some of the possible mutations in the CFTR locus, not one single drug has a permanent solution to the effects CF imposes. The suggestion that a sheep model of CF might be useful in advancing therapies was made in 1997 by Ann Harris, but this was not realized until the development of CRISPR technologies made it possible to target the sheep CFTR locus (Harris 1997).

A sheep model of cystic fibrosis generated by CRISPR/Cas9 disruption of the CFTR gene (Fan et al. 2018) describes the generation of homozygous CFTR sheep developed using CRISPR technology that disrupted the CFTR locus in fetal sheep fibroblasts. The most profound phenotype observed in the sheep models from loss of the CFTR gene was detection of intestinal obstruction (Fan et al. 2018). Differing from this study, intestinal obstruction is well-managed in other models through dietary changes and “gut correction” by the introduction of a functional CFTR gene (Stoltz et al. 2010). Similar to the homozygous CFTR pigs from past studies, all homozygous sheep models developed intestinal obstruction (Fan et al. 2018). Intestinal CFTR expression without pancreatic or hepatic correction was sufficient to prevent a blockage known as meconium ileus in homozygous CFTR pigs, and a similar approach is likely to be effective in CF sheep (Stoltz et al. 2010).

A comparison of the intestinal obstruction phenotype in CF sheep is of interest because their large size makes them most relevant for preclinical trials to test potentially promising drugs and gene replacement therapies in humans. At this point, it is unknown whether the CF sheep will replicate human lung CF lung disease over time. As observed in the results, the newborn CF sheep did not show signs of lung disease, but the sheep did show early signs of failure to clear harmful bacteria in the lungs. Failure to clear bacteria results in lung inflammation, tissue remodeling, and mucus accumulation (Fan et al. 2018). Both sheep and humans with CF share the pancreatic fibrosis phenotype which advances at birth. Further classification of this defect depends on natural breeding of heterozygous sheep to exclude the impact of pancreatic hypoplasia. This feature was seen in CFTR null and heterozygous sheep, so it is most likely associated with the somatic cell nuclear transfer (SCNT) technique (Fan et al. 2018).

When comparing sheep models to previous studies using newborn CF pigs, the newborn pigs show signs of pancreatic inflammation, fibrosis, abnormal tissue architecture, and liver and lung disease that is much more advanced than in human CF (Lamireau et al. 2004). These findings are associated with the findings from heterozygous CFTR sheep because the liver phenotypes are also more severe than in humans living with Cystic Fibrosis. Approximately 80% of the CF sheep showed periportal fibrosis, and over 85% had intrahepatic cholestasis. Heterozygote sheep did not reveal any liver lesions. About 80% of CFTR null sheep had a hypoplastic gallbladder, which is not shown in heterozygous CFTR sheep. The liver and gallbladder phenotypes were more pronounced in the CFTR null sheep than the null pigs (Fan et al. 2018).

Lambs produced by SCNT sometimes show kidney abnormalities which are often characterized by development of hydronephrosis (Dawson et al. 2004). Hydronephrosis is a condition characterized by excess fluid in the kidney due to a backup of urine (Nuraj P and Hyseni N. 2017). Hydronephrosis was observed in 87% of homozygous CFTR lambs and 80% of heterozygous lambs examined (Dawson et al. 2004). This phenotype is unrelated to the loss of the CFTR gene in the lambs, and natural breeding of heterozygous CFTR sheep is expected to eliminate this condition (Fan et al. 2018). Lastly, males with CF are of particular interest

due to the loss of the vas deferens and obstruction of the epididymis. CFTR homozygous male lambs showed aplasia or severe atrophy of the vas deferens. This loss of genital ducts is shown at birth which suggests an in-utero phenomenon, which could be obstruction of the ducts with mucus secretions (Fan et al 2018).

Benefits of genetically modified CF sheep have received little attention, but this greatly relates to the early developmental processes of Cystic Fibrosis. Since CF develops in utero, further studies of intrauterine interventions may provide possibilities for effective disease treatment. Sheep are most suitable for this type of research because of their similar size, similar anatomy to humans, and the length of gestation which allows a long-term in utero observation. Making in utero sheep available will enhance new opportunities to investigate the early disease process which is impossible to study in humans. These findings will help lead to further development of therapeutic strategies (Fan et al. 2018).

Phenotypes of CF rabbits generated by CRISPR/Cas9-mediated disruption of the CFTR gene (Xu et al. 2021) illustrated how rabbits are excellent CF models because of their similar CFTR expression in the lung with respect to humans. CF rabbits also make great research models because they exhibit high homology to humans, and sensitivity to potentiators developed for CF pharmacology. The use of CRISPR-mediated genetic modifications of rabbit CFTR have provided an ideal model for CF pathogenesis and therapeutical studies. Female CF rabbits are fertile; therefore, the generation of future generations is easily accomplished for these studies. Also, untreated CF rabbits do not experience perinatal meconium ileus or early mortality which is often observed in larger CF research models (Xu et al. 2021).

As seen in past studies and humans, GI obstruction is one of the most common phenotypes observed early in an animal model's life. Although the rabbits died due to GI obstruction, mode of death in CF rabbits appeared to reflect later-onset GI obstruction. This differs from other studies because the rabbits lived longer than previously studied models, which allowed for a more extensive observation before dying at adolescence. Freshly excised jejunum displayed bioelectric properties resulting from the loss of CFTR function. The bioelectric properties observed in the jejunum are predicted to be the cause of reduced volume/water secretion

in the gut. Another jejunum defect in young CF rabbits was the increase in sodium-dependent glucose transport. This abnormality may reflect response to malnutrition and have different effects on the GI tract of a CF rabbit. These effects include possible volume depletion and hyperconcentration of intestinal content in the gut lumen (Xu et al. 2021).

Intraluminal accumulation of fecal material was observed in one- to two-month-old CF rabbits which could reflect problems of hyperconcentrated material and/or gut dysmotility due to bacterial overgrowth. The high heterophil percentage is consistent with persistent inflammation, which was observed by impaction of the appendix. Abnormal pancreatic function, suggested by the low blood lipase levels, may have contributed to gut obstruction (Xu et al. 2021).

Airway structure contributes to a majority of the dysfunctions a modified CFTR gene imposes. From the study, CF rabbits of all ages exhibited the classic nasal abnormalities with CFTR dysfunction. The upper airways revealed abnormalities in the olfactory and respiratory epithelium. These abnormalities are associated with a CFTR expression pattern suggesting ionocyte localization in this region. The CFTR expression in lower airways and throughout the distal airway regions in the rabbits resembled that of humans due to the absence of anion secretion. Decreased anion production is predicted to dehydrate the airway surface, causing hyperactivation of mucus. Rabbit tracheal CFTR was responsive to modulators active on human CFTR, suggesting that rabbits with CF can be useful when studying CFTR pharmacology. The lower airways of CF rabbits exhibited no bacterial infection, but mucus flakes were observed in one year old rabbits, which is similar to human findings. The next step in testing whether CF rabbits exhibit vulnerability to inhaled foreign insults would be to raise the rabbits in a wild-like environment and expose them to experimental changes such as controlled aspiration (Xu et al. 2021).

In conclusion of the rabbit study, CF rabbit models offers CF researchers with a medium-sized model that is relevant to human pathogenesis. CF rabbits exhibit vigorous measurements of CFTR function for testing of pharmacology and genetic therapy. These measurements include: a. spontaneous development of upper

airway pathology, b. abnormal GI properties coupled with weight loss and mortality, c. a CFTR protein responsive to human CFTR modulators, and d. lower airway bronchiolar CFTR expression. In order to improve future studies, there should be gut-corrected, lung-specific, and maintained modulators available for CF-relevant challenges (Xu et al. 2021).

Both of the current studies have been successful in providing researchers with CFTR mutation abnormalities that are similarly seen in human CFTR mutations. Cystic Fibrosis is genetically passed through generations, so there is no way to prevent the disease from developing in the fetus. Although this may be the case, research trials from recent years have provided key insights for how to properly live with CF and described how CF is not as deadly as the disease once was years ago. CF is still a life-threatening illness, but animal studies have helped researchers to learn and understand more about the phenotypes the CFTR gene imposes and how to develop the proper therapies to combat these defective phenotypes. Animal models are vital to CF research since humans are unable to be used as subjects due to the severity of the disease. Animal models allow researchers to study specific organs and tissues without limiting the life of a human experiencing CF. If we continue heading down this path, it is anticipated that a permanent cure for this life-threatening genetic disease will be discovered due to the extensive research on genetically modified animal models.

Acknowledgments:

I would like to thank my thesis mentor, Professor Sarah Cooper, for her guidance during the semester. She has supported me throughout this entire process with her advantageous comments and responses. I would also like to thank my academic advisor, Dr. Maria Theodoraki, for her support during my four years at Arcadia University. She has helped me to determine my career path and encouraged me to always stay positive along the journey. Lastly, thank you to Michaela Hvizdak for her feedback during the peer-editing process.

Bibliography:

- Adler F, Aurora P, Barker D, Barr M, Blackwell L, Bosma O, Brown S, Cox D, Jensen J ...
Walsh W, Woo M, Liou T. 2009. Lung Transplantation for Cystic Fibrosis. 6(8): 619-633.
doi: 10.1513/pats.2009008-088TL.
- Bilton D, Robinson P, Cooper P, Gallagher C, Kolbe J, Fox H, Jaques A, Charlton B. 2011.
Inhaled dry powder mannitol in cystic fibrosis: an efficacy and safety study. 38(5): 1071-
1080. doi: 10.1183/09031936.00187510.
- Boyd C, Shuling G, Huang L, Kerem B, Oren Y, Walker A, Hart S. 2020. New approaches to
genetic therapies for cystic fibrosis. *Science Direct*. 19 (1): 54-59.
- Condren M and Bradshaw M. 2013. Invacaftor: A Novel Gene-Based Therapeutic Approach for
Cystic Fibrosis. 18(1): 8-13. doi: 10.5863/1551-6776-18.1.8
- Dawson A, King T, Wilmut I, Harkness L, Kelly B, Rhind S. 2004. Immunohistological
Characterization of Cloned Lamb Nephropathy. *Journal of Histochemistry & Cytochemistry*.
52(12): 1657-1664. doi: 10.1369/jhc.4A6367.2004.
- Dreano E, Bacchetta M, Simonin J, Galmiche L, Usal C, Slimani L, Sadoine J, Tesson L,
Anegon I, Concordet J, Hatton A, Vignaud L, Tondelier D, Sermet-Gaudelus I, Chanson M,
Cottart C. 2019. Characterization of two rat models of cystic fibrosis-KO and F508del CFTR-
Generated by Crispr-Cas9. 2(4): 297-311.
- Edmondson C and Davies J. 2016. Current and future treatment options for cystic fibrosis lung
disease: latest evidence and clinical implications. 7(3): 170-183.
doi: 10.1177/2040622316641352
- Fan Z, Perisse I, Cotton C, Regouski M, Meng Q, Domb C, Wettete A, Wang Z, Harris A, White
K, Polejaeva I. 2018. A sheep model of cystic fibrosis generated by CRISPR/Cas9 disruption
of the CFTR gene. *JCI Insight*. 3(19): 1-22. doi: 10.1172/jci.insight.123529
- Guilbault C, Saeed Z, Downey G, Radzioch D. 2006. Cystic Fibrosis Mouse Models. *ATS
Journals*. 36(1): 1-7.
- Harris A. 1997. Towards an Ovine Model of Cystic Fibrosis. *Oxford Academic*. 6(13): 2191-
2193. <https://doi.org/10.1093/hmg/6.13.2191>
- Kellerman D, Mospan A, Engels J, Schaberg A, Gorden J, Smiley L. 2008. Denufosol: A review
of studies with inhaled P2Y2 agonists that led to Phase 3. 21(4): 600-607.

- Lamireau T, Monnereau S, Martin S, Marcotte J, Winnock M, Alvarez F. 2004. Epidemiology of liver disease in cystic fibrosis: a longitudinal study. *National Library of Medicine*. 41(6): 920-925. doi: 10.1016/j.jhep.2004.08.006.
- Liessi N, Pesce E, Braccia C, Bertozzi S, Giraud A, Bandiera T, Pedemonte N, Armiotti A. 2020. Distinctive lipid signatures of bronchial epithelial cells associated with cystic fibrosis drugs, including Trikafta. 5(16):e138722. Doi: 10.1172/jci.insight.138722
- Maule G, Arosio D, Cereseto A. 2020. Gene Therapies for Cystic Fibrosis: Progress and Challenges of Genome Editing. *International Journal of Molecular Sciences*. 1-13.
- Nuraj P and Hyseni N. 2017. The Diagnosis of Obstructive Hydronephrosis with Color Doppler Ultrasound. *U.S. National Library of Medicine*. 25(3): 178-181. doi: 10.5455/aim.2017.25.178-181
- Riordan JR, Rommens, JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JK. 1989. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science*. 245:1066-1073.
- Rosen B, Chanson M, Gawenis L, Liu J, Sofoluwe A, Zoso A, Engelhardt J. 2018. Animal and model systems for studying cystic fibrosis. 17(2): 28-34.
- Santos V, Cardoso A, Lopes C, Azevedo P, Gamboa F, Amorim A. 2017. Cystic fibrosis- Comparison between patients in pediatric and adult age. 23(1): 17-21.
- Semaniakou A, Croll R, Chappe V. 2019. Animal Models in the Pathophysiology of Cystic Fibrosis. *Frontiers in Pharmacology*. 9: 1475.
- Stoltz D, Rokhlina T, Welsh M. 2013. Intestinal CFTR expression alleviates meconium ileus in cystic fibrosis pigs. *The Journal of Clinical Investigation*. 123(6): 2685-2693. <https://doi.org/10.1172/JCI68867>
- Wilke M, Buijs-Offerman R, Aarbiou J, College W, Sheppard D, Touqui L, Bot A, Jorna H, Jonge H, Scholte. 2011. Mouse models of cystic fibrosis: Phenotypic analysis and research applications. *Science Direct*. 10(2): 152-171.
- Wine J and Joo N. 2003. Submucosal Glands and Airway Defense. *ATS Journals*. 1(1): 1-7.
- Xu J, Livraghi-Butrico A, Hou X, Rajagopalan C, Zhang J, ... Gentzsch M, O'Neal W, Grubb B, Chen E, Boucher R, Sun F. 2021. Phenotypes of CF rabbits generated by CRISPR/Cas9-mediated disruption of the CFTR gene. *JCI Insight*. 6(1): 1-18. doi: 10.1172/jci.insight.139813
- Yan Z, Stewart Z, Sinn P, Olsen J, Hu J, McMray P, Engelhardt J. 2015. Ferret and Pig Models of Cystic Fibrosis: Prospects and Promise for Gene Therapy. 26(1): 38-49.