INTESTINAL DYSFUNCTION IN CYSTIC FIBROSIS

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Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

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<tbody>
<tr>
<td>ACE</td>
<td>Angiostensin 1 Converting Enzyme</td>
</tr>
<tr>
<td>ADIPOR2</td>
<td>Adiponectin Receptor 2</td>
</tr>
<tr>
<td>AGTR2</td>
<td>Angiotension type II Receptor</td>
</tr>
<tr>
<td>AHRR</td>
<td>Aryl-Hydrocarbon Receptor Repressor</td>
</tr>
<tr>
<td>APIP</td>
<td>APAF1-Interating Protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CBAVD</td>
<td>Congenital Bilateral Absence of the Vas Deference</td>
</tr>
<tr>
<td>CDH8</td>
<td>Cadherin 8</td>
</tr>
<tr>
<td>CDKAL1</td>
<td>CDK5 Regulatory Subunit Associated Protein 1-Like 1</td>
</tr>
<tr>
<td>CDKN2A/B</td>
<td>Cycin-Dependent Kinase Inhibitor 2 A/B</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic Fibrosis</td>
</tr>
<tr>
<td>CF1</td>
<td>Complement Factor 1</td>
</tr>
<tr>
<td>CFF</td>
<td>Cystic Fibrosis Foundation</td>
</tr>
<tr>
<td>CFRD</td>
<td>Cystic Fibrosis Related Diabetes</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic Fibrosis Transmembrane Conductance Regulator</td>
</tr>
<tr>
<td>DCTNA</td>
<td>Dynactin 4</td>
</tr>
<tr>
<td>DIOS</td>
<td>Distal Intestinal Obstruction Syndrome</td>
</tr>
<tr>
<td>EDNRA</td>
<td>Endothelin Receptor Type A</td>
</tr>
<tr>
<td>EEA1</td>
<td>Early Endosome Antigen 1</td>
</tr>
<tr>
<td>EHF</td>
<td>ETS Homologous Factor</td>
</tr>
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Guluronate
Geometric Center of Fluorescence
Gastroesophageal Reflux Disease
Gastrointestinal
Glutathion S-Transferase Pi 1
Genome Wide Association Study
Hypothesis Driven Genome Wide Association Study
Bicarbonate
Human Leukocyte Antigen
Intestinal Epithelium
Insulin-like Growth Factor I
Insulin-Like Growth Factor 2 MRNA Binding Protein 2
Immunoreactive trypsinogen
Knock-Out
Leucine-Rich α2 Glycoprotein
Mannuronate
Mannose Binding Lectin
Mast Cell Protease 1 and 2
Meconium Ileus
20 kDA Light Chain of Myosin
Myosin Light Chain Kinase
Membrane-Spanning Domain
Methionine Sulfoxide Reductase A
Muc20  Mucin 20
Muc4  Mucin 4
NBD  Nucleotide-Binding Domain
PGs  Prostaglandins
PI  Pancreatic Insufficiency
PKA  Protein Kinase A
PKG  Protein Kinase G
PS  Pancreatic Sufficiency
PSS  Physiological Saline Solution
R Domain  Regulatory Domain
SERPINA1  Serpin Peptidase Inhibitor, Clade A, Member 1
SIBO  Small Intestine Bacterial Overgrowth
SLC26A9  Solute Carrier Family 26 Member 9
SLC4A4  Solute Carrier Family 4 Member 4
SLC6A14  Solute Carrier Family 6 Member 14
SLC8A3  Solute Carrier Family 8 Member 3
SLC9A3  Solute Carrier Family 9 Member 3
SNPs  Single Nucleotide Polymorphisms
TGFβ1  Transforming Growth Factor β1
VDCC  Voltage-Dependent Calcium Channel
WT  Wildtype
ΔCT  Cycle Difference
Intestinal Dysfunction in Cystic Fibrosis

Abstract

By

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Cystic Fibrosis (CF) is an autosomal recessive genetic disease that is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The CFTR protein is an anion channel and loss of CFTR function impedes transepithelial chloride permeation leading to dehydration and the accumulation of thick, sticky mucus in vital organs. Many symptoms occur as a result of non-functional CFTR; however mucus accumulation is a hallmark of CF. CF is a systemic disease that most commonly affects the lungs, pancreas, and gastrointestinal system. Severe lung disease is the major cause of morbidity and mortality associated with CF. There have been many advances in CF diagnosis and treatment since CF was initially described and the continued development of treatments and therapies has increased the predicted survival of patients born today to over 40 years old. However, patients still develop symptoms of CF that decrease their quality of life.

GI symptoms of CF are a major concern for patients today as they may be lethal if untreated, affect patients of any age, and may greatly decrease the quality of life for patients. Intestinal complications of CF may include Meconium Ileus (~10-25%), Distal Intestinal Obstruction Syndrome (~10-20%), Small Intestine Bacterial Overgrowth (~30-50%), and failure to thrive (~ 25-30%). Many factors, such as pancreatic insufficiency, modifier genes, and the environment, may contribute to the incidence of intestinal
symptoms, thus the origin of CF intestinal manifestations is complex and not well understood.

Even though CF has been extensively studied, the exact mechanism behind the development of intestinal symptoms remains to be elucidated. It was previously hypothesized that absence of CFTR in the intestinal epithelium was the main cause of intestinal obstruction, however work in our lab showed that absence of CFTR is necessary, but not sufficient to be the sole cause of intestinal obstruction. I analyzed the role of CFTR in the smooth muscle via the use of conditional mouse models that had Cftr inactivated only in the smooth muscle. I determined that Cftr in the smooth muscle contributes to the development of intestinal obstruction. However, absence of Cftr in the intestinal epithelium and smooth muscle together contribute to the development of CF-like intestinal obstruction rates. I have shown that absence of Cftr in the intestinal epithelium leads to dehydration and mucus accumulation and absence of Cftr in the smooth muscle leads to decreased muscle contraction. The decreased muscle contraction may be due to an impairment in calcium reuptake in the sarcoplasmic reticulum. My work provided novel insight into the origin of CF intestinal manifestations.

It is known that CF symptoms, including intestinal obstruction are influenced by modifier genes. Genome wide association studies (GWAS) have identified variants in potential modifier genes, however these studies do not provide information about how these variants modify the symptoms or gene expression. Functional studies are required to test the identified variants and determine how they alter CF symptoms. A GWAS of Meconium Ileus (MI) in CF identified variants in the gene solute carrier family 26, member 9 (SLC26A9) as a potential modifier of intestinal obstruction. I utilized CF
mouse models and functionally tested the effects on reduction of $Slc26a9$ function. I determined that reduction of one copy of $Slc26a9$ increased intestinal obstruction rates in the CF mice. I also utilized human epithelial cells and showed that single nucleotide polymorphisms in $SLC26A9$ identified via GWAS of MI in CF correlated with decreased $SLC26A9$ mRNA expression. Decreased $SLC26A9$ expression may lead to increased intestinal cellular proliferation, which may contribute to the increase in intestinal obstruction seen in the CF mouse model with a reduction of $Slc26a9$. My work provided evidence that $SLC26A9$ may prove to be a potential therapeutic target for CF intestinal obstruction.

There are several treatment options available for patients with intestinal obstruction, however not all treatments alleviate complications in all patients and some treatments have negative side effects. It is necessary to develop and test new treatments that may safely alleviate CF patients of intestinal obstruction. I tested a novel guluronate oligomer, OligoG, to determine if drug treatment alleviated CF mouse models of intestinal manifestations. I determined that drug treatment did improve intestinal obstruction, decreased intestinal transit time, and decreased mucus accumulation in CF mice. OligoG may be working as a calcium chelator and sequestering calcium from the mucins, which allows for proper unfolding of the mucins. Proper unfolding of mucins then creates mucus that is easily removed from the lumen. My work provided evidence that OligoG may help alleviate patients of CF intestinal obstruction.

A better understanding of the mechanisms behind the development of CF intestinal obstruction, as well as how to alleviate patients of the obstruction, is necessary to improve the quality of life of CF patients. This thesis provides novel insight into the
origin of intestinal obstruction, how a potential modifier gene influences intestinal obstruction, and how a novel drug may be used to prevent intestinal obstruction in CF patients.
Chapter 1 – Background and Significance

1.1. CF and CFTR

Cystic Fibrosis (CF) is an autosomal recessive genetic disorder that affects ~30,000 individuals in the United States and ~70,000 individuals worldwide. While CF occurs in every ethnicity, it is most commonly seen in Caucasians with a disease incidence of ~1/3000 (1). CF is a systemic disease that can affect all organ systems, however commonly affected organs include the lungs, pancreas, and gastrointestinal (GI) tract. Since the disease was initially described in the 1930s there have been many significant advances in CF research. However, there are still many questions that need to be answered to fully appreciate every aspect of the disease and its symptoms.

CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The CFTR gene is located on chromosome 7, contains 27 exons, and spans over 230 kb (2–4). CFTR is composed of two motifs and each motif contains a membrane-spanning domain (MSD) that typically consists of six transmembrane segments and a nucleotide-binding domain (NBD) (5). The NBDs contain sequences that interact with adenosine triphosphate (ATP) (5–7). The two MSD-NBD motifs are linked together by the regulatory (R) domain, which contains multiple consensus phosphorylation sites and charged amino acids (5). The CFTR protein is found in many cell types throughout the body and functions as a chloride channel that is regulated by cyclic adenosine monophosphate (cAMP) and is gated by ATP (5–7). Epithelial cells lacking CFTR function fail to allow transcellular permeation of chloride and this is accompanied by elevated sodium absorption through the sodium channel, ENaC. Fluid secretion from the lumen of the airway is increased and the mucosa becomes dehydrated.
Fluid secretion is also impaired when the exchange of chloride and sodium ions is altered in the cell. Recent evidence has suggested that CFTR also functions as a bicarbonate channel. An impairment of bicarbonate secretion in the lungs or GI tract may lead to abnormal ionic and fluid transport across epithelial surfaces, altered mucin unfolding, mucus accumulation, and abnormal pH (8–18). CFTR is a protein expressed in many cells types in the body and alterations in its function lead to many disease symptoms. The severity of the symptoms can be related to the type of mutation found in the patient.

The CFTR gene was identified as the cause of CF in 1989 (2–4) and since then over 2,000 mutations in CFTR have been described (19–22). The residual activity of different mutations contributes to the variation in severity of symptoms observed in CF patients. The mutations have been grouped into different classes depending on how they alter protein production, trafficking, function, or stability. The traditional mutation classification method consists of six classes (23,24). Class 1 mutations often create a premature stop codon, leading to the synthesis of truncated CFTR protein and mutation examples include W1282X and R553X. Class 2 mutations result in misfolded CFTR protein, which leads to alterations in processing and trafficking or premature CFTR degradation. Mutation examples for this class include F508del and N1303K. Class 3 mutations produce CFTR protein that reaches the cell membrane, but the channel regulation is impaired due to improper ATP binding and includes mutations such as G551D and S549N. Class 4 mutations result in proteins that have faulty ion channel function and therefore have decreased conductance. Mutation examples for class 4 include R117H and D1152H. Class 5 mutations lead to reduced synthesis of CFTR protein and include mutations such as 3849+10kbC→T and 2789+5G→A. Class 6
mutations produce CFTR protein with decreased stability and include mutations such as 4326delTC and 4279insA. Mutations in classes 1-3 may lead to more severe disease symptoms since CFTR channel function is significantly decreased or non-existent. People who carry mutations in classes 4-6 tend to have less severe CF symptoms since there is some functional CFTR present, it is just at a reduced quantity. However, different factors such as modifier genes and the environment may influence CFTR mutations, therefore making it difficult to group the mutation classes based on disease severity. Researchers and clinicians have recently adopted an additional mutation classification system parsing mutations in CFTR in terms of how they affect quantity and/or function of the CFTR protein (25). This system allows grouping mutations based on how CFTR may be targeted directly to correct the protein deficit and create functional CFTR.

As mentioned previously, there are over 2,000 classified mutations in the CFTR gene. The most common CFTR mutation is F508del, in which there is a deletion of phenylalanine in position 508 of CFTR and protein degradation due to improper folding (26). Of the 86.4% of patients with the F508del mutation, 46.5% are homozygous for the mutation and 39.9% are heterozygous (23). The next most common CFTR mutation is G542X, which is a nonsense mutation that leads to a premature stop codon and is found in 4.6% of CF patients (23,27). Another common CFTR mutation is G551D, which is a glycine to aspartate change and is seen in 4.4% of CF patients (23,28). The R117H CFTR mutation is seen in 2.8% of patients and is a missense mutation (23). This mutation may lead to severe or mild CF symptoms depending on the length of the polypyrimidine tract in the splice acceptor site of intron 8 of the CFTR gene. When the R117H mutation is in cis with a splice acceptor containing seven thymidines (7T) full-length transcript is
created and milder symptoms are seen due to the production of partially functional CFTR protein (29). When the R117H mutation is in cis with a splice acceptor containing five thymidines (5T) then significantly lower levels of full-length CFTR transcript are produced and more severe symptoms are seen in patients (29). The variety of CFTR mutations and the effects on protein quantity and/or function are only partially responsible for the wide variation of CF symptoms seen in patients.

CF was distinguished from celiac disease and described as an independent disorder in 1938 (30). Dr. Dorothy Anderson first described CF as a disease that arose from abnormal mucus accumulation and affected the lungs, pancreas, and GI tract (30,31). Death was predominately due to malnutrition and lung infection and the life expectancy of a CF patient was only ~6 months (30). Early treatments for CF consisted of pancreatic enzyme replacement therapy to aid in proper digestion of nutrients in the GI tract (32–36) and the use of antibiotics, such as penicillin, to help decrease the lung infections (37). The Cystic Fibrosis Foundation (CFF) was created in 1955 and worked to establish CF centers that utilized aggressive and comprehensive treatment programs that focused on the nutritional needs of patients, airway clearance, and treatment of lung infection (30). The overall goal of the CFF was to work with researchers, clinicians, and patients to find a cure for CF, and a huge milestone toward their goal was the identification of the $CFTR$ gene in 1989. That discovery has allowed researchers today to develop drugs, such as Ivacaftor, that target CFTR mutations directly. There are many drugs, targeting CFTR and symptoms of CF, currently in various stages of the CF Drug Pipeline. Treatment for patients today still includes pancreatic enzyme replacement therapy and antibiotics to target the malnutrition and lung infections; respectively. In
addition, patients often take supplements, such as laxatives, to loosen stool and prevent constipation and intestinal obstruction. Patients also use high-frequency chest wall oscillation vests to loosen the mucus that has accumulated in the lungs so it may be expelled from the body. The collaborative effort of research scientists and clinical teams has increased the predicted median age of CF patients in the 21st century to over 40 years old. The continued development of novel treatments and therapies has played a huge role in increasing the lifespan of patients and improving the quality of life for everyone with CF.

Another result of the identification of the CFTR gene was improved diagnoses for patients. The vast majority of CF patients today are diagnosed via newborn screening, which allows for immediate treatment of the disease and improved disease prognosis (23,38). Nationwide newborn screening for CF was realized in 2010 and all states are required to screen newborns for CF, unless the parents opt out of the screen. However, the methods used for screens may vary from state to state. All states take a blood sample from the baby to check the levels of immunoreactive trypsinogen (IRT), a protein secreted by the pancreas (38). Some states only perform a test to analyze IRT levels (IRT-state) and other states follow up a positive IRT test with a DNA analysis to draw further diagnostic conclusions (IRT-DNA-state) (38). A positive newborn screen occurs when IRT levels are elevated in the blood, which may happen if the baby has CF, is a carrier for CF, was born premature, or had a stressful delivery (38). Therefore it is imperative that babies with a positive newborn screen go through a complete evaluation for CF. The standard procedure to confirm a diagnosis of CF is the sweat chloride test, which measures the amount of sodium and chloride in sweat. Sweat test results range
from normal (salt chemicals ≤39mmol/L) to intermediate (salt chemical 40-59mmol/L) to abnormal (salt chemical ≥60mmol/L) and any newborn with sweat chloride results that are intermediate or abnormal should undergo genetic testing for CF (38). Genetics evaluations test for the most common alleles and will identify the majority of CF patients in infancy, however because there are a large number of alleles reported it is possible to miss a rare genetic mutation (23,30). Newborn screening has allowed for early diagnosis and intervention; however patients still develop symptoms of CF.

CF is a systemic disease that affects many organ systems, yet not all patients experience the same type or severity of symptoms due, in part, to the variation in genotype. Commonly affected organs include the lungs, pancreas, and intestines. The GI symptoms of CF will be covered in more depth in chapter 1.2 on page 23.

Lung disease accounts for much of the morbidity and nearly all of the mortality associated with CF (6,30,31). Almost all CF patients experience chronic pulmonary infection by CF opportunistic bacteria and also have an increased incidence of obtaining seasonal viral infections (39). CF opportunistic bacteria include *P. aeruginosa*, *S. aureus*, and *H. influenza* (6,30,39,40). When these bacteria colonize in the lungs the body counters with an immune response to attack the pathogens. It is hypothesized that the chronic colonization of bacteria in the lungs leads to an abnormal immune response which creates a chronic inflammatory state, subsequently causing destruction of the tissue and lung disease (6,39,40). There is some debate in the CF research community as to when the inflammation occurs in the CF lungs. It has been hypothesized that airway inflammation begins early in the illness, prior to bacterial infection (41). However, it has also been hypothesized that airway inflammation follows respiratory infection and the
removal of pathogens from the airways improves the lung disease in young patients (42). Regardless of the timing of bacterial infection and inflammation, an abnormal immune response to bacterial and viral pathogens is an accepted aspect of CF lung disease and is present in patients of all ages (6,39–43).

The characteristic accumulation of thick, sticky mucus in the CF organs also contributes to the bacterial infections (6,30,39). The absence of CFTR leads to altered chloride transport, which in turn alters fluid levels and causes dehydration in the lungs (6,30,39). Non-functional CFTR also limits bicarbonate secretion, which prevents proper mucin unfolding and leads to abnormal mucus production (8–11,13,14,16–18). The buildup of mucus impairs the mucociliary and cough clearance and causes bacteria to become trapped in the viscous mucus layer, which stimulates the inflammatory response in the lungs (6). It is also hypothesized that P. aeruginosa and S. aureus have a mucoid phenotype in CF lungs and lead to further accumulation of material (6,8).

CF patients also experience a wide variety of symptoms associated with lung disease. Lung dysfunction is common and includes impaired intake and expiration of air, decreased lung capacity, and respiratory distress such as coughing and wheezing (39). Patients also experience high airway resistance (opposition to airway flow caused by friction in the lungs), air trapping, hyperinflation, and pulmonary exacerbations (39,44). There is no real definition for the pulmonary exacerbations that patients experience, but an exacerbation occurs when there is a steady decline in lung function (44). During pulmonary exacerbations patients are typically hospitalized and receive intravenous antibiotics (23,39,44). Patients may also experience abnormalities secondary to bacterial
infection, such as sinusitis, nasal polyposis, lung collapse, and reduced pulmonary tissue
elasticity (30,31,39).

Exocrine pancreatic insufficiency is commonly seen in CF patients. The CFTR
protein is expressed in the pancreatic epithelial cells and allows for anions and fluid to
enter into the lumen of the ducts. In the lumen, chloride is exchanged for bicarbonate and
the net result of the anion transfer is an increase of alkaline fluid in the lumen (45). The
increased fluid allows the highly concentrated proteins in the pancreas to remain in a
soluble state prior to secretion by the acinar cells (46–48). Non-functional CFTR
channels in the pancreatic epithelium prevent the transfer of chloride and bicarbonate
ions and lead to a decreased volume of fluid in the lumen with a more acidic pH (45–47).
It is hypothesized that the decreased pancreatic secretions with high protein concentration
will precipitate in the pancreatic ducts leading to obstruction, inflammation, and atrophy
of the pancreas (45,46,49). In some patients, the pancreatic destruction begins in utero
and the pancreas may be destroyed in the first few years of life (45). It has been
determined that the type of CFTR mutation plays a huge role in the occurrence of CF
pancreatic disease and its rate of progression (45,50). CF patient pancreatic status is
classified as either pancreatic insufficient (PI) or pancreatic sufficient (PS). A large
number of CF patients are PI (~70-90%) (6,30,31,39,45) and symptoms may include
greasy stools, flatulence, abdominal bloating, and poor weight gain (49). The pancreatic
destruction and fibrosis seen in PI patients leads to malnutrition and deficiencies in
vitamins A, D, E, and K, essential fatty acids, proteins, sodium, potassium, and zinc
(39,49). Pancreatic enzyme supplements help patients with PI obtain the necessary
nutrients and prevent malnutrition (49). CF patients that are PS have evidence of
pancreatic damage, but retain sufficient exocrine pancreatic function to maintain normal digestion (45). However, PS patients have an increased risk of pancreatitis, which will lead to diminished exocrine pancreatic function over time (51).

CF patients may also develop a type of diabetes referred to as CF-related diabetes (CFRD). The prevalence of CFRD typically increases with age and is a type of diabetes that shares features of type 1 and type 2 diabetes (6,30,31,39,52). In general, CFRD occurs when the pancreatic damage prevents the endocrine component of the pancreas from functioning properly. The pancreas is not able to produce enough insulin (a feature of type 1 diabetes) and patients become insulin deficient (53). Also, patients with CFRD are not able to respond to the insulin that is produced (a feature of type 2 diabetes), which leads patients to become insulin resistant (53). Symptoms of CFRD may include increased thirst and urination due to hyperglycemia, excessive fatigue, weight loss, and unexplained decline in lung function (45,53). CFRD causes increased morbidity in CF patients, as it is associated with malnutrition and decreased lung function, therefore patients need to be tested for CFRD on a regular basis and treated by monitoring and controlling blood sugar levels with insulin treatments (53).

Some CF patients also develop symptoms in other organ systems. Patients may develop biliary disorders, such as obstructive biliary cirrhosis (6,30,39). Fatty infiltration of the liver occurs in ~70% of older CF patients and in <10% of those patients the fatty infiltration will progress to biliary cirrhosis (6,49). Patients also have small and poorly functioning gallbladders (~30%) and may experience gall stones (~10-15%) (6,30). Male patients also experience congenital bilateral absence of the vas deferens (CBAVD) (~98%) due to the vas deferens becoming blocked by viscid secretions and then...
becoming reabsorbed (6,30,31). Female patients typically do not have fertility issues, however the cervical mucus may become dehydrated and may impair fertility (6,30). Clinicians and researchers have also begun to analyze the psychosocial and psychiatric states of CF patients. It has been hypothesized that CF patients suffer from an increased prevalence of psychiatric problems and recently data have been produced that support this hypothesis (54–56). Further research is necessary in this field to determine a treatment plan for the mental health of CF patients.

All of the manifestations of CF play an important role in the type of treatment the patients receive and affect their quality of life. Further research into any of the CF complications may provide the knowledge necessary to treat or cure the symptoms and may improve quality of life for patients. This thesis will focus on the intestinal symptoms of CF and will address hypotheses on how the intestinal symptoms develop and how they may be alleviated to improve the quality of life for CF patients.

1.2. Intestinal Symptoms of CF

Improved pulmonary care has led to improved survival of CF patients; therefore GI complications of CF have become an increasing concern for patients. Intestinal symptoms of CF may be considered one of the most important non-pulmonary manifestations of the disease, as GI symptoms affect patients of all ages and affect all aspects of the intestinal tract, are associated with malnutrition and decreased lung function, and may lead to morbidity and mortality if not properly treated. Complications in the GI tract are complex and CF patients may experience a wide range of GI symptoms
that occur from newborn to adulthood. These manifestations are described below (Figure 1.1).

Meconium Ileus (MI) is a form of intestinal obstruction that occurs in 10-25% of CF patients and typically develops in utero and presents shortly after birth (within the first 48 hours of life) (24,31,57–59). MI occurs when thick meconium blocks the GI lumen, typically at the terminal ileum (49,57,60–62). It is hypothesized that the meconium becomes thickened due to the lack of fluid transport as a result of non-functional CFTR (63). There are two types of MI that are seen in CF patients. Simple MI occurs when there is failure to pass meconium within the first 48 hours of life without other GI complications (64). Complex MI occurs when there is failure to pass meconium and one or more of the following complications occurs: intestinal atresia, microcolon due to failed passage of luminal contents, necrosis, and/or perforation of the intestine (64). MI is fatal if untreated and is highly associated with severe CFTR genotypes in full term infants (61,64,65). Treatment options for MI include hyperosmolar enemas, nasogastric decompression, antibiotics, and intravenous hydration (66). After the obstruction has been resolved, enzyme replacement therapy is required once the patient is able to ingest sufficient volumes of food and nutrients (63). If conservative treatment methods fail to resolve the MI then operative approaches are required. Surgical treatment methods include ileostomy and surgical decompression (63,64).

Distal Intestinal Obstruction Syndrome (DIOS) is a recurrent intestinal obstruction that typically begins in the ileocecal region of the intestine and extends through the colon (63). DIOS affects ~10-20% of CF patients and can occur in children and adults (67,68). Symptoms of DIOS include abdominal pain, vomiting, and distension
Diagnosis of DIOS includes a physical exam that reveals a palpable mass in the right lower quadrant of the abdomen and a radiologic study demonstrating intestinal obstruction (64). The cause of DIOS is most likely due to abnormal CFTR function, since CFTR regulates chloride and bicarbonate secretion, and abnormal ion secretions lead to dehydration and improper breakdown of materials in the intestine (63). In addition, CFTR regulates EnaC, NHE3, and anion exchangers and impairment of these ion channels leads to excess absorption and reduced fluid secretion, which promotes bowel obstruction (63,70,71). A wide variety of causes secondary to the dehydration brought about by impaired CFTR may lead to the development of DIOS and include: change in diet, immobilization, respiratory infection, bacterial overgrowth, use of medications that can cause constipation, and inadequate pancreatic enzyme replacement therapy (63). Of the CF patients that experience DIOS, ~90% also have PI and DIOS is more frequently seen with severe CFTR genotype (68,69). Up to 50% of patients with DIOS had MI as neonates; therefore research has been done to determine if there is a correlation between MI and DIOS. Groups have shown that MI is a risk factor for DIOS (65,68,69,72,73), while others have shown that there is no significant correlation between the incidence of MI and DIOS (67). Treatment options for DIOS include oral rehydration, stool softeners, and osmotic laxatives (69,74). Maintaining adequate hydration is one of the most important methods for management of and prevention of recurrent intestinal obstruction (63). CF patients need to be vigilant that dehydration does not occur, as that will increase the likelihood of obstruction occurring. Several medications are also available that will aid in the management of intestinal obstruction. Bulk-forming laxatives, such as Lactulose, are useful to maintain water in the bowels to prevent dry, hard stools (63).
Polyethylene glycol (Miralax and Golytely) may be used to treat CF patients and works to pull water into the intestine, increasing the volume of the intestinal lumen, and simulating peristalsis (63,75). Patients with recurrent DIOS who have not responded to typical treatment for obstruction utilize a Mucomyst “clean-out” (63). This consists of 3 doses of N-acetylcysteine mixed with soda, orange juice, or mineral oil, followed by a clear liquid diet for 24 hours (63). Gastrografin enemas are another type of therapy used for DIOS and are a last resort treatment to dissolve the obstruction prior to surgical intervention. Severe cases of intestinal obstruction require surgery to remove the obstruction.

Constipation is a common symptom experienced by ~40% of patients and results from increased viscosity of intestinal contents (76). Constipation is a concern for CF patients, as it may lead to intestinal obstruction if it is not treated. Deciphering between constipation and DIOS is complicated. An abdominal radiograph may aid in distinguishing between the two as constipation will show stool throughout the colon, while DIOS typically presents with a large obstructive mass (74). Treatment for constipation is similar to that of DIOS and includes the use of laxatives and stool softeners, lubricants, and osmotic agents (69,77). It may be beneficial to modify the diet to a high-fiber and high-fluid diet (77). Behavioral modifications, such as regular toilet sitting and keeping a calendar of bowel movements, may also be helpful to prevent constipation (63,77). If constipation leads to fecal impaction then enemas or oral/nasogastric polyethylene glycol solutions should be used to alleviate the obstruction (63).
GI motility refers to the movement of food from the mouth through the esophagus, stomach, small and large intestines, and out through the colon. GI motility disorders are frequently reported in CF patients (76,78). Intestinal transit has been monitored in patients via the use of a wireless motility capsule (79). Data from the capsule visually demonstrated a significant delay in the small intestinal transit in the CF patients as compared to the healthy controls (79). Treatment for altered, or slowed, GI motility consists of the use of laxatives and stool softeners to help keep materials hydrated and flowing through the intestine. More research on the cause of the delayed GI motility in CF patients is necessary to develop new or repurpose current drugs to target this symptom of CF.

Appendicitis is a problem for CF patients as a diagnosis may be difficult to make since appendicitis and DIOS present with similar symptoms. The incidence of complications due to appendicitis is high in CF patients due to a delay in diagnosis and treatment, therefore there is an increased morbidity associated with appendicitis in CF patients (63,64,80).

Intussusception is an intestinal condition in which part of the intestine telescopes, or slides, into itself. CF patients are 10x more likely to develop intussusception than healthy individuals (74,81). In children intussusception has an acute presentation, but in adults it presents with abdominal pain and may be mistaken for DIOS (45,81). The cause of intussusception is thought to be due to the thickened secretions in the lumen creating a lead point for the telescoping section of bowel (81). Also, slowed GI motility, altered bowel thickness, and appendicle dilation may also contribute to its occurrence (81). Intussusception may be chronic and recurrent and ~25% of CF patients with
intussusception also present with intestinal obstruction (45,63). Occasionally it is possible to push the intestine back into its proper place via an enema, but surgery is often required to correct the intussusception (64).

Rectal Prolapse occurs in ~20% of CF patients and typically presents in children between 1-2.5 years of age (82). In children there is no gender bias, however in adults women typically suffer from rectal prolapse more often than men (83,84). It is hypothesized to be the result of malnutrition, increased intra-abdominal pressure from coughing, and excess intestinal strain due to constipation (49,83). Treatments for rectal prolapse include manual reduction of the prolapse, correcting malabsorption, avoiding excess straining during defecation by using stool softeners, and improving the patient’s nutritional status (63).

Fibrosing Colonopathy occurs when there is hypertrophy of the muscularis mucosae of the colon and inflammatory infiltrate and collagen deposition in the submucosa layer of the colon (85). The mechanism for the fibrosis is hypothesized to be a result of ischemia and repair (85). Fibrosing colonopathy is typically seen in younger CF patients, but it has also been reported in adults and can cause significant morbidity and possibly mortality if not treated properly (86). Surgical resection is the main treatment option for this CF symptom (86). Fibrosing colonopathy was first reported in 1994 and soon found to be directly associated with large doses of pancreatic enzyme supplements (64). Treating patients with lower doses of pancreatic enzymes has essentially eliminated the existence of this condition (64).

Gastroesophageal Reflux Disease (GERD) is a type of acid reflux disease that is 6-8x more likely to occur in CF patients than in healthy individuals (87). GERD is
frequently seen in patients with CF throughout their childhood (63). It is hypothesized that GERD occurs as a result of altered gastric emptying, medications that alter the GI pH, and increased abdominal pressure from coughing and percussion therapy (87). To diagnose GERD the patient may undergo esophageal pH monitoring, endoscopy, or manometry (63). Treatments for GERD include: acid suppression, antibiotics (erythromycin), and avoidance of foods that cause the acid reflux symptoms (63,64). If medical interventions have failed to treat GERD and complications of GERD arise (failure to thrive, erosive esophagitis, esophageal strictures, and reflux-related lung disease), then surgical therapy may be necessary (63).

Peptic ulcer disease consists of mucosal injury involving the esophagus, stomach, or small intestine. There is an increased incidence of peptic ulcer disease in CF patients due to gastric hypersecretion and decreased bicarbonate production as a result of PI (49,60,74). CF patients are also hypothesized to have an increased incidence of duodenitis (49,60,74).

Recent data have determined that CF patients have altered GI pH as compared to healthy controls. It was determined that fasting gastric and duodenal pH were normal in all patients, however after eating there was a significant acidification in the duodenal pH that became more pronounced with successive meals (88). Overnight fasting during sleep allowed the duodenal pH values to return to normal (88). GI pH was also monitored via a wireless motility capsule. The capsule data detected a deficient buffering capacity, which is necessary to neutralize gastric acid in the proximal small bowel, in CF patients (79). A balanced pH is necessary throughout the body, however in the intestine proper pH is required for digestion to occur and for the intestinal tract to function normally. An
abnormal pH may lead to insufficient secretion of pancreatic and gastric enzymes, improper breakdown of materials in the intestine (which may contribute to intestinal obstruction) and accumulation of bacteria in the intestine. Further research on GI pH and how it is altered in CF is required to develop drugs and therapies that may target the manifestations that occur as a result of altered pH.

Small intestine bacterial overgrowth (SIBO) is a condition that occurs in ~30-50% of CF patients due to a large number of bacteria growing in the small intestine (89,90). The exact incidence and cause of SIBO are not well understood, however predisposing factors of SIBO include: the use of antibiotics, previous GI surgery with resultant stasis, and GI dysmotility (63). Symptoms of SIBO may include bloating, gas, diarrhea, and abdominal pain (64). The gold standard for SIBO diagnosis is the hydrogen breath test, but it is not the most accurate test as it may not identify non-hydrogen producing bacteria present in the intestine and testing is not possible if patients are being treated with antibiotics (63,91). Antibiotics to remove the excess bacteria is the main treatment for SIBO, however antibiotic treatment needs to be carefully monitored because removing all of the bacteria from the intestine will disrupt the normal function of the GI tract.

Failure to thrive, or falling outside of the parameters for growth for the appropriate age group, is seen in ~25-30% of patients (92,93). Failure to thrive is one of the most commonly seen symptoms in newly diagnosed CF patients (94). It is thought to occur as a result of malnutrition and PI that is commonly seen in CF. Current treatments include the use of pancreatic enzyme supplements and a high-fat diet.

Many factors may contribute to the occurrence of intestinal symptoms in CF patients and several of these factors have been studied. However, there are still many
questions regarding CF intestinal symptoms, specifically, what is the mechanism for the development of the GI complications and how symptom development may be targeted to prevent these symptoms from occurring. Animal models of CF have proved to be very useful tools to study CF. Modern techniques have provided the opportunity for researchers to create animal models that allow for a more in depth analysis of CF symptoms.

1.3. Animal Models of CF

Several different types of animal models have been utilized to study CF and include zebrafish, mice, rats, rabbits (unpublished), ferrets, and pigs. Each type of published animal model has been used in CF research and displays several symptoms similar to CF patients (Table 1.1.).

Zebrafish have been used to study CF. While zebrafish do not exhibit many of the same CF phenotypes as the other models or patients, they do have altered chloride transport as a result of non-functional \textit{Cftr} (95). Zebrafish have been used to study the biophysical properties of CFTR in vivo and to determine the in vivo role of CFTR during development (25,95). It has also recently been shown that \textit{Cftr} is expressed and localized in the apical membrane of the zebrafish pancreatic duct and loss of \textit{Cftr} function leads to destruction of exocrine pancreatic function (96). Therefore, zebrafish may also prove to be a useful model to study the pathophysiology of pancreatic disease in CF.

Rodents, such as mice and rats, have been used to study CF. While mice and rats have similar characteristics in appearance and lifestyle, they are different species and therefore have many genetic, reproductive, developmental, morphological, and
anatomical differences. The creation and utilization of mouse models to study CF will be discussed in a later chapter. A rat with non-functional $Cftr$ was created and displayed several characteristics of CF. CF rats have altered chloride transport, maldevelopment of the trachea, and males have abnormal vas deferens (97). Rats with non-functional $Cftr$ also develop intestinal obstruction after weaning and experience significant weight loss and decreased survival. Rat models may prove to be useful to study the developmental processes that affect disease severity, the impact of $Cftr$ on the airways, and mucus synthesis and secretion (25,97).

A CF ferret model has been created and displays phenotypes similar to CF patients. Ferrets with non-functional $Cftr$ are a good representation of human lung disease in that they have defective airway chloride transport, decreased submucosal gland fluid secretion, and a predisposition to lung infections in the early postnatal period (25,98). They also develop liver disease and males have abnormal vas deferens (98,99). CF ferrets are born with mild exocrine pancreatic disease and during the first few months of life damage occurs to the pancreas that leads to the progressive loss of exocrine and endocrine pancreatic function (99). During this time CF ferrets experience hyperglycemia and glucose intolerance, suggesting that CF ferrets have abnormalities in the regulation of insulin secretion by the endocrine pancreas and develop CFRD (99). Lung infections are the primary cause of mortality in the first week of life and after the first week severe malabsorption and intestinal obstruction are the main cause of mortality (98). Ferrets may prove to be a good model to study CF lung disease and CFRD; however there are some special circumstances to take into consideration when using ferrets as a model. There are small numbers of suppliers; therefore ferrets must be shipped long distances and require
proper transport and handling to minimize shipping stress (100). Also an expert, knowledgeable of ferrets is required for successful handling of pregnant and whelping ferrets and rearing neonates, as well as maintaining proper housing for the ferrets (100).

Pigs that display non-functional $Cftr$ have been created and develop CF phenotypes, such as MI (100% of newborn CF pigs), failure to thrive, exocrine pancreatic destruction, and abnormalities in the liver and gallbladder (101,102). While it seems that CF pigs develop more severe CF intestinal symptoms than humans, the lung disease that develops is similar to that of CF patients (101,102). CF pigs also develop diabetes mellitus, however the features of diabetes occur before the absence of substantial loss of insulin producing cells, which is dissimilar to humans (103). CF pigs may prove to be a useful model to study many aspects of CF, however they are an expensive model to maintain as they are large animals and require surgical intervention and drug treatment to correct the severe MI that occurs at birth and to sustain life (101,102).

Animal models are tremendously useful tools to study human disease. They allow researchers to learn about $Cftr$ and its functions and to develop methods to treat the disease. Each of the animal models of CF is unique in their own way and presents the opportunity to study various aspects of the disease. This thesis utilized mouse models to study the intestinal symptoms of CF.

1.4. Mouse Models of CF

Mice have been used as a model organism for scientific research since the early 1900s and are recognized today as the foremost model for modern genetic research (104). Mice have genetic and physiological similarities to humans, therefore human diseases
may be recreated and studied with relative ease. The mouse genome has been studied thoroughly and can easily be manipulated and analyzed. Mice have been used as a model of CF since 1992 and the \textit{Cftr} gene has been extensively manipulated in mice (105,106). Also, mouse models with non-functional \textit{Cftr} present with several of the same characteristics of the disease as CF patients. Mice and patients both experience intestinal symptoms, failure to thrive, and reduced fertility (31,106). Mice display several aspects of the lung disease that is commonly seen in patients, however the lung disease in mice is not as severe, takes more time to develop, and is not the major cause of mortality in this model (106–108). One of the major CF symptoms not observed in mice is pancreatic insufficiency (109). Overall, the CF mouse model is a useful tool to study the role of ion channels in the development of lung disease, the biology of intestinal obstruction, and to evaluate genetic modifiers (105,106,109,110).

There are several different types of CF mouse models that are used for research. Complete absence of function, or null, mouse models were created via disruption of the \textit{Cftr} coding region, which lead to no expression of \textit{Cftr} mRNA (106). Mutations that are commonly seen in CF patients, F508del, G551D, G480C, and R117H, have been introduced into mice to create clinically relevant models (106,109). Each of the different mutations expresses unique characteristics that mimic the human mutation.

The work in this thesis utilized several different types of CF mouse models. One type of model used expresses the F508del \textit{Cftr} mutation, which leads to the production of non-functional CFTR protein (106,109). These mice exhibit severe intestinal symptoms, which is the major cause of mortality in this model, and have a 10-20\% reduction in body weight as compared to controls (106,111). Mice with another severe \textit{Cftr} mutation,
S489X, were also used. No detectable Cftr mRNA is observed in mice with this mutation (105,106). These mice develop severe intestinal symptoms, including intestinal obstruction, which is the major cause of mortality in this model (105). These mice also have a 10-25% reduction in body weight (105). Mouse models that have a R117H mutation and express 5-20% of functional Cftr mRNA were also utilized (106,112). These mice develop mild intestinal symptoms with ~10% developing intestinal obstruction and have a 10-25% reduction in body weight as compared to controls (109,111,112). The work in this thesis also used CF mouse models that were created by targeting exon 10 of Cftr. Exon 10 of the Cftr gene was targeted to create a CF mouse model because this exon has been found to play an important role in CFTR protein function and it has been shown that the disruption of exon 10 in mice leads to the development of CF phenotypes (113–115). Several different mouse models of CF were used to address different questions to elucidate the mechanism behind the development of CF intestinal symptoms.

Traditional mouse models of CF, with systemically non-functional Cftr, are great tools to study CF and are helpful in analyzing the disease as a whole. However, such models lead to the development of overlapping phenotypes. Genes may play various roles in different cells types and it is necessary to determine what those roles are to further understand how they affect disease development. The two major questions in this thesis are, what are the effects of cell specific Cftr absence and how does this absence lead to the development of GI complications. Since this could not have been achieved through the use of conventional null mouse models, mice with a conditional allele for Cftr were
generated. The conditional model has allowed for the understanding of the role of \textit{Cftr} in individual cell types.

A common method, the Cre/\textit{loxP} system, was used to alter gene expression. This system used the recombinase activity of the Cre and \textit{loxP} sites, or recognition points, as genetic activation or inactivation switches to create conditional knock-out models (116). Cre is an enzyme from P1 bacteriophages that functions to recognize and catalyze the recombination between its recognition sites, short sequences of DNA called \textit{loxP} sites (116). A relevant part of the gene of interest is floxed, or flanked by \textit{LoxP} sites. The location and orientation of these \textit{LoxP} sites determine the result of recombination (117).

There are multiple ways the Cre/\textit{loxP} system may be used to manipulate genes of interest. The most common use for the Cre/\textit{loxP} system is to inactivate a DNA sequence. When the \textit{loxP} sites flox the gene of interest in the same orientation, deletion of the surrounding area occurs during recombination. In this thesis, the Cre/\textit{loxP} system was used to delete exon 10 of \textit{Cftr} and this type of mouse model was referred to as \textit{Cftr}\textsuperscript{Δ10} (Figure 1.2.A) (118). If the \textit{loxP} sites flox the gene of interest in opposite orientation, then the floxed region becomes inverted and function is restored when Cre recombinase is expressed (116,119). In this thesis, exon 10 was targeted for inversion, thus activating CFTR protein production upon expression of Cre recombinase (120). This type of mouse model was referred to as \textit{Cftr}\textsuperscript{fl'} (Figure 1.2.B) (120). These models allowed for the study of the role of \textit{Cftr} in individual cell types and helped determine the role \textit{Cftr} plays in the development of CF intestinal symptoms.

The mouse models with conditional alleles for \textit{Cftr} are innovative and have provided novel information on the development of CF symptoms for multiple reasons.
First, *Cftr* restoration models are novel and are rarely used in CF research. Second, the *Cftr*\[^{Δ10}\] and *Cftr*\[^{Δl}\] models are complementary approaches to analyze the development of CF symptoms. This approach is more powerful than relying on a single model to analyze the gastrointestinal complications of CF. I utilized these conditional mouse models in order to determine the role of *Cftr* in the smooth muscle and if it contributes to the development of intestinal obstruction in CF.

1.5. CFTR Expression and its Role in Individual Cell Types

CF is a systemic disease most likely due to the wide expression of CFTR throughout the body. CFTR is usually found on the apical surface of epithelial cells in tissues such as the lungs (121–127), salivary glands (125), GI tract (125,127–129), pancreas (121,125,129,130), liver (125,131), gall bladder (125), sweat glands (121,127,129,132,133), kidneys (129), and male and female reproductive tracts (121,125,134). Recently is has been shown that CFTR is also expressed in several non-epithelial tissues such as the heart (135), brain (136), smooth muscle (137–140), skeletal muscle (141,142), and lymphocytes (143). The wide distribution of CFTR throughout the body makes it difficult to determine the role of CFTR in each of the individual cell types. To further analyze the phenotypes of CF one must be able to analyze the role of CFTR in a cell specific manner, which would provide a further understanding of the disease and allow for improvements in treatment and therapies.

The effect of *Cftr* function in the intestinal epithelium has previously been analyzed by our lab via mouse models that have *Cftr* conditionally inactivated and restored in the intestinal epithelium (IE) (118). It was hypothesized that if *Cftr* in the IE
played a necessary role in the development of intestinal obstruction, then the mice with $Cftr$ inactivated in the IE (IE $Cftr^{A10}$) would display intestinal obstruction at rates similar to $Cftr$ null mice. These data showed that the IE $Cftr^{A10}$ mice developed intestinal obstruction and goblet cell hyperplasia, but the incidence of obstruction was half that of the null mice (120). The mice that had $Cftr$ function restored in the IE (IE $Cftr^{fl'}$) were protected against obstruction and goblet cell anomalies (120). Next intestinal sections were analyzed and the presence of mucus and goblet cells were recorded. Intestinal sections from wild type mice showed normal levels and distribution of goblet cells, while sections from null mice displayed an increased number of goblet cells (120). Intestinal sections from IE $Cftr^{A10}$ mice displayed goblet cell numbers similar to the null samples and sections from IE $Cftr^{fl'}$ mice displayed results similar to the wild type samples (120). Since the IE $Cftr^{A10}$ mice showed a decreased incidence of obstruction, as compared to the nulls, it was concluded that absence of $Cftr$ in the IE is necessary for intestinal obstruction to occur, but it is not sufficient. Since the IE $Cftr^{A10}$ mice histologically resembled the null mice, it was hypothesized that $Cftr$ inactivation in the intestinal epithelium may result in decreased fluid secretion and an overproduction of mucus and that $Cftr$ inactivation in another tissue may contribute to the improper removal of the accumulated material. These data led me to analyze the function of $Cftr$ in the intestinal smooth muscle.

While the Cftr channel is usually located on apical membranes of epithelial cells, it has recently been shown to be expressed in other cell types. It has been shown that $Cftr$ is expressed and plays a functional role in regulating vascular tone in rat aortic smooth muscle cells and smooth muscle cells cultured from rat intrapulmonary arteries.
It has also been demonstrated that \textit{Cftr} is present in aortic vascular smooth muscle cells and smooth muscle cells isolated from \textit{Cftr} null mice showed an increase in muscle contraction when treated with vasoactive reagents as compared to smooth muscle cells isolated from wild type mice (138). It has also been determined that the CFTR channel is expressed in human airway bronchial tissue and in cultured airway smooth muscle cells and plays a functional role in calcium release (140).

It is hypothesized that altered intestinal smooth muscle function may aid in the occurrence of intestinal obstruction, but the cause of smooth muscle dysfunction is unknown. It has been observed that impaired intestinal smooth muscle function leads to slowed GI motility (144,145) and promotes erratic contractions of the muscle (146). These symptoms could be a result of altered muscle contraction and/or relaxation that affect peristalsis enough to prevent the proper removal of waste, thus elevating the incidence of obstruction. It is hypothesized that \textit{Cftr} absence in the smooth muscle may directly lead to muscle dysfunction, thus leading to the occurrence of intestinal obstruction. It is also hypothesized that the absence of \textit{Cftr} may lead to altered levels of prostaglandins (PGs), which in turn may lead to intestinal smooth muscle dysfunction. Smooth muscle peristalsis is influenced by PGs, thus elevated or altered PG levels may lead to intestinal dysfunction and subsequently obstruction (146,147). The role of \textit{Cftr} in the intestinal smooth muscle is not well understood and further research is required to determine if the absence of \textit{Cftr} in the smooth muscle directly leads to intestinal obstruction.

\subsection*{1.6. Gastrointestinal Environment in CF}
A recent publication suggested that a lack of \textit{Cftr} in the smooth muscle does not directly cause intestinal dysfunction, but rather smooth muscle dysfunction occurs indirectly as a result of other environmental effects (147,148). The results showed that mouse intestinal smooth muscle was functional at postnatal day 5 and that dysfunction began to develop around postnatal day 7-14, suggesting that the smooth muscle becomes dysfunctional after other GI environmental factors become altered (147). GI environmental factors that could influence how the smooth muscle functions include: pH, bicarbonate, and mucus.

The GI tract plays a crucial role in digesting the food people eat. The pH throughout the GI tract needs to be specifically regulated to carry out this function. The stomach is normally acidic, which aids in the breakdown of foods and the intestines are a more neutral pH, which allows the proper enzymes to be created that further breakdown fats and proteins into usable substances. Alterations in pH levels of the GI tract may lead to intestinal dysfunction as proper digestion may not be able to occur in an altered environment. Previous data have shown that wild type mice have acidic gastric conditions, with a fasting pH of $\sim$4.0 (149) and a more basic intestinal pH around 6.47 (150). Similar to humans, the CF mouse intestinal pH ($\sim$6.15) is significantly more acidic than the wild type pH ($\sim$6.45) (150,151). A decreased pH, meaning a more acidic environment, in the proximal small intestine may lead to delayed enzyme release or inhibited activity of the enzymes (88). It has been shown in CF mice that an abnormally acidic duodenum leads to increased signaling to the pancreas to stimulate bicarbonate secretion, which adds stress to the pancreas and causes inflammation (151).
CFTR typically functions as a chloride channel, but it has been shown that CFTR may also function as a bicarbonate (HCO₃⁻) channel (13,15,152). HCO₃⁻ assists in the digestion of food by breaking down materials in the stomach and neutralizing the acidic chyme that enters the intestines (151). Another suggested function of HCO₃⁻ is aiding in the normal development of mucins, thus the reduction or absence of HCO₃⁻ secretion may contribute to the accumulation of the thick, sticky mucus that is characteristic of CF (9,11). Mouse models have been used to analyze the link between Cftr inactivation and mucus accumulation. Previous data have shown that basal and stimulated secretion of duodenal mucosal HCO₃⁻ is reduced in CF mice as compared to wild type mice (11–13). Currently, the link between Cftr loss, bicarbonate secretion, and mucus production is not well understood, but it is hypothesized that bicarbonate is required for the production of normal mucus (14).

Mucus is a normal component of the human body and serves to cover and protect the epithelial surfaces lining the respiratory, GI, and reproductive tracts (153). Mouse models have been utilized to analyze the effect of Cftr absence on mucus production. As compared to wild type models, the null mice display increased expression of several intestinal mucins (the main structural component of mucus), which corresponded to the accumulation of mucus seen in CF (154–156). Further analyses on these topics are required to further understand how exactly Cftr affects mucin production and mucus secretion.

If the intestinal symptoms seen in CF were all due to alterations in the GI environment, then our lab would have seen a greater incidence of intestinal obstruction in the IE CftrΔ10 mouse models previously analyzed. Our data suggested that Cftr in the IE
plays a necessary role in the development of CF intestinal symptoms, but is not sufficient to be the sole cause of the symptoms. I hypothesize that the development of CF intestinal symptoms is complex and multiple factors are involved. I also hypothesize that there is cross talk between CFTR in several different cell types and absence of CFTR in multiple cell types diminishes these interactions. When the cross talk between the different cell types is reduced or stopped altogether, than CF intestinal symptoms develop.

1.7. Modifier Genes of CF

While the causative gene for CF, *CFTR*, was identified in 1989 (2–4), there has become increasing evidence that other factors play a substantial role in disease severity. Many factors may create phenotypic variation in a monogenic disorder, such as different mutations in the disease causing gene, genetic modifiers, environmental factors, random events, and interactions among and between any of those listed above (157). It was hypothesized that gene-gene and/or gene-environment interactions likely explained the variation seen in CF symptoms (157). Researchers began to analyze the relationship between *CFTR* genotype and disease phenotype to determine if the variation in severity of the disease was due to *CFTR* mutation or other factors (50,158). It was determined that there was a strong correlation between *CFTR* genotype and pancreatic status, however there was poor correlation between *CFTR* genotypes and the severity of lung disease (50,158). These data supported the previous hypothesis and concluded that CF lung disease severity is influenced by environmental factors and secondary genetic factors.

Once the initial genotype-phenotype analyses determined that other factors play a role in CF symptoms, the next step was to determine if the disease variation was due to
environmental factors or secondary genetic factors (157). Traditionally, twin and sibling studies have been used to determine the degree of heritability of various phenotypes. These types of studies are able to determine the contribution of genetics to disease symptoms of interest. Monozygotic twins, who share 100% of their genes, and dizygotic twins or typical siblings, who share 50% of their genes, are used in such studies. An estimate of heritability of the traits of interest is determined when the similarities between monozygotic twins are compared to the similarities between dizygotic twins or siblings. Heritability estimates range from 0 to 1 and a heritability value of 1 indicates that variation is due to genetic factors and a heritability value of 0 indicates that none of the symptom variation is genetic. Twin and sibling studies are helpful to separate out the genetic and environmental causes of disease symptoms. One group utilized data collected from the European CF Twin and Sibling Study and analyzed weight predicted for height (representative of nutritional status) and pulmonary status (159). Overall, this study determined that there was discordance between weight predicted for height and pulmonary status for the monozygotic twin pairs, which indicated that the disease severity was influenced by secondary genetic factors (159). Additional twin studies have supported the finding that CF lung disease severity is influenced by genetic factors other than \textit{CFTR} (157,158,160). Twin and sibling studies have also been conducted to determine if modifier genes play a role in CFRD in patients. One group determined that the concordance rates in monozygotic CF twins (75% in 12 pairs) and dizygotic CF twins and CF siblings (14% in 71 pairs) generated heritability estimates that indicated a strong role of secondary genetic factors in the development of CFRD (161). Another study analyzed the heritability of MI and DIOS in CF patients. The aim of the study was to
determine the contribution of genetic and non-genetic factors on the development of CF intestinal obstruction (65). This group utilized data collected by the CF Twin and Sibling Study and analyzed the samples for MI status, significant covariates, and genome-wide linkage (65). The study concluded that there was greater concordance for MI in monozygous twins than in dizygous twins and siblings (heritability value was close to 1), thus modifier genes play a significant role in the incidence of MI in CF patients (65). However, the study showed the opposite result for DIOS, suggesting that non-genetic factors play a role in its incidence in CF.

After it was shown that some symptoms of CF are influenced by genetic factors, analyses were done in order to identify potential modifier genes that influence CF disease severity. This has been done via genome wide association studies (GWAS) and linkage studies. GWAS allow researchers to scan across the DNA of many individuals and identify genetic variants which may be associated with particular traits. In the case of CF, researchers utilize test subjects that all have a particular trait of CF (eg. DF508 CFTR mutation, pancreatic status, or intestinal obstruction) and then determine if variants associate with the aspect of the disease being studied. Conventional GWAS may analyze ~500,000 SNPs, however statistical analyses may be carried out on the initial GWAS data in order to provide more power to the study and obtain more detailed information. Linkage studies have also been helpful in identifying loci that associate with disease symptoms. Linkage studies are used to determine the physical segments, or chromosomal location, of the genes that are associated with the trait of interest. GWAS and linkage analyses are very useful in identifying genetic variations that may contribute to complex disease symptoms, however additional functional studies are required to determine if the
variants associated with nearby genes are the cause of the manifestation or simply linked to the causative variant. Substantial progress has already been made in determining the contribution of genetic factors to the variability of CF symptoms. Association and linkage studies have been used by many groups to identify several potential modifier genes that may play a significant role in CF symptom development (Table 1.2.) (162).

Studies have been conducted to elucidate the genetic components associated with the severity of CF lung disease. Many genes have been proposed to be potential modifiers of CF lung disease and this section will highlight several of those candidate genes (163–165). Variants near the gene transforming growth factor β1 (TGFβ1), which plays a role in regulating airway inflammation and remodeling (166), have been previously identified as a potential modifier region that may alter disease severity in asthma and chronic obstructive pulmonary disease (166). Therefore, TGFβ1 has been repeatedly studied as a potential modifier gene of CF lung disease. Variants in TGFβ1 have been associated with decreased lung function in two independent studies monitoring lung disease in patients homozygous for the F508del mutation (167,168) and it was determined that alleles in the promoter (-509) and first exon (codon 10) of TGFβ1 also are associated with variation in lung disease severity in CF (167,168). It was also reported that a novel TGFβ1 haplotype (opposite to the haplotype associated with disease severity) had a beneficial effect on the pulmonary phenotype (168). However, a small study did not report the same association of TGFβ1 and CF lung disease (162). Variants near ETS homologous factor (EHF) and APAF1-interating protein (APIP) were both identified via GWAS and linkage studies to have an association with decreased lung function in F508del homozygous patients (164,165). EHF works to regulate epithelial differentiation under stress conditions and
inflammation and APIP inhibits apoptosis (169). There was significant association for CF lung disease severity found on a locus on the X chromosome and may contain the genes angiotension 2 type II receptor (AGTR2) and solute carrier family 6 member 14 (SLC6A14) (164,165). The AGTR2 gene has been shown to mediate apoptosis and play an important role in the early developmental stages of life. AGTR2 has also been suggested to play a role in pulmonary function and is a risk factor for CF lung disease severity (164,165,170,171). SLC6A14 encodes an amino-acid transporter and variants in this gene have been reported to modify risk for lung disease severity (165,172). The human leukocyte antigen (HLA) gene family, which aid in making the proteins that are present on immune system cells, have been determined to be associated with lung disease severity in non-CF and CF individuals (173–176). Variants near the HLA genes have been identified via GWAS and linkage studies as potential modifiers of CF lung disease severity (164,165,173). Variants near the following genes, early endosome antigen 1 (EEA1) which plays a role in intracellular trafficking of CFTR (177), aryl-hydrocarbon receptor repressor (AHRR) which is involved in regulation of cell growth and differentiation, and cadherin 8 (CDH8) which mediates calcium-dependent cell-cell adhesion, have all been identified via GWAS and linkage analysis as potential modifier genes of CF lung disease severity (164). A locus near the genes mucin 4 (Muc4) and mucin 20 (Muc20) have also been identified as possible modifier genes of CF lung disease (165). Both genes are present in airway mucus and encode mucins that are ‘tethered’ on ciliated airway mucosal surfaces (178–180). It is hypothesized that Muc4 and Muc20 play a role in airway mucus secretion, removal of mucus from the airway epithelium, and mucociliary clearance (178). Modification of these genes may allow
mucus and bacteria to accumulate in the lungs and subsequently lead to impaired lung function. Solute carrier family 9 member 3 (SLC9A3) is a sodium/hydrogen exchanger that is involved in pH regulation and epithelial ion transport (181) and several studies have identified variants near this gene to have a correlation with CF lung disease severity (165,172,182). Variants within the solute carrier family 8 member 3 (SLC8A3) gene, which is a sodium/calcium exchanger that controls calcium homeostasis, have also been identified to have a correlation with CF lung disease (164). Variants near the gene mannose binding lectin (MBL2), which plays a role in innate immunity, and MBL have been associated with decreased lung function in CF (183–189). GWAS also identified a locus near the endothelin receptor type A (EDNRA) gene, which plays a role in vasoconstriction, to have an association with decreased pulmonary function in CF patients (190). GWAS have identified several loci that may contain genes that correlate with lung disease severity in CF patients, however additional functional studies are required to determine if alterations in the potential modifier genes do indeed alter CF symptoms.

It has been hypothesized that an early infection with *P. aeruginosa* leads to an increase in lung disease severity; therefore several GWAS and linkage analyses were conducted to identify regions that associated with an early *P. aeruginosa* infection (187). A locus near the gene dynactin 4 (DCTNA), which plays a role in intracellular transport, was shown to significantly associate with early age of chronic *P. aeruginosa* infection (191). Variants near the genes MBL2, SLC26A14, and SLC9A3 were also identified to have strong associations with early *P. aeruginosa* infection (172,182,186–188). Variants within the pathways involving HLA Class II genes have also been associated with *P.*
*aeruginosa* infection (176). Future studies will aid in determining if lung disease severity is influenced by the variants identified via GWAS for lung infection.

Pancreatic disease in CF, specifically exocrine pancreatic function, has been determined to be strongly influenced by *CFTR* (50,192,193). However, it has been noted that newborns with the same *CFTR* genotype display variable levels of pancreatic disease at birth, which suggests a role for additional genetic or environmental influences (194). IRT levels may be used to monitor acinar destruction postnatally, which is a marker for exocrine pancreatic damage and could be used to predict the development of PI in newborns (195,196). A study monitoring IRT levels in sibling pairs with severe disease reported there was a high proportion of shared variation in predicted IRT levels, thus suggesting that IRT is heritable and genetic modifiers may play a role in CF pancreatic disease (197). GWAS have identified a locus near the gene solute carrier family 26 member 9 (*SLC26A9*), that is strongly associated with prenatal exocrine pancreatic disease (172,193). Further analyses are necessary to determine if they are additional modifier genes of CF exocrine pancreatic disease.

CF patients develop CFRD, which has similar disease characteristics and strong genetic components of Type 1 and Type 2 diabetes (198,199). A GWAS of patients with CFRD identified several regions that associated with CFRD (200). Variants in *SLC26A9* were identified as having a strong association with CFRD (200). Loci in genes that are commonly associated with type 2 diabetes in the general population were also identified as potential modifier genes of CFRD. The potential modifier genes identified include: protein coding genes CDK5 regulatory subunit associated protein 1-like 1 (*CDKAL1*), cyclin-dependent kinase initiator 2 A/B (*CDKN2A/B*), and insulin-like growth factor 2
MRNA binding protein 2 (IGF2BP2) (200). Additional studies are required to determine if the potential modifier genes of CFRD identified do indeed alter the CF manifestation.

A small percentage of CF patients (~3-5%) develop severe liver disease with portal hypertension. It was hypothesized that secondary genetic factors may influence the risk of liver disease in CF patients and several genetic studies were performed to identify variants that had an association with liver disease in patients. An association study identified variants near the gene serpin peptidase inhibitor, clade A, member 1 (SERPINA1), which is a serine protease inhibitor, as a possible risk factor for CF liver disease (201,202). Another association study determined that loci within the genes angiotensin l converting enzyme (ACE), which helps catalyze angiotensin II, and TGFβ1 had strong association with liver disease in CF patients (203). Another study identified variants near glutathione S-transferase Pi 1 (GSTP1), an enzyme necessary for detoxification, as a potential risk factor for severe liver disease (202,204). Variants in the gene MBL2 was also identified to have an association with CF liver disease (205). Functional analyses will provide insight into the role of the identified variants in the development of liver disease in CF patients.

There is also evidence that secondary genetic factors play a role in the development of intestinal obstruction in CF patients (65,206). One group performed a genome-wide linkage analysis to identify regions that may contain modifier genes of MI (65). This group identified several regions that had suggestive linkage for modifier genes of MI and these regions were located on chromosome 4q35.1, 8p23.1, and 11q25 (65). Another study found a strong association between variants near the methionine sulfoxide reductase A (MSRA) gene, located on chromosome 8, and MI in CF patients (110). An
association was also identified between incidence of MI and a locus on chromosome 12p13.3 near the adiponectin receptor 2 (*ADIPOR2*) gene (207). Another GWAS identified five single nucleotide polymorphisms (SNPs) at 2 loci near the genes *SLC6A14* at Xq23-24 and *SLC26A9* at 1q32.1 (206). This same group utilized a hypothesis driven GWAS (GWAS-HD) to obtain a more in depth analysis of regions that may contain modifier genes of MI. A GWAS-HD incorporates biological hypotheses into the prioritization and interpretation of the initial GWAS data. This test re-evaluates the data and adds statistical significance based on the hypothesis, in addition to improving the power of the test. The GWAS-HD analyzing MI in CF incorporated the knowledge that mutations in *CFTR* alter electrolyte and fluid transport across cell surfaces into the data analyses and identified associations with the same SNPs initially identified (*SLC6A14* and *SLC26A9*) and a identified variants in another region near the gene *SLC9A3* (206).

Genome-wide analyses have also identified regions or genes that may protect against the development of MI. A genome-wide linkage analysis identified several regions that had suggestive linkage for modifiers genes that may protect against MI and these regions were located on chromosome 20p11.22 and 21q22.3 (65). In addition, another study identified a locus mapped to chromosome 4q13.3, including the sodium bicarbonate cotransporter solute carrier family 4 member 4 (*SLC4A4*), to have a protective association with the incidence of MI (207).

CF is a monogenetic disorder that becomes complex when manifestations of the disease are influenced by secondary genetic factors. It is interesting to note that several of the potential modifier gene listed above further complicate the disease by having pleiotropic effects on CF symptoms. Several loci near genes that associated with lung
disease severity in patients also were identified as risk factors for infection with *P. aeruginosa* (MBL2, SLC9A3, SLC6A14, and HLA-DRA) and severe liver disease (*TGFβ1* and *MBL2*). Variants in a few of the solute carrier genes were pleiotropic and showed strong association with exocrine pancreatic disease and CFRD (SLC26A9) and MI (SLC26A9, SLC6A14, and SLC9A3).

GWAS have identified several loci near genes that may modify CF symptoms. Variants have been found to associate with CF lung disease severity and infection with *P. aeruginosa*, exocrine pancreatic insufficiency and CFRD, liver disease, and MI. Variants have been identified in many different types of genes, but the genes that play a role in inflammation or immune system regulation, cell differentiation/growth, and anion transport are of interest as CF patients and *Cftr* null animal models report abnormalities in these processes in multiple organs. Inflammation and proliferation have been reported in the lungs, intestine, and pancreas and leads to a worse disease phenotype (6, 39–43, 46, 48, 49, 208–210). Alterations in anion transport which lead to dehydration are a hallmark of CF and affect many organs. These potential modifier genes add to the complexity of CF in a couple ways. A few of the variants have pleiotropic effects and may influence multiple CF manifestations. In addition, the CF symptoms are influenced by one another. For example, decreased lung function typically correlates with malnutrition or intestinal obstruction, therefore variants that alter one CF symptom may be influencing another symptom indirectly. While GWAS are useful tools to identify variants in genes that may modify CF manifestations, they do not provide information on gene expression or mechanisms of gene modification. Functional studies must be conducted to determine the variant’s effect on disease symptoms and to provide insight
into pathways that may play a role in the incidence of the symptom. Chapter 3 of this thesis functionally analyzed a locus on chromosome 1 near the gene \textit{SLC26A9}, which was identified via a GWAS of MI in CF.

1.8. \textbf{Solute Carrier Family 26 Member 9 – SLC26A9}

Solute Carrier Family 26, Member 9, or \textit{SLC26A9}, was recently identified as a potential modifier in a GWAS analyzing MI in CF patients. SNPs associated with genome-wide significance from two regions were identified and included \textit{SLC26A9} located on chromosome 1 and \textit{SLC26A14} on the X chromosome (206). I focused on \textit{SLC26A9} and functionally analyzed the gene to determine if it modified the risk of CF intestinal obstruction.

\textit{SLC26A9} is a member of the \textit{SLC26} gene family, which encodes several multifunctional anion exchangers and anion channels with distinct physiology (211,212). \textit{SLC26A9} is located on chromosome 1 and encodes an anion transporter (206). This transporter has three distinct functions, operating as a chloride-bicarbonate exchanger, a chloride channel, and a sodium co-transporter (212,213). It is hypothesized that these distinct functions allow \textit{SLC26A9} to perform a wide array of physiological functions and allow for dynamic physiological regulation in tissues where \textit{SLC26A9} is expressed (212). \textit{SLC26A9} has been shown to be strongly expressed in the epithelial lining of the stomach and lung and has also been detected in the brain, heart, kidney, small intestine, thymus, and ovary (212,214). It has been determined that SLC26A9 and CFTR are co-expressed in several of the previously mentioned tissues and that the two proteins interact with one another (214). This interaction is not very well understood and there are several
conflicting hypotheses about the interaction in the literature, which suggests that there may be many layers involved in the interaction between SLC26A9 and CFTR. One group hypothesized that there is a reciprocal regulation between CFTR and a few members of the SLC26 family. It was determined that two members of the SLC26 family, SLC26A3 and SLC26A6, interact with the R-region of CFTR via their Sulfate Transporter Anti-Sigma (STAS) domains (215,216). The reciprocal regulation between CFTR and the SLC26 family members is crucial to maintain control of epithelial chloride and bicarbonate transport in the cell (216,217). One group hypothesized that, in human bronchial epithelial cells, SLC26A9 functions as a constitutively active chloride channel that is regulated by CFTR (213). This hypothesis was based on data that showed that SLC26A9 had decreased chloride transport when CFTR activity was blocked with a pharmacologic agent (213). Another group hypothesized that SLC26A9 and CFTR have a physical interaction and SLC26A9 stimulates wildtype CFTR protein expression, but is ineffective on mutant CFTR protein expression (218). Another hypothesis agrees with the others in that the R-region of CFTR interacts with the STAS domain of SLC26 family members, however this group suggests that the protein-protein interactions are not the same for all of the SLC26 family members (214). It is hypothesized that the SLC26A9-STAS domain binds to the R-region of CFTR, however this binding interaction prevents the function of SLC26A9 ion transport activity (110). These results imply that SLC26-STAS domains may all interact with the R-region of CFTR; however the physiological outcome is specific to SLC26 proteins. There are many different cell types that have been used to analyze the interaction between SLC26A9 and CFTR, which may contribute to the controversy about the interaction. The functional interaction between CFTR and
SLC26A9 in polarized epithelial cells and in non-polarized cells expressing CFTR and SLC26A9 was examined and it was determined that the interaction is dependent on cell type (219). In polarized airway epithelial cells it was determined that SLC26A9 acts as a constitutively active chloride channel when wildtype CFTR is present and contributes to the calcium and chloride secretions (219). It was determined that SLC26A9 chloride channel activity was inhibited during activation of CFTR (219). Overall, these data show that there is still much to learn about the interaction between SLC26A9 and CFTR and that the interaction may have different functions depending on the cell type.

To understand the role of Slc26a9, a Slc26a9 KO mouse model was created and the phenotypes were analyzed. Initial reports on the examination of the KO mice suggested that they appeared healthy and displayed normal growth patterns (220). Upon closer examination of the GI tract, several abnormalities were discovered. The Slc26a9 KO mice had a more basic gastric pH as compared to controls, which would lead to improper digestion of materials in the GI tract (220). The altered gastric pH was determined to be caused by a decrease in gastric acid secretion due to the loss of tubulovesicles in parietal cells, which are the acid secreting cells in the stomach (220). In younger Slc26a9 KO mice (3-4 weeks of age) histological alterations, such as elongated villi and crypts, and reduced proximal duodenum bicarbonate and fluid secretion were observed (221). Each of the alterations observed may play a role in the occurrence of intestinal obstruction by preventing proper mucus secretion and allowing the accumulation of mucus in the lumen, impairing the secretion of digestive enzymes, or by altering the cross-talk between the cells that allows for proper GI tract function to occur (220,221).
To analyze the role of \textit{Slc26a9} in the CF context, a \textit{Cftr} and \textit{Slc26a9} KO mouse model was created (221). The \textit{Cftr} and \textit{Slc26a9} KO mouse model had significantly increased rates of intestinal obstruction (221). The enhanced rate of intestinal obstruction seen in the CF mice lacking \textit{Slc26a9} may be due to any of the previously mentioned phenotypes of the \textit{Slc26a9} KO mouse model. It is hypothesized that the absence of \textit{Cftr} and \textit{Slc26a9} together further exacerbates the phenotypes seen in the \textit{Slc26a9} KO mouse. Further research is required to elucidate the mechanism behind \textit{Slc26a9}'s modification of CF intestinal obstruction.

\textbf{1.9. Summary/Research Aims}

The overall goal of this thesis was to test various hypotheses that might explain the mechanisms behind the development of CF intestinal symptoms, specifically intestinal obstruction. Another aim of this thesis was to determine if intestinal obstruction may be alleviated by targeting a modifier gene of MI in CF and by treating CF mice with a novel drug, OligoG.

CF intestinal obstruction and factors hypothesized to cause obstruction have been extensively studied in CF mouse models; however the origin of intestinal obstruction remains to be elucidated. It was previously hypothesized that \textit{Cftr} in the intestinal epithelium was the origin of the intestinal obstruction. Previously published work by our lab showed that absence of \textit{Cftr} in the intestinal epithelium is necessary for intestinal obstruction to occur, but it is not sufficient to be the sole cause. It was then hypothesized that \textit{Cftr} in another cell type may be contributing to the development of the obstruction. Chapter 2 of this thesis focused on determining the role of \textit{Cftr} in the intestinal smooth
muscle and how \textit{Cftr} in this cell type contributes to the development of intestinal obstruction in CF. The smooth muscle is a major part of the GI tract and any alterations in it may lead to abnormal peristalsis and may contribute to the occurrence of several CF intestinal symptoms. CFTR has recently been found to be expressed in vascular, airway, and intestinal smooth muscle of CF animal models and patients and studies testing the smooth muscle in \textit{Cftr} null animal models have been conducted. However, CF is a complex disease and an analysis of a \textit{Cftr} in a null model may make determining the origin of obstruction difficult. This thesis utilized a novel approach to target \textit{Cftr} exclusively in the smooth muscle in mouse models and performed intestinal assays to determine how absence of \textit{Cftr} in the smooth muscle altered the function of the intestine. I determined that absence of \textit{Cftr} in the smooth muscle is involved in the development of CF intestinal obstruction and absence of \textit{Cftr} in the intestinal epithelium and smooth muscle together contribute to the development of CF-like intestinal symptoms. I have shown that absence of \textit{Cftr} in the intestinal epithelium contributes to the dehydration and mucus accumulation in the intestine and absence of \textit{Cftr} in the smooth muscle decreases smooth muscle contractions. This decreased muscle contraction may be due altered calcium mobilization in the smooth muscle cell. It has been shown in airway smooth muscle cells in pigs that there is decreased reuptake of calcium in the sarcoplasmic reticulum after stimulation and a similar issue may be occurring in the intestinal smooth muscle in CF mouse models and mouse models with \textit{Cftr} inactivated in the smooth muscle.

While Cystic Fibrosis is a monogenic disease, it has been shown that modifier genes are responsible for the variation in disease severity seen in CF patients. GWAS
have identified many loci associated with several genes that influence CF lung disease severity, CFRD, and MI. These potential modifier genes add to the complexity of CF as some of the genes have pleiotropic affects and influence multiple symptoms. In addition, CF symptoms in one organ system are influenced by the severity of symptoms in another organ system. For example, severe CF lung disease is often correlated with malnutrition or intestinal obstruction, so a potential modifier gene influencing the incidence of intestinal obstruction may also indirectly modify lung disease severity. Chapter 3 of this thesis focused on functionally testing variants in \textit{Slc26a9} identified in a GWAS of MI in CF. \textit{Slc26a9} is a anion channel that is strongly expressed in the stomach and lungs. A \textit{Slc26a9} knockout mouse model has been created and had a more basic gastric pH due to decreased gastric acid secretion and decreased bicarbonate secretion. A CF mouse model with absent \textit{Slc26a9} was also created and it was shown that absence of \textit{Slc26a9} in the CF context led to increased intestinal obstruction. Overall, the work published on the CF mouse lacking functional \textit{Slc26a9} was not done very well, as it utilized just one type of CF mouse model that had complete absence of \textit{Slc26a9} and did not correlate the work back to patients. My work utilized two different types of CF mouse models, CF mice that were heterozygous for the \textit{Slc26a9} mutation (more similar to patients), and utilized human data. I determined that a reduction of one copy of \textit{Slc26a9} in CF mice with mild intestinal symptoms led to increased rates of intestinal obstruction, which were not due to slowed GI transit or a more basic gastric pH. I also showed that \textit{Slc26a9} mRNA expression is more prevalent in newborns and significantly decreases at 21 days and 6-8 weeks of age and hypothesize that \textit{Slc26a9} may have developmental significance. I analyzed nasal epithelial cells from patient volunteers and determined that samples with
an increased number of risk alleles in SNPs in *SLC26A9* associated with MI in CF correlated with decreased *SLC26A9* mRNA expression. Decreased *SLC26A9* expression may lead to increased intestinal cellular proliferation, which may contribute to the increased intestinal obstruction seen in the CF mice with a reduction of *Slc26a9*. *SLC26A9* may prove to be a therapeutic target to prevent CF intestinal obstruction.

Chapter 4 of this thesis tested a potential new drug treatment for intestinal obstruction in CF. I am interested in CF intestinal obstruction and ways to alleviate patients of this CF symptom. A drug company, AlgiPharma, had a drug they wanted to test on CF mouse models to determine if it unfolded mucins in the intestine and prevented intestinal obstruction. The drug, OligoG, is a specifically designed low weight oligomer made of >85% guluronate acid and <15% mannuronic acid. Previous studies determined that OligoG is a calcium chelator that may act as bicarbonate and remove calcium from folded mucins, thus allowing them to properly expand. It was also shown that OligoG has antibacterial and antifungal properties that allow it to disrupt complex biospheres, such as CF lung mucus. OligoG has also been shown to decrease mucus viscosity and alter the mucin interlinking networks. My work determined that OligoG treatment improved GI complications and increased the survival in the CF mice. This work showed that OligoG may be a useful drug treatment for CF patients to alleviate intestinal obstruction.

Overall, the main goal of this work was to provide information on intestinal dysfunction in CF. I determined that *Cftr* in the smooth muscle plays a role in the development of CF intestinal obstruction, however absence of *Cftr* in the intestinal epithelium and smooth together contribute to the development of CF-like intestinal
obstruction. I have shown that an absence of \textit{Cftr} in the intestinal epithelium leads to dehydration of the intestinal lumen and mucus accumulation. I hypothesize that absence of \textit{Cftr} in the smooth muscle leads to decreased calcium reuptake in the sarcoplasmic reticulum. Together the dehydration/mucus accumulation and decreased calcium reuptake are enough to increase the incidence of intestinal obstruction. My work also showed that a reduction of a potential modifier gene of MI in CF, \textit{Slc26a9}, in CF mouse models had a detrimental effect on survival. I also showed that SNPs in \textit{SLC26A9} that were identified to associate with MI in CF correlated with decreased \textit{SLC26A9} mRNA expression in human epithelial cells. I hypothesize that \textit{SLC26A9} may prove to be a potential therapy to prevent intestinal obstruction in CF patients. Lastly, I showed that treatment of a novel drug improved the intestinal symptoms in CF mice. Drug treatment in CF mouse models increased intestinal transit time, decreased mucus accumulation, and decreased intestinal obstruction rates. The drug has been shown to aid in mucin unfolding by chelating calcium in mucus in CF lungs. I hypothesize that OligoG is working in a similar manner to improve CF intestinal symptoms. I believe this work has contributed to the knowledge of CF intestinal obstruction and provides a foundation for additional studies to further elucidate the origin of CF intestinal obstruction.
Figure 1.1. Overview of CF intestinal symptoms. There are many GI complications that affect CF patients. This figure shows several common CF intestinal symptoms and the part of the GI tract that is affected.
Table 1.1. Comparison of CF animal models. Several types of animal models are used to study CF and each model develops manifestations of the disease. ↑ indicates that the manifestation is seen in that model and ↑↑ indicates an increase in severity of the symptom as compared to other models.

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Adapted from Cutting 2014
Figure 1.2. Schematic of conditional Cftr alleles. A) Conditional Cftr allele created by loxP sites surrounding exon 10 (Cftr<sup>fl</sup>). Cre recombinase expression allows for recombination between the loxP sites, resulting in deletion of exon 10 (Cftr<sup>Δfl</sup>) and nonfunctional Cftr. B) Conditional Cftr allele created by loxP sites surrounding inverted (non-functional) exon 10 (Cftr<sup>invfl</sup>). Cre recombinase expression allows for recombination between the loxP sites, resulting in the inversion of exon 10 and restoration of Cftr function (Cftr<sup>fl'</sup>).
### Table 1.2. Modifier genes of CF symptoms.

Many modifier genes have been identified to have an association with CF symptom severity. Table A lists the genes that have been identified to be associated with lung disease severity and early lung infection with *Pseudomonas aeruginosa* (P.A). Table B lists the modifier genes identified to have an association with exocrine pancreatic disease, cystic fibrosis related diabetes, liver disease, and meconium ileus.

#### Table A

<table>
<thead>
<tr>
<th>Symptom</th>
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#### Table B

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<th>Symptom</th>
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<td>Exocrine Pancreatic Disease</td>
<td>SLC26A9</td>
<td>Li et al 2014, Miller et al 2015</td>
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Chapter 2 – Absence of CFTR in Smooth Muscle Contributes to the Etiology of Intestinal Obstruction in Cystic Fibrosis

This work will be submitted for publication in the American Journal of Physiology: Gastrointestinal and Liver Physiology.

2.1. Introduction

Cystic Fibrosis (CF) is an autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. CFTR is an anion channel that affects epithelial fluid secretion and absorption in various organs throughout the body. Absence of CFTR function leads to a range of CF manifestations including lung disease, pancreatic insufficiency, growth reduction, and intestinal dysfunction consisting of malabsorption and obstruction. Intestinal obstruction syndromes such as meconium ileus (MI), distal intestinal obstruction syndrome (DIOS) and constipation are common in CF patients. MI, a prenatal obstruction in the small intestine, occurs in 13-20% of CF newborns (67,76,209). DIOS, a postnatal ileocecal obstruction, occurs in 7-8% of the CF pediatric population and in 14-16% of CF adults, with those numbers increasing as patient life expectancy improves (67). Constipation, a gradual impaction of the colon, has been reported in nearly half of CF patients (76). Absence of CFTR in the intestine leads to decreased anion and fluid transport resulting in a dehydrated intestinal lumen and the accumulation of thick sticky mucus. This accumulation of mucus, which impedes movement of intestinal contents, is thought to be a major cause for intestinal obstruction in CF (209). This presence of excess and improperly unfolded mucus in the lumen is also thought to contribute to the slowed transit time, bacterial overgrowth and
inflammation observed in the CF intestine, which are all factors that contribute to intestinal obstruction in CF (209).

While CFTR is expressed in many cell types throughout the body (143), absence of CFTR in the intestinal epithelium has been hypothesized to be the origin for CF intestinal obstructions (209,222,223). Support for this hypothesis comes from several CF animal models in which CFTR function is restored specifically in the intestinal epithelium leading to the absence of CF intestinal obstruction and normal intestinal histology (98,120,223,224). In addition, absence of CFTR specifically in the intestinal epithelium of a conditional CFTR knockout leads to goblet cell hyperplasia and intestinal obstruction (120). Interestingly, the incidence of intestinal obstruction in the intestinal epithelium specific CFTR knockout mouse was less than half that of constitutive CFTR KO mice (25% vs. 60% obstruction) (120). These data suggest that loss of CFTR function in the intestinal epithelium is necessary for the occurrence of intestinal obstruction, but is not sufficient to be the sole cause. Absence of CFTR function in other cell types must contribute to the incidence of CF intestinal obstruction.

Recent studies have suggested a functional role for CFTR in the contraction and relaxation of smooth muscle and thus absence of CFTR in intestinal smooth muscle has been hypothesized to contribute to gastric dysmotility and intestinal obstruction. CFTR is present in smooth muscle cells throughout the body, including vascular (137–139) and airway (140,225,226) smooth muscle. CFTR in smooth muscle cells has been shown to respond to pharmacologic activators and inhibitors of CFTR in the same manner as CFTR in epithelial cells (137–140,225). In the airway, lack of CFTR decreases the capacity for contraction of smooth muscle in the trachea (225,226), possibly due to
cartilage malformation observed in CF, but in the lungs absence of CFTR leads to increased contractility and hyperresponsiveness (227–229). The effect of CFTR’s absence in intestinal smooth muscle is still unclear. One study has observed that absence of CFTR in smooth muscle increases contraction (230), while another set of studies suggests that loss of CFTR in epithelium, not smooth muscle, indirectly decreases contraction of smooth muscle (146,147). In addition, in vitro data support a direct role for CFTR in relaxing smooth muscle since activation of CFTR in pre-constricted smooth muscle cells leads to relaxation, while absence of CFTR in these cells impairs relaxation (137,231). Even though these studies suggest an important role for CFTR in smooth muscle, no study to date has shown a direct in vivo consequence of loss of CFTR in smooth muscle.

In this study, I tested the hypothesis that absence of CFTR in smooth muscle contributes to intestinal obstruction in CF. To accomplish this goal, I utilized a conditional Cftr knockout (KO) mouse model to produce mice in which CFTR was absent specifically in smooth muscle, intestinal epithelium or in both cell types. Growth, intestinal obstruction, histology, motility, and contraction for each of these models were compared to normal littermates and CF mice.

2.2. Materials and Methods

2.2.1. Mouse Strains

The creation of the conditional Cftr KO mouse model (Cftrfl/fl), in which mouse exon 11 (also referred to as exon 10 in legacy numbering of exons) is surrounded by loxP target sites for Cre recombinase, has been previously described (118). To create a smooth
muscle specific $Cftr$ KO strain, the $Cftr^{fl/fl}$ mouse was crossed to either the $Sm-Cre$ strain (stock# 017491; Jackson Laboratory) or the $Myh-Cre$ strain (stock # 007742; Jackson Laboratory) which express Cre recombinase in smooth muscle cells from the smooth muscle 22-alpha promoter or the smooth muscle myosin heavy chain promoter respectively (232,233). These strains were referred to as $Cftr^{fl/fl};Sm-Cre$ and $Cftr^{fl/fl};Myh-Cre$. To create an intestinal epithelium specific $Cftr$ KO strain, the $Cftr^{fl/fl}$ mouse was crossed to the $Vil-Cre$ strain (stock # 004586; Jackson Laboratory) which expresses Cre recombinase in intestinal epithelium from the villin promoter (234). This strain was referred to as $Cftr^{fl/fl};Vil-Cre$. $Cftr^{fl/fl}$ littermates, in which no Cre was present, display normal CFTR function and were used as wildtype controls. $Cftr^{-/-}$ mice, in which the exon 11 was absent in all cells throughout the mouse, and there was no detectable CFTR function were used as CF mice. Animals were monitored on a daily basis, and weight was assessed every 5 days from 10 to 40 days of age. Autopsies were performed on any mice that died during this period to determine cause of death. Length of 7-wk-old euthanized mice was assessed from nose to anus by use of digital calipers. Animals were housed in standard polysulfone microisolator cages in ventilated units with corncob bedding. Mice were given ad libitum access to chow (Harlan Teklad 3000; Harlan Teklad Global Diets, Madison, WI) and sterile water. All animals were maintained on a 12-h light, 12-h dark schedule at a mean ambient temperature of 22°C. All animals used in this study were cared for according to a Case Western Reserve University approved protocol and Institutional Animal Care and Use Committee guidelines.

2.2.2 PCR and RTPCR
Genotyping was completed by PCR analysis using DNA extracts from tissue biopsies as described previously (118). The amplified portion of DNA for the \(Cftr^{\beta}\) allele was 408 bp and the \(Cftr^{-}\) allele was 148 bp. Human smooth muscle intestinal RNA (Sciencell Research Laboratories) was reverse transcribed into cDNA (QScript cDNA synthesis kit; Quanta Biosciences) and PCR amplified with primers 5'-TCTGCGCATGGCGGTCACTC-3' and 5'-TCCCCAAATCCCTCCTCCAGA-3' to human \(CFTR\) exons 8 and 10 respectively giving a 186 bp product.

2.2.3. Intestinal Histology

Ileal sections from mice were removed and fixed in methacarn fixative solution (60% methanol, 30% chloroform, and 10% glacial acetic acid) for four hours, washed in phosphate buffered saline and stored in 70% ethanol until sectioning. The intestines were paraffin embedded, were cut into 5 \(\mu\)m thick longitudinal sections, placed on glass slides, and stained with Nuclear Fast Red and Alcian Blue. Histology sections were sampled from each ileum in two distinct regions of at least 100 \(\mu\)m apart. The slides were scanned via a Leica SCN400 slide scanner and images were analyzed via VisiomorphDP software (Visiopharm). Alcian Blue and Nuclear Fast Red staining was designated in the software and pseudo-colored to indicate the call of each pixel by the software. Mucus staining was indicated by green pseudo-color and non-mucus staining was indicated by purple pseudo-color. The percentage of the whole area covered by mucus was calculated as the area of mucus staining divided by the whole area. Intestinal smooth muscle thickness was measured in these sections in 6 unique areas and averaged for each mouse. The inner
circular smooth muscle and the outer longitudinal smooth muscle, as well as the total smooth muscle thickness, were evaluated.

2.2.4. Measurement of Gastrointestinal Motility

Gastrointestinal motility was analyzed as previously described (144). Briefly, mice were fasted overnight and were allowed free access to water. In the morning mice were given 100μl of 25 mg/mL rhodamine labeled dextran (Sigma-Aldrich) solution by gavage. 25 minutes after gavage, the mice were sacrificed and the gastrointestinal tract, from stomach to cecum, was removed and placed in cold saline. The small intestine was divided into 10 equal sections and each segment, in addition to the stomach, was flushed with 2 mL of saline. The flushed contents were centrifuged at 500 rpm for 10 minutes and 200 ul of the supernatant from each section was placed in a 96 well plate. The quantification of the fluorescent signal in the supernatant from each segment was determined utilizing a multi-well fluorescence plate reader (FLUOstar Omega plate reader;BMG Labtech; excitation 545 nm and emission 590 nm). The distribution of the fluorescent signal in the intestinal segments was used to calculate the geometric center of fluorescence (GCF). GCF was determined by calculating the fraction of fluorescence per segment multiplied by the segment number (1-10) and adding all segments together. GCF can range from 1 to 10 with a higher number indicating a faster motility and shorter intestinal transit time.

2.2.5. Intestinal Smooth Muscle Contraction
The small intestine was removed in 37°C physiological saline solution (PSS). A 0.5 cm ring of ileum was taken and mounted vertically with one end to a fixed hook and the other to a hook attached to a force transducer (Grass Technologies) in an organ bath filled with 15 ml of PSS that was maintained at 37°C and continuously gassed with 95% O₂/5% CO₂. Isometric force data were recorded using Powerlab/4SP and LabChart software (ADInstruments). The tension was adjusted to 0.5 g and the tissue was equilibrated for 1 h. The buffer was changed every 30 min throughout the experiments. High potassium (80mM) solution was added to each bath for 2 min and then washed with PSS. High potassium was repeated 2 additional times per intestinal segment. 30 min after the final high potassium wash, the cholinergic agonist carbachol (CCh, Sigma) was added to the bath at 2-min intervals in cumulative concentrations of 10⁻⁹ - 10⁻⁴ M to stimulate activity. At the end of an experiment, the ileal ring was blotted on paper and the final wet weight was used to normalize the force data.

2.2.6. Statistical Analysis

Results are expressed as the mean +/- SEM. Differences between groups were determined using a one-way Anova with post-hoc Tukey test. Kaplan-Meier survival curves were evaluated using a log-rank test. A P value of <0.05 was considered significant.

2.3. Results

2.3.1. CFTR in intestinal smooth muscle
The presence of CFTR has been reported in smooth muscle in various parts of the body but no report has specifically shown CFTR expression in intestinal smooth muscle. RNA from human intestinal smooth muscle cells was used to verify expression of CFTR in this tissue (Figure 2.1.A). To determine if the absence of CFTR in smooth muscle contributes to CF intestinal manifestations, a conditional Cftr KO mouse model was utilized to specifically inactivate CFTR function in smooth muscle. This smooth muscle specific Cftr KO mouse, referred to as Cftr<sup>β/β</sup>; Sm-Cre, displayed both the functional allele Cftr<sup>β</sup> and the nonfunctional allele Cftr<sup>-</sup> in various tissues throughout the body, which is expected given the ubiquitous nature of smooth muscle (Figure 2.1.B). Specifically in the intestine, the Cftr<sup>-</sup> allele was more prominent when epithelium was removed and was the only allele when intestinal smooth muscle cells were isolated (Figure 2.1.B). As expected, the absence of CFTR in smooth muscle had no effect on CFTR function across epithelium as assessed in the airway and the intestine (Figure 2.1.C-D). In the intestine, Cftr<sup>β/β</sup>; Sm-Cre mice and control littermates with no Cftr deletion, referred to as Cftr<sup>β/β</sup>, displayed normal CFTR function with cAMP-induced peak increases in short-circuit current of 85.3 ± 25.1 and 104.7 ± 25.8 µA/cm² respectively. In contrast, CF mice with no CFTR function, also referred to as Cftr<sup>-/-</sup>, displayed an absence of a cAMP-induced peak increase in short-circuit current (0.9 ± 2.5 µA/cm²; P< 0.05 vs. Cftr<sup>β/β</sup>) (Figure 2.1.C). Similarly, in the airway, the nasal potential difference in response to low-chloride and forskolin for Cftr<sup>β/β</sup> and Cftr<sup>β/β</sup>; Sm-Cre mice were similar with values of -13.8±4.3 mV and -14.3± 3.6 respectively. CF mice however had significantly increased nasal potential difference of 2.5 ± 2.0 mV compared to control mice (Figure 2.1.D; P< 0.05).
2.3.2. Survival and Growth

To determine if the absence of CFTR in smooth muscle contributed to the intestinal obstruction phenotype in CF mice, conditional Cfr KO mice and control mice were observed daily from birth to 40 days of age. Survival of Cfr^	ext{fl/fl} mice (97.3%) and Cfr^	ext{fl/fl}; Sm-Cre mice (96.1%) was normal with no intestinal obstruction observed (Figure 2.2.A). CF mice had significantly decreased survival due to intestinal obstruction (40.9%; P< 0.005). Similar to our previous report, 81.5% of intestinal epithelium specific Cfr KO mice, referred to as Cfr^	ext{fl/fl}; Vil-Cre, survived to 40 days of age with 18.5% succumbing to intestinal obstruction which was significantly different from both control and CF mice (P< 0.005). Since loss of CFTR in the intestinal epithelium is necessary but not sufficient to reproduce the high incidence of intestinal obstruction and CFTR has been suggested to have a functional role in smooth muscle, our lab created a conditional Cfr KO mouse with loss of CFTR in both the intestinal epithelium and smooth muscle, referred to as Cfr^	ext{fl/fl}; Vil-Cre/Sm-Cre. The Cfr^	ext{fl/fl}; Vil-Cre/Sm-Cre mice had reduced survival due to intestinal obstruction similar to CF mice (52.8%; P < 0.005 vs. Cfr^	ext{fl/fl}).

Growth for the control and conditional Cfr KO mouse models was also observed. CF mice displayed significantly decreased weight at every time point compared to Cfr^	ext{fl/fl} mice (P<0.01). Cfr^	ext{fl/fl}; Vil-Cre/Sm-Cre displayed decreased weight from 25-35 days of age compared to control (P<0.01) but were not different at earlier time points or at 40 days of age. At 7 weeks of age, CF male and female mice displayed decreased length compared to control mice (Males 8.4±0.2 vs. 7.7±0.2 and Females 7.7±0.2 vs. 6.9±0.3;
P<0.01). All conditional \(Cfr\) KO strains were not different in length compared to controls (Figure 2.2.C).

2.3.3 Intestinal Histology

Intestinal histology for the control and conditional \(Cfr\) KO mice was assessed (Figure 2.3.A-D). Upon initial inspection, several of the mouse strains had increased amount of goblet cells and mucus between the villi. To quantify the amount of mucus, intestinal sections from each strain were assessed using a software program that differentiates area based on color. Cells were stained in red and mucus in blue (Figure 2.3.A-B) with the epithelial area indicated by outlining the area in the program. The area was then pseudo-colored by the program with epithelial cells in purple and mucus in green (Figure 2.3.C-D). To quantify the amount of mucus in each area, the area in green was divided by the total area (green and purple). The percent of intestinal mucus staining in \(Cfr^{+/+}\) and \(Cfr^{-/-}\); \(Sm-Cre\) mice were 7.2% and 9.3% respectively and were not significantly different. CF, \(Cfr^{+/+}; Vil-Cre\) and \(Cfr^{-/-}; Vil-Cre/Sm-Cre\) mice all displayed significantly increased intestinal mucus staining with 17.2%, 14.1% and 19.1% respectively (p<0.01 vs. \(Cfr^{-/-}\); Figure 2.3.E).

Smooth muscle thickness was assessed in control and conditional \(Cfr\) KO mice. The thickness of longitudinal, circular and total smooth muscle was measured (Figure 2.4.A). \(Cfr^{-/-}\) mice displayed significantly thicker intestinal smooth muscle content compared to controls (78.0±2.7 vs. 57.4±3.8 µM; P < 0.01). Much of the difference in smooth muscle thickness was due to an increased thickness of circular smooth muscle in the CF mice (51.5±1.9 vs. 37.4±1.8 µM; P< 0.05). There were no differences in intestinal
smooth muscle thickness in $Cftr^{fl/fl}$; $Sm-Cre$ or $Cftr^{fl/fl}$; $Vil-Cre$ mice compared to $Cftr^{fl/fl}$ mice. $Cftr^{fl/fl}$; $Vil-Cre/Sm-Cre$ did display a significantly increased smooth muscle thickness compared to control mice and similar to $Cftr^{-/-}$ mice (88.0±4.6 for total thickness; $P<0.01$) (Figure 2.4.B).

2.3.4. Gastrointestinal Motility

GI motility was assessed in the control and conditional $Cftr$ KO mouse models to observe how loss of CFTR in different intestinal cell types may affect intestinal motility. Intestinal motility was quantified by calculating the average geometric center of fluorescence (GCF) for each model as described in the methods. CF mice displayed a significantly slower motility with a decreased average GCF compared to control mice (2.5±0.2 vs. 4.4 ±0.2; $P<0.001$). While $Cftr^{fl/fl}$; SM-Cre and $Cftr^{fl/fl}$; $Vil-Cre$ mice had normal motility that was not significantly different than $Cftr^{fl/fl}$ mice, $Cftr^{fl/fl}$; $Vil-Cre/Sm-Cre$ mice displayed a significantly slower motility with a decreased average GCF compared to $Cftr^{fl/fl}$ mice (2.6±0.2; $P<0.001$).

2.3.5. Intestinal Smooth Muscle Contraction

Intestinal smooth muscle contraction in control and conditional $Cftr$ KO mice was assessed *ex-vivo* using an organ bath system with an attached force transducer. First, a high potassium solution was used to give maximal contraction of each intestinal section (Figure 2.5.A). CF mice had decreased peak force compared to $Cftr^{fl/fl}$ intestines (1.9 ±0.3 vs. 4.0 ± 0.2 mN/mg; $P < 0.01$). Intestinal rings from $Cftr^{fl/fl}$; $Vil-Cre/Sm-Cre$ mice also displayed decreased peak force compared to those from $Cftr^{fl/fl}$ mice (2.0±0.5; $P<
0.01). Cftr\textsuperscript{fl/fl}; Sm-Cre and Cftr\textsuperscript{fl/fl}; Vil-Cre intestines did not have significantly different peak force contraction compared to Cftr\textsuperscript{fl/fl} intestines (Figure 2.5.B). A difference in intestinal contraction pattern was also observed upon exposure to high potassium with Cftr\textsuperscript{fl/fl} mice displaying a steady increase in force while CF mice displayed an irregular pattern of contraction before peak force was attained (Figure 2.5.A). This irregular contraction pattern was significantly more prevalent in the intestines from Cftr\textsuperscript{--}; Cftr\textsuperscript{fl/fl}; Sm-Cre and Cftr\textsuperscript{fl/fl}; Vil-Cre/Sm-Cre mice compared to intestines from Cftr\textsuperscript{fl/fl} and Cftr\textsuperscript{fl/fl}; Vil-Cre mice (Table 2.1). Carbachol (CCh) was also used in a stepwise fashion to observe tissue reactivity to a different contractile agonist (Figure 2.5.C). Intestines from Cftr\textsuperscript{fl/fl} mice displayed a stepwise increase in basal contraction when exposed to increasing amounts of CCh. This increase in basal contraction was nearly absent in intestines from Cftr\textsuperscript{--} mice (Figure 2.5.C). Maximal basal contraction was decreased in Cftr\textsuperscript{--} and Cftr\textsuperscript{fl/fl}; Vil-Cre/Sm-Cre mice compared to control. (0.6±0.1 and 1.2±0.3 vs. 3.4 ± 0.7; P <0.01 vs. Cftr\textsuperscript{fl/fl}) (Figure 2.5.D). Intestines from Cftr\textsuperscript{fl/fl}; Sm-Cre and Cftr\textsuperscript{fl/fl}; Vil-Cre mice displayed similar maximal basal contraction as intestines from Cftr\textsuperscript{fl/fl} mice.

2.3.6. Replication of Results Utilizing an Additional Smooth Muscle Cftr KO Mouse

To verify that the intestinal effects observed were specific to loss of CFTR in smooth muscle, an additional smooth muscle Cre recombinase strain was used to replicate the above findings. The Myh-Cre strain was utilized in a similar manner as the Sm-Cre strain in the above experiments. This Cre recombinase transgene also expresses eGFP, facilitating the visualization of Cre expression in smooth muscle (Figure 2.7.A). The Cftr\textsuperscript{fl/fl};Myh-Cre mouse displayed a similar phenotype as the Cftr\textsuperscript{fl/fl};Sm-Cre mouse in
that normal survival, growth and epithelial CFTR function was observed. However, when the $Cftr^{+/+};\text{Myh-Cre/Vil-Cre}$ mice were generated they displayed a high incidence of intestinal obstruction that was comparable to CF and $Cftr^{+/+};\text{Vil-Cre/Sm-Cre}$ mice (44% survival $P<0.001$ vs. $Cftr^{+/+}$; Figure 2.7.B).

2.4. Discussion

The goal of this work was to analyze the role of $Cftr$ in intestinal smooth muscle and determine if the absence of $Cftr$ in this cell type contributes to the development of intestinal obstruction. CFTR has been previously shown to be expressed and functional in smooth muscle cells, but the role of this protein in these cells is unknown. $Cftr$ has also been shown to be expressed and functional in rat and mouse aortic muscle cells, rat intrapulmonary arteries, piglet vascular smooth muscle cells, mouse and human airway smooth muscle cells, and now human intestinal smooth muscle (137–140,235,236). To fully analyze the effect of absence of $Cftr$ in the smooth muscle, conditional $Cftr$ KO mouse models were utilized that allow for inactivation of $Cftr$ in a cell specific manner (118). Based on previous work from our lab and others, I hypothesized that loss of $Cftr$ in the smooth muscle would impair smooth muscle function and contribute to the development of intestinal obstruction. Our previous work showed that absence of CFTR in the intestinal epithelium is necessary for intestinal obstruction to occur but not sufficient to recapitulate the high incidence (120). Conditional KO mouse models were created in which CFTR was either absent in the intestinal epithelium, absent in the smooth muscle or absent in both the intestinal epithelium and smooth muscle. These conditional $Cftr$ KO models were compared to normal littermates and CF mice with
respect to survival, growth, intestinal histology, GI motility and intestinal muscle contractility.

The absence of CFTR specifically in smooth muscle alone did not produce any intestinal obstruction in mice. When \( Cftr \) was absent in smooth muscle in either model that was generated, \( Cftr^{\beta/\beta}; Sm-Cre \) or \( Cftr^{\beta/\beta}; Myh-Cre \), there was no effect on survival, growth, intestinal histology or GI motility. This finding was not altogether surprising since the previous data showed that loss of CFTR in the intestinal epithelium is necessary for intestinal obstruction to occur in CF (120). The \( Cftr^{\beta/\beta}; Vil-Cre \) mice displayed increased intestinal obstruction incidence (19.5%) but significantly less than CF mice (59.1%). Intestinal mucus content, as assessed by histology, was similar between \( Cftr^{\beta/\beta}; Vil-Cre \) and CF mice supporting the idea that absence of CFTR in the intestinal epithelium is the origin of the increased goblet cell number and mucus in the lumen (11,120,209). Even with similar amounts of intestinal mucus as CF, \( Cftr^{\beta/\beta}; Vil-Cre \) mice did not have altered growth, smooth muscle thickness, GI motility or intestinal contractility that CF mice do have, suggesting absence of CFTR in other cell types contribute to these phenotypes. Interestingly, when CFTR was absent in both the intestinal epithelium and smooth muscle cells in either the \( Cftr^{\beta/\beta}; Vil-Cre/Sm-Cre \) or the \( Cftr^{\beta/\beta}; Myh-Cre/Vil-Cre \) model, intestinal obstruction occurred at a similar rate to that of CF mice and they also displayed similar histology, GI motility and intestinal smooth muscle contractility as CF mice. I conclude that absence of CFTR in smooth muscle cells does contribute to intestinal obstruction but only when CFTR is also absent in the intestinal epithelium.
How does loss of CFTR in both intestinal smooth muscle and epithelium lead to intestinal obstruction? One model would suggest that the absence of CFTR in the intestinal epithelium leads to the well characterized overproduction and improper unfolding of mucins, resulting in the viscous mucus in CF (9,10,16). The absence of Cft in the intestinal epithelium alone can create an intestinal environment that leads to intestinal obstruction, but the majority of mice are able to survive and pass their intestinal contents with this loss. While absence of CFTR in smooth muscle did not cause any intestinal obstruction or other intestinal manifestations, the intestines of these mice did have an irregular response to high potassium induced hyperpolarization (Table 2.1.). This irregular pattern of contraction may not have any effect on normal intestinal muscle function in the absence of excess mucus or material that could cause blockage. The absence of CFTR in both the intestinal epithelium and smooth muscle may alter the intestinal smooth muscle enough to make the majority of mice succumb to intestinal obstruction.

Altered smooth muscle contractility may originate from a physical change of the muscle. In CF mice, smooth muscle thickness has been analyzed, but several studies present contradictory results. While two studies have reported no change in smooth muscle thickness (146,230), other studies have observed clear increases in thickness (237,238). These inconsistent results may be due to different methods for histological assessment, different mouse models or different diet. In my study, I observed an increase in smooth muscle thickness only in the CF mice and mice with CFTR absent in both smooth muscle and intestinal epithelium (Figure 2.4.). One hypothesis is that the absence of Cft in smooth muscle changes smooth muscle thickness and this alters the ability to
contract. My data do not support this hypothesis since smooth muscle Cftr KO mice did
not show any changes in muscle thickness. It is interesting to note that the smooth muscle
thickness does seem to be caused by hypertrophy of cells versus hyperproliferation (237).
This hypertrophy could be due to an adaptive response to intestinal obstruction, as this is
observed in induced intestinal obstruction models as well (239). Essentially the constant
contraction of the intestinal smooth muscle may cause bigger muscle cells and alter the
ability to contract. Another hypothesis is that increased muscle thickness is due to
increased bacterial load observed in CF patients and mice (144,209,237). Intestinal
infections have been shown to induce hypertrophy in rat intestine but this also caused
hyperplasia (240), which was not noted in one study in CF mice (237). Since I did not
observe increased smooth muscle thickness in either Cftr^{fl/fl};Vil-Cre or the Cftr^{fl/fl};Sm-Cre
model these data suggest that the increase in muscle thickness is an adaptive response to
the obstruction that is occurring in the other models.

Alterations in intestinal function in CF have been hypothesized to be due to
impaired smooth muscle function as a result of the altered intestinal environment rather
than lack of Cftr in the smooth muscle cells (146,147). These studies showed that mouse
intestinal smooth muscle had normal capacity to contract at postnatal day 5 but
dysfunction began to develop around postnatal day 7-14. This suggests that smooth
muscle becomes dysfunctional after the gastrointestinal environment is altered by factors
such as bacterial overgrowth, intestinal pH and mucus accumulation. This altered GI
environment leads to the increased production of prostaglandins (PGs) from the intestinal
epithelium. PGs are hormone-like compounds that, once released from the intestinal
epithelium, target receptors on the smooth muscle to trigger contraction or relaxation
Alterations in PG levels may lead to impaired muscle function and subsequently intestinal obstruction (146,148). The interpretation of these studies may need to be altered with the addition of my study. Smooth muscle function early in the life of a CF mouse may indeed be normal due to the absence of excess mucus and the buildup of material. It may not be until 7-14 days that the absence of CFTR in smooth muscle becomes apparent. This result is much like my result of no major alteration in muscle function when CFTR is only absent in smooth muscle. In addition, while the PG levels may indeed be altered in CF, my study suggests that those alterations are not enough to affect smooth muscle function until CFTR is absent in the smooth muscle as well. More research on the intestinal environment of these conditional Cftr KO mice is necessary to reconcile these studies with my own in vivo results.

An explanation of how absence of CFTR in smooth muscle alters smooth muscle contraction may come from recent work in the CF porcine airway (229). CFTR was present in porcine airway smooth muscle as reported in other studies, but the localization to the sarcoplasmic reticulum had not previously been shown. This study observed that loss of CFTR in the pig airway regulates airway smooth muscle tone. This alteration in tone was hypothesized to be due to the delayed calcium reuptake following cholinergic stimulation and increased myosin light chain phosphorylation in smooth muscle cells that was altered in the CF pig (229). They suggest that loss of CFTR specifically in the airway smooth muscle cells may contribute to the airflow obstruction observed in human CF. A similar model may explain how absence of CFTR in intestinal smooth muscle cells contributes to the intestinal obstruction phenotype in CF.
It has been previously shown that several players that regulate smooth muscle cell contraction and relaxation pathway are altered in CF. It is known that the absence of \textit{Cftr} in smooth muscle cells alters chloride transport and chloride ions play a role in calcium transport in these cells (137,242). Several groups have shown that calcium regulation is altered in CF nasal and airway epithelial cells, further suggesting that the absence of \textit{Cftr} chloride channels alters calcium mobilization (243,244). Alterations in the vascular smooth muscle of CF piglets have been found and determined to be due to alterations in calcium regulation (235). The most recent findings that delayed reuptake of calcium to the sarcoplasmic reticulum, which in turn would lead to increased myosin light chain phosphorylation and inability to relax, may lead to long term inability for normal smooth muscle function. I hypothesize that the alterations observed in the intestinal epithelium and smooth muscle \textit{Cftr} KO model may be due to the same impaired calcium handling in the smooth muscle cells. Isolation of intestinal smooth muscle cells will be necessary to visually monitor calcium mobilization and myosin light chain phosphorylation and determine if these alterations are due to absence of \textit{Cftr} in smooth muscle cells.

The goal of this work was to analyze the role of \textit{Cftr} in the intestinal smooth muscle and determine if its absence led to impaired muscle function in the intestine. In addition, I asked whether the absence of CFTR in intestinal smooth muscle contributed to the development of intestinal obstruction and related intestinal manifestations that are commonly observed in CF patients and CF animal models. By utilizing conditional \textit{Cftr} KO mouse models I observed that there were minor alterations in smooth muscle function but no intestinal phenotype was present when CFTR was absent in smooth muscle alone. My data show that absence of CFTR in the intestinal epithelium is
responsible for the increased mucus in the intestine but does not produce the high rate of obstruction or the altered contractility or GI motility. The whole CF intestinal phenotype is recapitulated only when CFTR is absent from both the smooth muscle and intestinal epithelium. I observed this phenomenon utilizing two independent conditional smooth muscle Cftr KO models. While other studies have suggested an important role for CFTR in smooth muscle, my study is the first in vivo study to show that absence of CFTR in smooth muscle does contribute to intestinal manifestations in CF but only if CFTR is also absent in the intestinal epithelium. Both the increase in the amount of viscous mucus along with the alterations in smooth muscle function creates the CF intestinal manifestations. Further research is necessary to determine if CF intestinal smooth muscle function is altered due to complications in calcium mobilization or prostaglandin production. Once an altered pathway has been elucidated then therapeutic targets may be employed to counteract the issue caused by Cftr absence in the smooth muscle, thus improving the quality of life for CF patients that suffer from gastrointestinal symptoms.
Figure 2.1. Deletion of Cfr in smooth muscle. (A) CFTR expression is observed in human intestinal smooth muscle as assessed by RT-PCR. A single band of the expected 186 bp size is present in the + RT sample and absent in the - RT control. (B) PCR amplification of the DNA region surrounding the conditional Cfr allele. A 408 bp product is expected from Cfrfl/fl DNA and a 148 bp product is expected from Cfr−/− DNA due to the deleted exon. DNA from a Cfrfl/fl;Sm-Cre mouse indicates the presence of both the Cfrfl allele and the Cfr− allele. DNA from whole intestine (i), uterus (u), lung (lu) and heart (h) have more prominent bands at 148 bp compared to the liver (li) and brain (b). DNA from ileum (il) with epithelium removed displayed both alleles prominently and dissected intestinal smooth muscle (sm) displayed almost exclusively the Cfr− allele. (C) cAMP-induced peak increase in intestinal short-circuit current (ΔIsc). (D) Nasal potential difference (PD) of mice in response to low-chloride forskolin. (*P < 0.05 vs. Cfrfl/fl; n≥4.)
Figure 2.2. Survival and growth of conditional Cftr KO mice. (A) Percentage of mice surviving up to 40 days of age. (n≥25 per group; *P <0.005) (B) Weight of mice every 5 days from 10-40 days of age. Cftr−/− mice were significantly lighter from Cftrβ/β at every age ( P <0.001) *Both Cftrβ/β;Vil-Cre and Cftrβ/β;Vil-Cre/Sm-Cre mice were significantly lighter than Cftrβ/β; P<0.01; ^Cftrβ/β;Vil-Cre/Sm-Cre mice were significantly lighter than Cftrβ/β;P<0.01; n≥16 per group with equal number of males and females. (C) Length of mice at 7 weeks of age. (*P<0.05 vs. Cftrβ/β; n≥5 per group)
Figure 2.3. Intestinal mucus. Representative ileal sections from (A) a $Cftr^{0/0}$ mouse and (B) a $Cftr^{-/-}$ mouse stained with Nuclear Fast Red and Alcian Blue with blue staining indicating mucus in goblet cells and the lumen. (C-D) Same sections as A and B with pseudo-colored colored areas (mucus in green and epithelium in purple) indicating designation of pixels by software. (E) The percentage of mucus in the indicated area was calculated by dividing the area in green by the total area (green and purple) ($n \geq 6$ per group; *$P < 0.01$ vs. $Cftr^{0/0}$).
Figure 2.4. **Intestinal smooth muscle thickness.** Representative ileal sections from (A) a *Cftr* <sup>fl/fl</sup> mouse (left) and a *Cftr* <sup>−/−</sup> mouse (right) stained with Nuclear Fast Red with circular (CM) and longitudinal (LM) muscle thickness indicated by bars. (B) Average intestinal smooth muscle thickness from the control and conditional *Cftr* KO mouse strains. (n≥6 per group; *P < 0.005. ^ P < 0.05 vs. *Cftr* <sup>fl/fl</sup>. Scale bar is 15 µM)
Figure 2.5. Intestinal motility. Average GCF value for control and conditional Cфг KO mice. (n≥10. *P=0.001 vs. Cфгflo/flo)
Figure 2.6. Intestinal smooth muscle contraction. (A) Representative isometric force traces of ileal intestinal sections from $Cftr^{fl/fl}$ and $Cftr^{-/-}$ mice in response to the addition of high potassium solution (arrow). (B) Peak force response of ileal intestinal sections from control and conditional Cftr KO mice to high potassium solution. (C) Representative isometric force traces of ileal intestinal sections from $Cftr^{fl/fl}$ and $Cftr^{-/-}$ mice in response to increasing concentration of carbachol (CCh) with basal contraction (BC) indicated. (D) Maximal basal contraction response of ileal intestinal sections from control and conditional Cftr KO mice to carbachol. (*P < 0.01 vs. $Cftr^{fl/fl}$; n ≥ 16 per group)
Figure 2.7. Replication of the smooth muscle $Cftr$ KO data. (A) Image of intestinal section from a $Myh$-$Cre$ mouse that also expresses eGFP as a marker for Cre recombinase expression. Nuclei are stained with DAPI (Blue) and eGFP (green) marks Cre recombinase expression in smooth muscle (arrow) and vascular smooth muscle within the villi. No Cre expression is observed in the intestinal epithelium (arrowheads). (B) Percentage of mice surviving up to 40 days of age. ($n\geq25$ per group; $*P <0.001$ vs. $Cftr^{+/\mu}$)
Table 2.1. Ileal contraction response to high potassium in control and conditional $Cftr$ KO strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>% Normal</th>
<th>% Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Cftr^{fl/fl}$</td>
<td>91</td>
<td>9</td>
</tr>
<tr>
<td>$Cftr^{-/-}$</td>
<td>4</td>
<td>96</td>
</tr>
<tr>
<td>$Cftr^{fl/fl}; (Sm-Cre)$</td>
<td>16</td>
<td>85</td>
</tr>
<tr>
<td>$Cftr^{fl/fl}; (Vil-Cre)$</td>
<td>82</td>
<td>18</td>
</tr>
<tr>
<td>$Cftr^{fl/fl}; (Vil-Cre/Sm-Cre)$</td>
<td>5</td>
<td>92</td>
</tr>
</tbody>
</table>

* $P<0.001$ vs. $Cftr^{fl/fl}$; n$\geq$25
Chapter 3 – Reduction of SLC26A9 Increases Intestinal Obstruction in Cystic Fibrosis

This work will be submitted for publication in the American Journal of Physiology: Gastrointestinal and Liver Physiology.

3.1. Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) gene was identified as the causative gene of cystic fibrosis (CF) in 1989 (2–4). While CF is a monogenic disease, variability in disease severity is observed in patients with the same CFTR genotype (50,65,157–160,245). Genotype-phenotype studies have determined that environmental and genetic factors both contribute to the variety in disease severity (50,246). However, some symptoms are more heavily influenced by genetic contributors than others. For example, the incidence of Meconium Ileus (MI), a type of intestinal obstruction that presents shortly after birth in ~10-25% of CF patients, has been determined to be highly correlated with certain genetic loci (24,31,57–59,65,206). Genome wide association studies (GWAS) and linkage analyses have identified several genes that have an association with risk of MI in CF (65,110,206,207). I further analyzed one particular gene identified, solute carrier family 26 member 9 (SLC26A9), to determine the mechanism of how the gene might contribute to the incidence of MI in CF.

SLC26A9 is an anion exchanger that functions as a chloride channel, chloride-bicarbonate exchanger, and sodium co-transporter (212,213). SLC26A9 is strongly expressed in the stomach and lung and detectable in the brain, heart, kidney, small intestine, thymus, and ovaries (212,214). To gain a better understanding of the role of
Slc26a9, a mouse model with complete absence of Slc26a9 was created. Given the high expression of SLC26A9 in the stomach, the gastrointestinal (GI) tract was evaluated and it was determined that the Slc26a9 KO mice had a more basic gastric pH as compared to controls. This was found to be due to a decrease in gastric acid secretion (220). The Slc26a9 KO mouse also had decreased bicarbonate secretion and elongated villi and crypts in the duodenum (221). Despite these GI abnormalities, Slc26a9 knock-out (KO) mice appeared healthy and displayed normal growth patterns (220).

Since SLC26A9 was identified to correlate with incidence of MI in CF, it was necessary to determine how this gene modified CF intestinal symptoms. In a previous study, a CF mouse model with complete absence of Slc26a9 function was created. The CF mouse with non-functional Slc26a9 had significantly decreased survival, which was due to an increased incidence of intestinal obstruction (221). There were several limitations to this study including the use of multiple genetic backgrounds for the various mouse models tested (a congeneric S129/svj background for the Slc26a9 KO model and a mixed FVB/N and S129/svj background for the Cftr and Slc26a9 double KO mouse models), the use of only one type of CF mouse model, and limited experimental data on the double KO mouse model (221).

To determine the role of Slc26a9 in the incidence of MI in CF, the lab created two different types of CF mouse models that had alterations in the Slc26a9 gene. These crosses kept the alterations in Slc26a9 constant, while the Cftr mutation was varied, which allowed me to determine the effect of Slc26a9 alteration on the occurrence of intestinal obstruction. Growth, gastric pH, GI motility, anion transport, and Slc26a9
expression were monitored in the mouse models to determine the mechanism of how
Slc26a9 modifies the prevalence of intestinal obstruction in CF.

3.2. Materials and Methods

3.2.1. Mouse Models

Two different types of CF mouse models were utilized in these experiments. One
CF mouse model had the well characterized F508del mutation (115) and the other had the
R117H mutation (111). Both CF mice were maintained on the C57BL/6J background.
The establishment of the Slc26a9 KO mouse model on the S129/svj background has been
previously described (220,221). The mutation was backcrossed 5 generations to C57Bl/6J
background making this mouse over 98% C57Bl/6J.

I crossed the two CF mouse models to the Slc26a9 KO model and created mice
that were homozygous for the Cftr mutation and heterozygous for the Slc26a9 mutation.
These mice are referred to as CF mice with a reduction of Slc26a9, meaning they are
heterozygous for the Slc26a9 gene. The absence of both Cftr and Slc26a9 was not
conducive to life after birth. Slc26a9 KO mice were also crossed to obtain mice that were
wildtype for Cftr and were either heterozygous or homozygous for the Slc26a9 mutation.
Ear clippings were obtained from mice used in the experiments and were digested in a
sodium hydroxide-tris solution. PCR methods were used to genotype mice for the
F508del and R117H mutations (Integrated DNA Technologies CFTR probes) and for the
Slc26a9 mutation (Integrated DNA Technologies SLC26A9 primers).

All of the mice utilized for experiments were maintained on a normal mouse
chow and allowed free access to water. An equal number of males and females, aged 3-6
weeks, were used for experiments. The mice were observed everyday starting at birth and weight was measured every 5 days until the mice reached 40 days old. Upon death, necropsy was performed to determine the cause of death. All of the mice were bred and genotyped in accordance with the Institutional Animal Care and Use Committee (IACUC) approval at Case Western Reserve University.

3.2.2. SLC26A9 Expression

RNA was isolated from mouse tissues using Ribozol (Amresco). One microgram of the RNA was reverse transcribed into cDNA (Quanta Qscript cDNA Synthesis Kit) and $Slc26a9$ mRNA expression was determined for each sample (Applied Biosystems taqman assay). Mouse $\beta$-Actin (Applied Biosystems taqman assay) was used as an endogenous control.

3.2.3. Gastric pH

Gastric pH was measured via methods previously described (149,247). Gastric pH was analyzed in the different models via a pH microprobe (Lazar Research Laboratories, Inc. PHR-146B). Prior to every use the pH probe was calibrated with pH 4.0, 7.0, and 10.0 buffers. When not in active use the microprobe was kept submerged in diluted pH 7.0 buffer. Less than 45 minutes prior to pH measurement the mice were injected subcutaneously with histamine to stimulate gastric acid secretions (248). To measure the pH of the gastric contents, the stomachs were dissected from each sample and the gastric contents were flushed in 5mL of normal saline solution. The pH of the normal saline solution was recorded for each experiment to ensure consistency of the pH of the flushing
solution. The gastric contents and saline were then transferred to a 15mL conical tube and the sample was pelleted via centrifugation. The micro pH probe was used to pH the supernatant of the sample.

3.2.4. Measurement of Gastrointestinal Motility

Gastrointestinal motility was analyzed as previously described (144,145). Briefly, mice were fasted overnight and were allowed free access to water. In the morning mice were given 100µl of 25 mg/mL rhodamine labeled dextran (Sigma-Aldrich) solution by gavage. 25 minutes after gavage, the mice were sacrificed and the gastrointestinal tract, from stomach to cecum, was removed and placed in cold saline. The small intestine was divided into 10 equal sections and each segment, in addition to the stomach, was flushed with 2 mL of saline. The flushed contents were centrifuged at 500 rpm for 10 minutes and 200 ul of the supernatant from each section was placed in a 96 well plate. The quantification of the fluorescent signal in the supernatant from each segment was determined utilizing a multi-well fluorescence plate reader (FLUOstar Omega plate reader;BMG Labtech; excitation 545 nm and emission 590 nm). The distribution of the fluorescent signal in the intestinal segments was used to calculate the geometric center of fluorescence (GCF). GCF was determined by calculating the fraction of fluorescence per segment multiplied by the segment number (1-10) and adding all segments together. GCF can range from 1 to 10 with a higher number indicating a faster motility and shorter intestinal transit time.

3.2.5. Bioelectric Measurements
Electrolyte measurement were obtained as previously described (118). Anion transport was measured in the intestine of mice 3-4 weeks of age that were fasted overnight. Mice were sacrificed and the large and small intestines were removed and immediately placed in ice-cold HEPES-buffered Ringer’s solution (in mM: 138 NaCl, 5 KCl, 2.5 Na$_2$HPO$_4$, 1.8 CaCl$_2$, 1.0 MgSO$_4$, and 10 HEPES-NaOh, pH 7.4) for dissection. The intestine was cut longitudinally, and the contents were removed with a stream of Ringer’s solution. The tissue was stretched and pinned in an Ussing chamber with an aperture of 0.125 cm$^2$. Tissues were bathed on both sides by 6-8 ml of mammalian Kreb-Ringer bicarbonate solution in [mM: 115 NaCl, 25 NaHCO$_3$, 5 KCl, 2.5 Na$_2$HPO$_4$, 1.8 CaCl$_2$, 1.0 MgSO$_4$, and 10 glucose, pH 7.4 (mannitol replaced glucose in the apical bathing solution)], which was warmed to 37°C and circulated with 95% O$_2$–5% CO$_2$ through gas lifts. Transepithelial electrical voltage difference (Vms) was measured between two Ringer–agar bridges. Calomel cells connected the bridges to a high-impedance voltmeter (DVC 1,000 Voltage/Current Clamp, World Precision Instruments). Current from an external DC source was passed by silver–silver chloride electrodes and Ringer–agar bridges to clamp the spontaneous Vms to zero. The current required (short-circuit current, Isc) was corrected for solution resistance between the tips of the voltage-sensing electrodes and recorded. Tissues were maintained with Vms clamped to zero (short-circuit conditions). At 60-sec intervals, Vms was clamped to 12 mV for three sec to calculate transepithelial resistance (Rms). Tissues were mounted and observed until the Isc stabilized (generally 10–15 min), at which time experimental maneuvers were begun.

3.2.6. Human Nasal Epithelial Cell Acquisition and Culture
IRB approval was obtained prior to the start of the experimental procedure and recruitment of volunteers. CF and non-CF volunteers were recruited for donation of nasal epithelial cells and informed consent was obtained prior to acquiring samples. Primary nasal epithelium cells were obtained via nasal cell brushing with a cytology brush. Once obtained, the primary cells were dislodged from the cytology brush and dispersed in cell culture media. The primary epithelial cells were seeded onto irradiated 3T3 fibroblast feeder layers and the epithelial cells were allowed to grow as well-defined colonies surrounded by the 3T3 fibroblasts. After ample expansion time, the epithelial cells were collected by differential detachment and then seeded onto permeable filter supports (Snapwell or Millicell-CM). The epithelial cells were maintained as air liquid interface cultures for 35-42 days.

DNA was isolated from the nasal epithelial cells (Quiagen Blood and Tissue Kit) and the genotype for risk alleles in SNPs in SLC26A9 that were associated with MI in CF was determined. RT-PCR was utilized to genotype the epithelial cells for the following SNPs in SLC26A9: rs4077468, rs7512462, and rs7419153 (Applied Biosystems SNP taqman genotyping assays).

RNA was isolated from the nasal epithelial cells (Quiagen RNeasy Mini Kit) and 500 ng of RNA reverse transcribed into cDNA (Applied Biosystems High Capacity cDNA Reverse Transcription Kit). Quantitative RT-PCR was utilized to determine SLC26A9 mRNA expression (Applied Biosystems taqman assay). Human β-Actin (Applied Biosystems taqman assay) was used as an endogenous control.

3.3. Results
3.3.1. Reduction of \textit{Slc26a9} Leads to Increased Intestinal Obstruction in the R117H CF Mouse Model

Of the two CF mouse models utilized in this study, the F508del CF mouse model had high rates of mortality due to intestinal obstruction (~50% survival) and the R117H CF mouse model had low rates of mortality associated with intestinal obstruction (~94% survival), similar to previously published data (111,115). To determine the effects of reduced \textit{Slc26a9} on CF intestinal obstruction, I crossed each of the two CF strains, F508del and R117H, to the \textit{Slc26a9} KO strain to produce CF mice that had only one copy of \textit{Slc26a9}. These two mouse models allowed me to determine if reduction of \textit{Slc26a9} expression alleviated or exacerbated the rate of intestinal obstruction. I found that a reduction of one copy of \textit{Slc26a9} in the F508del mice had no significant effect on obstruction rates (wildtype \textit{Slc26a9} = 50% survival and reduction of \textit{Slc26a9} = 63% survival; p=0.3564) (Figure 3.1.A) and that reduced \textit{Slc26a9} in the R117H CF model led to increased obstruction (wildtype \textit{Slc26a9} = 94% survival and reduction of \textit{Slc26a9} = 57% survival; p=0.0005) (Figure 3.1.B). Necropsy was performed on all deceased mice and the cause of death was determined to be intestinal obstruction. Figure 3.1.C depicts an intestine from a R117H CF mouse with wildtype \textit{Slc26a9} and it may be noted that the intestine is clear of obstruction. Figure 3.1.C also shows an intestine from a R117H CF mouse with reduced \textit{Slc26a9} and the arrow points to the obstruction occurring in the mid-portion of the intestine. Taken together, these data showed that a reduction of \textit{Slc26a9} had a detrimental effect on the R117H CF mice and led to increased rates of intestinal obstruction.

3.3.2. Reduction of \textit{Slc26a9} has no Effect on Growth in CF Mice
Growth deficiencies are well characterized in CF; however the exact mechanism behind failure to thrive is unknown. Nutritional status has been correlated with long-term survival, lung function, and overall health, therefore it is necessary to determine how the growth manifestations of CF develop (249–252). Intestinal dysfunction has been linked to malnutrition and growth deficits in CF (238,253–255), therefore I wanted to determine if the increased incidence of intestinal obstruction observed in the CF mice with a reduction of $Slc26a9$ also had an effect on the growth phenotype. I found that there were no significant alterations in growth in the mouse models with a reduction of $Slc26a9$ or in the $Slc26a9$ KO models as compared to controls (data not shown). The R117H mice with wildtype levels of $Slc26a9$ had significantly decreased growth ($p=<0.05$) as compared to the controls and a reduction of $Slc26a9$ in the R117H mice had no effect on growth (Figure 3.2.).

### 3.3.3. Gastric pH is Not Altered in CF Mice with Reduced $Slc26a9$

Gastric pH is an important part of GI tract function and pH control is necessary for proper digestion to occur. Abnormal digestion of materials in the stomach may lead to downstream intestinal obstruction (8,146,256). $Slc26a9$ has been determined to play a role in pH regulation in the GI tract via secretion of bicarbonate (212,220,221,257) and a $Slc26a9$ KO mice have been reported to have significantly more alkaline gastric pH as compared to controls (220). I assessed gastric pH in the mouse models and determined that gastric pH was significantly more alkaline only in the $Slc26a9$ KO group (Control=3.2, Slc26a9/-=5.9, Slc26a9+/-=3.7, mild CF=3.1, mild CF Slc26a9+/-=2.96;
p=<0.0001) (Figure 3.3.). Absence of one copy of Slc26a9 in the CF mice does not alter gastric pH.

3.3.4. Reduction of Slc26a9 Does Not Alter GI Motility in CF Mouse Models

GI motility rates were analyzed in mouse models to determine if absence of or reduction of Slc26a9 had an effect on GI motility. It is known that CF mice have slowed GI motility rates (145,258) and I wanted to test if absence of one copy of Slc26a9 modified this phenotype. I found no significant alterations in GI motility with a reduction (GCF=4.16) or complete absence (GCF=5.37) of Slc26a9 as compared to the control group (GCF=4.35) (Figure 3.4.). The mild intestinal symptom R117H CF model had significantly slowed GI motility (GCF=2.33; p<0.01) as compared to controls (Figure 3.4.). The reduction of Slc26a9 did not alter motility in the R117H CF mice (GCF=2.95) as compared to the R117H model, but motility was still significantly decreased as compared to the control group (p<0.05) (Figure 3.4.). Absence of Slc26a9 in non-CF mice or reduction of Slc26a9 in CF mice does not have a significant effect on GI transit.

3.3.5. Reduction or Complete Absence of Slc26a9 Does Not Alter Anion Transport in Non-CF Mice

Slc26a9 is a known anion exchanger (206,211–213) and I wanted to determine if non-functional Slc26a9 altered anion transport in the small intestine of non-CF mice. I found that there were no significant differences in the change in short circuit current across the intestinal epithelium after stimulation with IBMX and forskolin as compared to controls (Figure 3.5.). Absence or reduction of Slc26a9 in non-CF mice does not alter intestinal anion transport.
3.3.6. Slc26a9 Detection and mRNA Expression Decreases with Developmental Age

To understand the mechanisms behind Slc26a9’s modification of intestinal obstruction, I wanted to developmentally analyze Slc26a9 expression in several tissues. I analyzed expression in the lung, pancreas, proximal duodenum, and ileum in non-CF mice at 3 different time-points, newborn, 21 days, and 6-8 weeks. First, I analyzed the number of samples in which Slc26a9 mRNA was detected. In the lung, 100% of the samples tested at every time point had detectable Slc26a9. In the ileum, 100% of the newborn samples had detectable levels, however only 40% of the 21 day old samples had detectable Slc26a9 and none of the 6-8 week old samples had Slc26a9 expression. In the proximal duodenum, 100% of the newborn samples had detectable Slc26a9 expression, only 66% of the 21 day old samples had detectable expression, and none of the 6-8 week old samples had Slc26a9 expression. In the pancreas, 100% of the newborn and 21 day old samples had detectable Slc26a9 expression and none of the 6-8 week old samples had Slc26a9 expression. Next, I analyzed Slc26a9 mRNA expression levels in each of the tissues tested. Figure 3.6. shows Slc26a9 mRNA expression in the lungs of wildtype mice. Slc26a9 expression is graphed as a % of newborn expression. The data show that Slc26a9 expression significantly decreases at 21 days and 6-8 weeks (p<0.001). The other tissues analyzed, ileum, proximal duodenum, and pancreas, had similar expression profiles with decreased Slc26a9 expression with developmental age (data not shown).

3.3.7. SNPs in SLC26A9 Associated with MI in CF Correlate with Decreased SLC26A9 mRNA Expression in Human Epithelial Cells
A recent GWAS identified SNPs in *SLC26A9* that associated with incidence of MI in CF patients (206) and I wanted to determine if risk alleles for these SNPs correlated with lower levels of *SLC26A9* mRNA levels. The SNPs analyzed were rs4077468, rs7512462, and rs7419153. **Figure 3.7.A** depicts the *SLC26A9* gene and indicates the location of each of the SNPs analyzed. The distance between the SNPs is also indicated in **Figure 3.7.A**. Information from dbSNP and the corresponding HapMap data was used to create a HapMap of *SLC26A9*. The HapMap shows individual HapMap blocks, or groups of SNPs that have been identified to have high linkage disequilibrium with one another. The $R^2$ values are also listed on the HapMap. $R^2$ is a commonly used method to summarize linkage disequilibrium and ranges from 0 to 1. A $R^2$ value of 0 indicates that the SNPs are in perfect equilibrium and inheritance is completely independent of one another. A $R^2$ value of 1 means that the SNPs provided identical information and the SNPs are inherited together. **Figure 3.7.B** depicts the HapMap created for *SLC26A9* and the HapMap insert zooms in on the region where the SNPs of interest are located. The generated HapMap data showed that SNPs rs7512462 and rs4077468 are located at opposite ends of block 4; however block 4 does not show very strong association throughout the entire area and there are several spots where there are no data reported and areas where the $R^2$ values are below 1. The $R^2$ value for SNPs rs7512462 and rs4077468 is 0.819. The HapMap data also showed that the SNP rs7419153 is located in a separate HapMap block, block 6, than the other two SNPs. The $R^2$ values for rs7419153 and rs407768 is 0.409 and for rs7419153 and rs7512462 is 0.365. The $R^2$ values indicate that SNP rs7419153 is not strongly linked with the other two SNPs. The inserted table in **Figure 3.7.B** shows the $R^2$ for each of the sets of SNPs.
According to the available resources, the SNPs analyzed in this thesis do not appear to be strongly linked with one another.

To relate my earlier findings in mouse models to CF patients, I wanted to determine if risk alleles associated with MI in CF correlated with decreased \textit{SLC26A9} expression. I obtained data from 15 human nasal epithelial cell samples, 5 from wildtype (WT) volunteers and 10 from CF volunteers. Absence of CFTR has been shown not to have an effect on SLC26A9 expression (213,219); therefore I utilized samples from WT and CF volunteers. All of the samples were genotyped for SNP risk alleles and the risk allele frequencies from the sample population corresponded with the reported allele frequencies for all of the SNPs tested (data not shown). \textit{SLC26A9} mRNA expression levels were evaluated and \textit{SLC26A9} expression is represented as the normalized difference, or the cycle difference (ΔCT) between \textit{SLC26A9} and human $\beta$-Actin (Figure 3.7.C). A large ΔCT value corresponds to a decrease in mRNA expression and a small ΔCT value corresponds to an increase in expression. Each dot represents an individual sample and samples are grouped based on the number of risk alleles (0-6) identified via genotyping. The trend line indicates decreased \textit{SLC26A9} expression as the number of risk allele increases ($R^2 = 0.4343$). The data may also be analyzed in terms of expression. \textbf{Figure 3.7.D} shows the number of risk alleles per sample and the % \textit{SLC26A9} expression relative to the sample with the highest expression. Again, the trend line suggests decreased A9 expression as the number of risk alleles increases ($R^2 = 0.1982$).

\textbf{3.4. Discussion}
Despite significant efforts to develop drugs and therapies to alleviate patients of CF symptoms, they are still suffering from manifestations of the disease. Specifically, patients still suffer from intestinal complications of CF such as Meconium Ileus (MI). MI is a form of intestinal obstruction that presents within 48 hours after birth and occurs in ~10-25% of CF patients (31,57,58). Modifier genes have been hypothesized to play a role in the incidence of MI in CF and a recent GWAS identified associations between SNPs in \textit{SLC26A9} and MI in CF (206). It was hypothesized that \textit{SLC26A9} may be a possible modifier gene of CF intestinal obstruction (206). \textit{SLC26A9} is an anion channel that has been shown to work as a chloride channel, chloride-bicarbonate exchanger, and sodium co-transporter and alterations in channel function may further contribute to the abnormal anion transport seen in CF, which may lead to more mucus accumulation and dehydration and an increase in intestinal obstruction rates (212,213). Functional analyses are required to determine the role of SLC26A9 in the intestine and to determine how the gene modifies intestinal obstruction in CF. A \textit{Slc26a9} KO mouse was created and was reported to be healthy and displayed normal growth patterns (220). The \textit{Slc26a9} KO mice had a more basic gastric pH, decreased gastric acid secretion, decreased bicarbonate secretion and elongated villi and crypts in the duodenum, suggesting that \textit{Slc26a9} may play a role in pH and anion secretion in the intestine (220,221). A CF mouse model with absence of \textit{Slc26a9} was created and was reported to have decreased survival due to increased rates of intestinal obstruction (221). However, the mechanism of how \textit{Slc26a9} modifies CF intestinal obstruction remains to be elucidated.

The main goal of my work was focused on elucidating a mechanism for how \textit{Slc26a9} is modifying CF intestinal symptoms. I monitored two different types of CF
mouse models and found that a reduction of one copy of \textit{Slc26a9} in the mild intestinal symptom, R117H, CF mouse model had detrimental effects on survival and increased the incidence of intestinal obstruction. I found that a reduction of \textit{Slc26a9} in the CF mice did not significantly affect growth in the mice. I determined that gastric pH and GI motility rates were not altered in the CF mice when \textit{Slc26a9} function was reduced. \textit{Slc26a9} mRNA expression was found to decrease with age, thus suggesting that \textit{Slc26a9} may have developmental significance. In addition, it was determined that risk alleles associated MI in CF correlate with decreased \textit{SLC26A9} mRNA expression in human epithelial cells. Together, the data suggest that \textit{Slc26a9} may be a therapeutic target to alleviate CF patients of intestinal obstruction.

Growth deficiency is a manifestation of CF that is typically measured in CF studies as alterations in growth are linked to intestinal abnormalities. It has been hypothesized that intestinal symptoms may lead to abnormal digestion and in turn malnutrition, which may contribute to the growth reduction observed in CF (92,251,253,254). I found no significant differences in growth between CF mice with WT \textit{Slc26a9} and CF mice with a reduction of one copy of \textit{Slc26a9}. If intestinal obstruction played a major role in the occurrence of growth deficiency then I would have expected the CF mice with reduced \textit{Slc26a9} and increased intestinal obstruction to be smaller than the CF mice with WT \textit{Slc26a9}, however this was not the case. In addition, other recent reports have utilized CF animal models and have shown that improved intestinal obstruction has no significant effects on the growth phenotype seen. Transgenic mouse models have been created that have restored CFTR function in the intestine and these mice displayed little to no intestinal obstruction, however the growth phenotype was not
alleviated (120). Intestinal obstruction was ameliorated in the CF pig by restoring CFTR function in the GI tract, yet growth was unaltered (224). The data showed that intestinal obstruction is not the sole contributor to growth deficiencies in CF and the origins of this symptom are complex.

Alterations in gastrointestinal pH are not very well studied in CF patients. Gastric pH produced by the stomach plays an important role in barrier function by preventing infection by pathogens that enter into the gut (259–261). The pH in the stomach is typically very low and the low pH is required to inactivate or kill the majority of ingested microbes (262). Wireless motility capsule studies have recently been conducted in CF patients to monitor the GI profile. It was determined that patients were unable to properly neutralize the gastric acid once it was released into the proximal small intestine, therefore leading to a more acidic duodenum than controls (79). An analysis of CF mouse models determined the CF mice also have a more acidic duodenal pH than controls (151). It was also determined that the altered intestinal pH increased signaling from the intestine to the exocrine pancreas, which led to increased stress and inflammation of the pancreas, which may contribute to the pancreatic disease seen in patients (151). An additional study determined that CF mice have impaired gastric acid secretions as compared to control animals, which may lead to issues digesting food and improper signaling to the pancreas and intestine (247). Slc26a9 KO mice have been reported to have a reduction in the number of gastric aid secreting cells in the stomach, parietal cells, and loss of gastric acid secretion (220). However, these mice have been reported to be healthy and display normal growth (220). Since GI pH alterations have been reported in CF and Slc26a9 KO mice, I evaluated the gastric pH in CF mouse models with decreased Slc26a9 and
determined if gastric pH was altered. I found no significant differences in the gastric pH between the CF mice with WT Slc26a9 and CF mice with a reduction of Slc26a9. I was surprised not to find a significant difference in gastric pH in the CF mice with reduced Slc26a9; since mice with reduced Slc26a9 have previously been shown to have decreased acid secretory rates in the stomach (220). I also did not observe significant differences in gastric pH in the mild intestinal symptom, R117H, CF mouse model, which may be because there is still some CFTR function in this model. The gastric pH difference may become apparent in the CF mice if a more severe intestinal symptom mouse model is utilized. My data showed that alterations in gastric pH were not the cause of the increased intestinal obstruction observed in the CF mice with absence of one copy of Slc26a9.

I analyzed GI motility in the mouse models since CF mice have significantly slowed GI transit times as compared to controls (145,258). I hypothesized that a reduction of Slc26a9 in the R117H CF mice may enhance the slowed GI motility and would lead to an increase in intestinal obstruction. The mild intestinal symptom CF model (R117H) had significantly slowed GI motility (GCF=2.33; p<0.01) as compared to controls, which was similar to my findings for the severe intestinal symptom CF model (GCF=2.47). I had not expected to see these results since the obstruction rates in the R117H CF model is not as high as the F580del CF model. I found no significant differences in GI transit between the R117H CF mice and the R117H CF mice that had a reduction of Slc26a9. Overall, the data suggest that alterations in GI motility are a factor for the development of intestinal obstruction, but are not solely responsible for the increase in intestinal obstruction rates seen in the CF model with reduced Slc26a9.
*Slc26a9* functions as an anion channel (212,213) and altered anion transport has been previously reported in the *Slc26a9* KO mice. Basal and forskolin stimulated bicarbonate secretion in the proximal duodenum of the *Slc26a9* KO mouse (6 weeks – 6 months of age) was found to be significantly reduced, however there were no alterations in the distal duodenum (221). In younger *Slc26a9* KO mice (3-6 weeks of age) the proximal duodenum displayed significantly decreased bicarbonate secretion and had abnormal fluid absorption as compared to controls (221). I analyzed anion channel function in the *Slc26a9* KO mice. My data showed that reduction or complete absence of *Slc26a9* did not alter intestinal anion transport in non-CF mice. The differences observed in my work may be due to mouse genetic background. As stated previously, my *Slc26a9* KO mice were maintained on a C57Bl/6J background, where the *Slc26a9* KO mouse previously reported was maintained on a mixed background. I hypothesize that the differences in genetic background explain the differences in the results.

Previous studies have shown that *Slc26a9* may be altering intestinal properties in younger mice (220,221), therefore I wanted to developmentally analyze the role of *Slc26a9*. *Slc26a9* has been shown to be strongly expressed in the stomach and lung and to some extent in the proximal duodenum, but not in the more distal GI tract in mice 3 weeks to 6 months of age (212,221). There is low *Slc26a9* expression in the pancreas and nothing was found in the liver (212,221). I measured mRNA expression levels at several time-points during development and found that *Slc26a9* levels were increased early in life (newborn) and expression decreased by 21 days of age. The data suggest that *Slc26a9* may play a role earlier in life and may have developmental significance.
To relate my findings in mouse models to CF patients, I utilized human epithelial cells to determine how alterations in \( SLC26A9 \) affected mRNA levels in human samples. I found that risk alleles associated with MI in CF patients correlated with decreased \( SLC26A9 \) mRNA expression in human nasal epithelial cells. While these SNPs may not be the causative SNPs, it may be stated that alterations in \( SLC26A9 \) correlate with decreased mRNA production in human epithelial cells. Further research will help determine if \( SLC26A9 \) may be a therapeutic target to decrease CF intestinal symptoms.

A limitation of this study was the use of human airway epithelial cells to study a gene that was identified in a GWAS of MI in CF. Nasal epithelial cells were utilized in this study because they are an easily accessible cell type and have high levels of \( SLC26A9 \) expression. Ideally, intestinal sections from healthy individuals would be utilized to study how SNPs associated with MI in CF affect \( SLC26A9 \) expression. However, intestinal sections from healthy individuals are difficult to obtain. In addition, it has been determined that \( Slc26a9 \) expression decreased with age in mice; therefore it may have a similar affect in patients. The ideal sample would then be intestinal sections from healthy newborns, which would be very difficult to obtain.

Future directions for this work include determining the mechanism behind the modification of intestinal obstruction via \( Slc26a9 \). One hypothesis is that a reduction of \( Slc26a9 \) in CF mice is enhancing cellular proliferation in the intestine. An increase in intestinal cellular proliferation may lead to an increase in material present in the lumen and may also alter the structure of the crypt and villi, thus preventing them from functioning properly. Also, it has been previously reported that CF mice and younger \( Slc26a9 \) KO mice have enhanced intestinal cellular proliferation (221,263). I plan to
monitor proliferation in duodenum sections from mice 3-5 weeks of age via either a proliferating cell nuclear antigen (PCNA) stain or a BrDU assay. Another hypothesis is that Slc26a9 may be playing a significant role in early development. I plan to characterize the intestinal symptoms in younger Slc26a9 KO mice as well as in the CF context, to determine if the effects of modification are occurring early in life. I also plan to determine if developmental abnormalities are occurring as a result of reduced or absent Slc26a9.

Another future direction for this work includes an in-depth analysis of the CFTR Slc26a9 double KO mice. I observed a large amount of death, unrelated to early MI (221), in the CF mice with a reduction of Slc26a9 and a complete absence of Slc26a9 in the early days of life and very few CF Slc26a9 double KO mice survived to weaning age (~20 days). I am in the process of analyzing the cause of this early death and hypothesize that the survival rates I observed differ from the reports previously published due to differences in mouse genetic background. These data suggest that Slc26a9 may play a role earlier in life and may explain why CF mice with complete absence of Slc26a9 have been found to die very soon after birth and do not survive to 20 days of age. I utilized mice on a congenic C57Bl/6J background, as opposed to a mixed background previously used (221), which may also explain the survival differences seen in my models. This future direction is discussed in more depth in Chapter 5.

Overall, my data have provided evidence that Slc26a9 modifies CF intestinal obstruction in mouse models. I observed an increased intestinal obstruction rates in the mild intestinal symptom, R117H, CF mouse models when one copy of Slc26a9 was reduced. The reduction of Slc26a9 in the CF mice and increase in intestinal obstruction
had no effect on the growth phenotype seen in CF, therefore suggesting the origins of the growth symptoms in CF are complex. Gastric pH and GI motility were found to be unaltered in the CF mice with a reduction of Slc26a9; therefore additional factors must contribute to the development of the intestinal obstruction observed in this mouse model. I analyzed Slc26a9 mRNA expression and found that expression is greater in younger mice and hypothesize that Slc26a9 may play a developmental role. It was also determined that risk alleles in SNPs in Slc26a9 that have been identified to associate with MI in CF patients correlate with decreased SLC26A9 mRNA in human epithelial samples. The data suggest that SLC26A9 may be a therapeutic target to treat CF intestinal obstruction. The exact mechanism of the modification of Slc26a9 on CF intestinal obstruction rates remains to be elucidated; however this work provides novel information on Slc26a9 in the CF context.
Figure 3.1. Reduction of Slc26a9 leads to increased intestinal obstruction in the R117H CF mouse model. A) Obstruction of F508del mice with wildtype levels of Slc26a9 and reduction of Slc26a9. B) Survival of R117H mice with wildtype levels of Slc26a9 and reduction of Slc26a9. C) Image shows an intestine from a R117H mouse with wildtype levels of Slc26a9 and an intestine from a R117H mouse with reduction of one copy of Slc26a9.
**Figure 3.2. Reduction of *Slc26a9* has no effect on growth.** The R117H CF mice with wildtype levels of *Slc26a9* have significantly decreased survival at day 10 and days 20-40 as compared to controls (p<0.05). The reduction of *Slc26a9* in the R117H CF mice has no significant effect on the growth manifestations.
Figure 3.3. Gastric pH is not altered in CF mice with reduced \textit{Slc26a9}. Gastric pH was assessed in the mouse models. A significantly more alkaline pH was seen only in the \textit{Slc26a9} null mouse model. Reduction of \textit{Slc26a9} in the R117H model had no effect on gastric pH. (Control=3.2, \textit{Slc26a9} -/-=5.9, \textit{Slc26a9} +/-=3.7, R117H -/-=3.1, R117H -/- \textit{Slc26a9} +/-=2.96; p=<0.0001)

* Significant as compared to WT, p < 0.0001. n ≥ 4.
Figure 3.4. Reduction of *Slc26a9* does not alter GI motility in CF mouse models. No alterations in GI motility were seen in mouse models with reduced or completely absent *Slc26a9*. The R117H model with wildtype *Slc26a9* and with a reduction of *Slc26a9* both had significantly decreased motility rates as compared to the control group (p<0.05), however there was no difference observed between the two groups. (Control=4.35, *Slc26a9* +/-=5.37, *Slc26a9* +/+=4.16, R117H -/-=2.33, and R117H -/- *Slc26a9* +/-=2.95; p<0.05)

* Significant as compared to WT, p < 0.05. n ≥ 4.
Figure 3.5. Reduced or complete absence of *Slc26a9* does not alter anion transport in non-CF mice. No significant differences were found when the change in short circuit current across the intestinal epithelium after stimulation with IBMX and forskolin was analyzed in duodenum samples from control, *Slc26a9* +/-, and *Slc26a9* -/-.

(Control=33.2, *Slc26a9* +/-=26.8, and *Slc26a9* -/-=34.9)

n ≥ 3
Figure 3.6. *Slc26a9* detection and mRNA expression decreases with developmental age. *Slc26a9* mRNA expression levels were analyzed in several tissues at three distinct time points, newborn, 21 days, and 6-8 weeks. This graph shows the expression data for the lungs as a % of newborn lung expression. *Slc26a9* expression significantly decreases at 21 days and 6-8 weeks. * Significant as compared to newborn, p<0.001. n ≥5.
A

17.714bp
2,552bp
15,162bp

rs7419153
rs4077468

rs7512462

5’
-4079
-2157
Intron 5

3’

SLC26A9

B.

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Figure 3.7. SNPs in *SLC26A9* associated with MI in CF. A) A depiction of the *SLC26A9* gene. The SNPs identified via GWAS to have an association with MI in CF are labeled on the gene and the distance between each SNP is indicated. One of the SNPs is located within an intron and the other two SNPs are located in the 5’ UTR. B) A HapMap was created to analyze *SLC26A9* using data from dbSNP and the corresponding HapMap data. The HapMap spans regions upstream and downstream of the gene. Individual HapMap blocks are indicated on the graph. The colors on the graph correlate with $R^2$ values and degree of linkage (Red = $R^2$ close to 1 and strong linkage, Light Red to Pink = $R^2$ written in square and some association, White = $R^2$ written in and low association, and Blue = Not enough HapMap data available). The SNPs analyzed are boxed and the linkage disequilibrium $R^2$ values for each are listed in the inserted table. The HapMap insert zooms in on the *SLC26A9* to show the SNPs analyzed and their HapMap boxes. C) *SLC26A9* expression graphed as the cycle difference, $\Delta CT$, between *SLC26A9* and human $\beta$-Actin. Each dot is representative of a sample and samples are grouped by the number of risk alleles. Red dots are WT samples and black dots are CF samples. D) % *SLC26A9* expression per sample relative to the sample with the highest *SLC26A9* expression. Each dot represents one sample and samples are grouped by the number of risk alleles. Red dots are WT samples and black dots are CF samples.
4.1. Abstract

Background: Cystic Fibrosis (CF) patients experience intestinal complications characterized by the accumulation of thick viscous mucus. CF mice were utilized to determine if a novel guluronate oligomer, OligoG, may be a potential therapy in reducing intestinal mucus and subsequent CF-related intestinal manifestations.

Methods: Intestinal motility, intestinal histology, survival and growth were examined in wildtype and CF mice on regular water and OligoG.

Conclusions: OligoG improves intestinal motility and survival in CF mice by reducing the accumulation of intestinal mucus likely through its ability to chelate calcium and promote CF mucin expansion.

Keywords: Cystic Fibrosis, Intestinal Motility, Mucus, Genetically Modified Mouse

4.2. Introduction

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The absence of functional CFTR, a cAMP-regulated anion channel, leads to decreased anion and fluid transport resulting in the accumulation of thick sticky mucus within organs including the lung, pancreas and intestine (153,209). This mucus contributes to intestinal manifestations observed in CF, including slowed transit time, small intestinal bacterial overgrowth (SIBO) and intestinal obstructions
Intestinal obstruction is common in CF patients with 13-20% of patients experiencing meconium ileus (MI) at birth, 10-20% of patients experiencing distal intestinal obstruction syndrome (DIOS) postnatally and half of all patients experiencing constipation throughout life (76). Common treatment for these obstructions are rehydration, enemas, oral or intestinal osmotic laxatives and, in rare cases, surgery (63,76). More effective therapy options are necessary for the treatment and prevention of these CF intestinal complications.

The CF mouse displays similar intestinal manifestations as observed in CF patients. The CF mouse displays the accumulation of intestinal mucus, reduced intestinal motility, SIBO, reduced body weight and a high incidence of intestinal obstruction (115,120,144). The CF mouse has been utilized to identify new insights into CF intestinal pathophysiology and to test potential therapies for CF intestinal manifestations (209,222,264,265). While the pathophysiology of CF intestinal manifestations is complex, both the accumulation of mucus, due to decreased fluid secretion, and improper mucin expansion are suggested to be primary factors in the CF-related intestinal symptoms (16,209).

Alginites are linear polymers of (1,4) linked –L-guluronate (G) and –D-mannuronate (M) residues that are found as biopolymers in brown algae and bacteria (266). Alginate is safe for human consumption and administration (US FDA reference #21CFR184.1724) and is a commonly used ingredient in foods and medicines (267,268). The ability to generate these polymers with defined molecular weight and G/M composition may provide new disease therapies. OligoG is a specifically engineered (>85% G) low molecular weight alginate oligomer (269). OligoG displays antibacterial
properties in that it disrupts and inhibits biofilm formation as well as potentiates the action of certain antibiotics through its ability to directly bind to the bacterial surface (269–272). Additionally, G oligomers have been shown to disrupt interactions in complex mucus by reducing cross-linking within mucus (273). The current study was undertaken to determine whether OligoG would decrease CF intestinal manifestations in CF mice through its ability to disrupt complex interactions within mucus.

4.3. Materials and methods

4.3.1. Mice

The CF mouse model utilized in these experiments contained the well characterized F508del mutation (Cftr<sup>imlkh</sup>) (115). The mutation is congenic on the C57BL/6J background and wildtype littermates were used as controls. All mice were allowed unrestricted access to chow (Harlan Teklad 7960; Harlan Teklad Global Diets). Wildtype (WT) and CF mice treated with OligoG (AlgiPharma AS) were allowed access to a 2% OligoG/4% sucrose solution in sterile water. The sucrose was added to make the experimental compound more palatable. For appropriate controls, WT and CF mice not treated with OligoG were allowed access to 4% sucrose solution in sterile water; however, no differences were observed in WT or CF mice with or without sucrose in the water. The specific structure of OligoG and its synthesis have previously been published (269). All mice were maintained on a 12 h light, 12 h dark cycle at a mean ambient temperature of 22 °C. The Institutional Animal Care and Use Committee of Case Western Reserve University approved all animal protocols.
4.3.2. Measurement of Gastrointestinal Motility

Gastrointestinal motility was analyzed as previously described (144). Briefly, mice were fasted overnight and were allowed free access to water. In the morning mice were given 100µl of 25 mg/mL rhodamine labeled dextran (Sigma-Aldrich) solution by gavage. 40 minutes after gavage, the mice were sacrificed and the gastrointestinal tract, from stomach to cecum, was removed and placed in cold saline. The small intestine was divided into 10 equal sections and each segment, in addition to the stomach, was flushed with 2 mL of saline. The flushed contents were centrifuged at 500 rpm for 10 minutes and 200 ul of the supernatant from each section was placed in a 96 well plate. The quantification of the fluorescent signal in the supernatant from each segment was determined utilizing a multi-well fluorescence plate reader (FLUOstar Omega plate reader; BMG Labtech; excitation 545 nm and emission 590 nm). The distribution of the fluorescent signal in the intestinal segments was used to calculate the geometric center of fluorescence (GCF). GCF was determined by calculating the fraction of fluorescence per segment multiplied by the segment number (1-10) and adding all segments together. GCF can range from 1 to 10 with a higher number indicating a faster motility and shorter intestinal transit time.

4.3.3. Intestinal Histology and Mucus Measurements

Ileal sections from mice were removed and fixed in methacarn fixative solution (60% methanol, 30% chloroform, and 10% glacial acetic acid) for four hours, washed in phosphate buffered saline and stored in 70% ethanol until sectioning. The intestines were paraffin embedded, 5 µm thick longitudinal sections were cut, placed on glass slides and
stained with Fast Red and Alcian Blue. The slides were scanned via a Leica SCN400 slide scanner and images were analyzed via VisiomorphDP software (Visiopharm). To determine mucus coverage between villi, mucus staining (Alcian blue) and non-mucus staining (white) was then assigned in the software and pseudo-colored to indicate the call of each pixel by the software. The percentage of the whole area covered by mucus between each villi was calculated as the area of mucus staining divided by the whole area. Histology sections were sampled from each ileum in two distinct regions of at least 100 µm apart.

4.3.4. Statistical Analysis

Results are expressed as the mean +/- SEM. Differences between groups were determined using a one-way ANOVA with post-hoc Tukey test. Kaplan-Meier survival curves were evaluated using a log-rank test. A P value of <0.05 was considered significant.

4.4. Results

4.4.1. Intestinal motility

To examine whether OligoG could be a potential therapy for the prevention of CF intestinal obstructions, intestinal motility was assessed. 6-8 week old wildtype (WT) and CF mice were given either regular water or water containing 2% OligoG for 7 days, after which the intestinal motility was determined. As shown in Figure 4.1., the fluorescein labeled dextran travelled farther in WT mice than CF mice (GCF= 5.92 vs. 2.78; P<0.001) indicating decreased intestinal motility and slower intestinal transit in CF mice.
similar to previous studies (144). Treatment with OligoG increased intestinal motility and shortened transit time in CF mice (GCF= 6.52 vs. 2.78; P<0.001), with results similar to WT levels. OligoG treatment did not significantly alter intestinal transit time in WT mice compared to non-treated WT mice (GCF= 5.92 vs. 6.66). There were no differences in any experimental parameters examined between WT mice on regular water and WT on OligoG, thus these groups are combined throughout the rest of the results section.

4.4.2. Survival

During the 7 day treatment with either regular water or OligoG, 100% of the WT mice (n=22) and 100% of the CF mice on OligoG (n=10) survived compared to only 50% of the CF mice on regular water (n=20; P<0.05 compared to treated CF mice). The CF mice that did not survive were determined to have succumbed to intestinal obstruction. To determine if OligoG is protective against obstruction and improves long term survival of CF mice, 4 week old WT and CF mice were weaned on regular water or water with OligoG and monitored daily for 25 days. As shown in Figure 4.2., 100% of WT mice and only 29% of CF mice survived after weaning while on regular water. The CF mice that did not survive were determined to have succumbed to intestinal obstruction. In contrast, 82% of CF mice on OligoG survived 25 days after weaning indicating a significantly improved survival (P<0.001) compared to CF mice on regular water.

4.4.3. Intestinal Histology

To determine if there were any mucus accumulation differences in the intestine between OligoG treated and non-treated mice, intestinal sections were collected and
examined. Blinded gross examination indicated a possible difference in mucus present between villi (Figure 4.3.A, column 1). To quantify the presence of mucus between villi, the software program VisiomorphDP was utilized. This software allows for quantitative image analysis in which the user indicates the area of interest and what to quantify. In these experiments, the space between the villi was marked and areas containing mucus were designated as blue stained areas while areas not containing mucus were designated as white or non-stained areas. The software pseudo-colored each pixel of the image green or black to indicate the software call (mucus vs. non-mucus) (Figure 4.3.A, column 2 and 3). The percentage of mucus in each area between villi was calculated as the area that was green divided by the whole area (green plus black). There was a significant difference in the area in which mucus was present between WT and CF intestines (37.7±3.6 vs. 65.2±7.4). While there was an increase in the average area in which mucus was present between from WT and CF Oligo intestines (37.7±3.6 vs 51.4±7.1) the difference was not significant (Figure 4.3.B).

4.4.4. Growth

To determine if treatment with OligoG improved weight gain in CF mice over the 25 days post-weaning period compared to CF mice on regular water, each mouse was weighed at weaning and every 5 days after. Similar to previous reports (115,118,120), WT mice weighed significantly more than either CF group throughout the 25 days post-weaning (Figure 4.4.A). This significant difference in weight was evident in both sexes (Figure 4.4.B; Females- WT=18.2±0.78, CF 13.5±1.36, CF on OligoG 14.0±1.24; Males- WT=24.0±0.58, CF 16.7±0.85, CF on OligoG 16.9±0.65). There were no
significant differences between CF mice on OligoG and non-treated CF mice (Figure 4.4.A and B).

4.5. Discussion

Mucus secretion and removal serves a protective role in the healthy lung and intestine (153). However, the overabundance of viscous mucus found in CF, also referred to as mucoviscidosis, turns the normally protective mucus barrier into a building block for disease progression. The absence of CFTR leads to altered anion flow and a dehydrated luminal surface. In addition, the loss of bicarbonate secretion through CFTR impedes normal mucus formation which requires bicarbonate to sequester calcium from condensed mucins so they can fully expand (9,14,16). The abnormal mucus in CF is thought to lead to bacterial overgrowth and an inflammatory response that leads to the overproduction of more viscous mucus (153). This accumulation of tightly packed mucus eventually leads to the majority of complications observed in the CF intestine (209).

Osmotic laxatives and stool softeners are widely prescribed to treat intestinal obstructions such as DIOS and constipation. However, common side effects of these treatments include diarrhea, vomiting and dehydration with multiple treatments usually required (63). Alternative therapies are needed for CF-related intestinal obstructions.

The aim of this study was to determine if a novel guluronate oligomer, OligoG, could be used to reduce CF intestinal manifestations in a CF mouse model. Based on previous studies in which OligoG could disrupt both bacterial biofilm and mucus in vitro (271–273), I hypothesized that OligoG would display a similar role in disrupting intestinal mucus accumulation in vivo and reduce CF intestinal complications. While I
observed no significant changes in any parameters between treated and non-treated WT mice, the administration of OligoG to CF mice had a significant impact on the CF intestinal phenotype. Intestinal motility was normalized in CF mice treated with OligoG suggesting that intestinal contents could more easily move through the small intestine and consequently shorten intestinal transit time. Moreover, treatment with OligoG did not significantly alter transit time in WT mice, so it was not simply functioning as a laxative. Both short (7 days) and long term (25 days) treatment of CF mice with OligoG resulted in a significantly decreased incidence of intestinal obstruction and improved survival. Improved survival was most likely due to the decreased amount of accumulated mucus that was observed in the intestinal histology: There was significantly more mucus between villi in CF mice than WT mice, while the CF mice treated with OligoG were not significantly increased compared to WT mice. The calcium chelating properties of the OligoG (discussed below) may have assisted in the unfolding of the mucus and reduced its accumulation. These results indicate that OligoG does significantly improve CF intestinal symptoms.

An additional parameter that is commonly measured in CF studies with regards to intestinal phenotype is growth. I observed that OligoG administration did not improve CF mouse body weight over the 25 day treatment period. Initially, this finding may seem surprising. Alleviating or even reducing intestinal obstruction should improve growth in these CF mice. However, understanding the origin of the reduced growth in CF is difficult given the systemic nature of CF. For instance, both pancreatic insufficiency and lung disease can clearly affect growth in CF patients (274,275). By contrast, the CF mouse has mild lung disease and pancreatic pathology (107,276,277); however it still
manifests the significantly reduced growth observed in CF patients (115,120). In the past, the intestinal obstructions in the CF mouse have been hypothesized to be the origin of the observed reduced growth. However, recently, multiple reports using CF animal models have alleviated intestinal obstruction with no improvement in growth. In the CF mouse, two different transgenic models have been utilized to restore CFTR function in the intestine. While these models display little to no intestinal obstruction, growth was not improved (120). Similarly, in the CF pig, correction of CFTR in the gut did not improve growth even in the absence of intestinal obstruction (224). Endocrine abnormalities in CF may be an alternative origin that contributes to the reduced growth in CF patients and animal models. Insulin-like growth factor I (IGF-1), an important endocrine growth hormone, is reduced in CF patients, mice and pigs, which suggests non-intestinal origin(s) for the reduced growth (278–281). Clearly, any growth effects will be difficult to evaluate in these models given the complex systemic nature of the disease.

OligoG is a novel alginate derived from seaweed that has been specifically engineered and composed of low molecular weight guluronic acid oligomers (269). OligoG has antibacterial and antifungal properties and potentiates specific antibiotics and antifungals (269,282). These actions are thought to be due to the ability of OligoG to bind to the bacterial and fungal surfaces, modulate the surface, induce aggregation, inhibit motility and disrupt biofilm formation (270–272). In addition, there is also evidence that these short G-rich oligomers could disrupt the complex mucus gels through electrostatic interactions and thus could play a similar role in dispersing mucus (273). Nevertheless, calcium is a major factor in pre-secreted mucin and removal of calcium leads to mucin release, unfolding and expansion (283). Several studies have shown that mucus
aggregates dissolve in calcium chelators (9,283). In a study specific to CF, the combination of bicarbonate with the calcium chelator EDTA increased the detachment of preformed CF mucus in *ex vivo* explants (284). Although alginates do have calcium chelating ability, guluronate oligomers specifically have an increased ability to bind calcium *in vitro* compared to M oligomers (285). G oligomers specifically have been shown to disrupt complex mucus systems leading to the weakening of the cross linking in mucus (273). Specific to CF, G oligomers were shown to weaken CF sputum as assessed by rheology which would decrease mucus viscosity and theoretically make it easier to clear (273). These previous findings may explain the ability of OligoG to decrease mucus accumulation and improve CF-related intestinal complications in the CF mice. OligoG may be acting as calcium chelating agent which substitutes for the missing bicarbonate in the CF intestinal environment. OligoG may be sequestering calcium away from the packed mucins that are secreted into the intestine, allowing the mucus to expand, mature and disperse.

In conclusion, this is the first study to show that administration of OligoG improves CF-related intestinal complications *in vivo*. In CF mice, OligoG reduced intestinal mucus accumulation, shortened intestinal transit time and improved survival. While prevention of intestinal obstruction by OligoG is evident in my data, further studies are necessary to assess whether OligoG could be used to resolve an existing obstruction. Further studies are needed to examine the effect of OligoG on additional intestinal alterations in CF that are downstream of mucus accumulation, such as the incidence of SIBO, changes in the intestinal microbiota and intestinal inflammation (144,237). Alginates are safe and routinely utilized in products consumed by and
administered to humans. OligoG is currently in PhaseIIb clinical trials as an inhaled therapy in CF (NCT02157922; NCT02453789). The data from my study suggest that OligoG may also be a potentially promising therapy for intestinal complications in CF patients.
Figure 4.1. Intestinal motility in WT and CF mice. (A) Examples of rhodamine-labeled dextran (bright white areas) in the small intestines of fasted WT and CF mice on regular water or OligoG. Two examples are shown for the CF mice treated with OligoG to show variability in drug treatment. All intestines are oriented with the duodenum at the top and ileum at the bottom. GCF for each displayed animal was 5.3, 2.9, 4.7 and 8.5 from left to right. (B) Average GCF value for WT and CF mice on regular water or OligoG. n≥8. *P=0.001 vs. WT.
Figure 4.2. Survival of WT and CF mice on regular water or OligoG. Mice were placed on water or OligoG at time of weaning and surviving was monitored for 25 days after weaning.

* p < 0.001 compared to WT or CF on OligoG. n ≥ 17.
Figure 4.3. Intestinal histology and assessment of relative intestinal mucus amount in WT and CF mice on regular water or OligoG. (A) Intestinal sections from WT, CF and CF mice on OligoG are shown stained with nuclear fast red (red) to stain cells and Alcian blue (blue) to stain mucus (first column). Assessment of blue stained mucus was made between pairs of villi (second column). The space between villi was indicated within the software which then pseudo-colored mucus green and empty space black (third column) to calculate percent of space between the villi that contained mucus. Scale bar = 50µm (B) Percentage of area between villi that is mucus filled from intestinal sections in WT, CF and CF mice on OligoG.
* p < 0.05 compared to WT. n ≥ 10.
Figure 4.4. Growth of WT and CF mice on regular water or OligoG. (A.) Weight of WT, CF and CF mice on OligoG was measured from weaning (day 0) to 25 days post-weaning. WT mice were significantly heavier at all time points measured p<0.05. Equal numbers of males and females were included. (B) Weight of WT, CF and CF mice on OligoG at 25 days post-weaning separated by sex.

* p < 0.05 compared to WT. n ≥ 10.
5.1. Rationale and Summary of Conclusions

Over the past several years research has provided the opportunity for the development of treatments and therapies for CF that have made remarkable improvements in patients’ quality of life. Patients today have the ability to go to college, have a career, get married, and have their own children. While CF patients today are doing better than ever, the disease is still prominent in their lives and still leads to a shortened lifespan. CFTR genotype, environmental influences, secondary genetic factors, and stochastic events all contribute to the development of CF manifestations and several of these aspects have been analyzed in depth to determine how they contribute to symptom development. This thesis analyzed the gastrointestinal (GI) manifestations of CF and determined how the tissue specific expression of CFTR and a modifier gene influenced the occurrence of the intestinal symptoms. Despite great efforts to determine how the GI manifestations in CF develop, there are still many questions regarding the intestinal symptoms. Overall, knowing the mechanism for the development of the GI complications and how the factors that contribute to symptom development can be targeted to prevent the symptoms from occurring is the main goal of this thesis.

The data presented in this thesis provide insight into the development of GI complications by analyzing several aspects that contribute to the incidence of intestinal obstruction. In chapter 2, the role of CFTR in different intestinal cell types was analyzed. It was determined that absence of Cftr in the intestinal epithelium and smooth muscle together contributed to the development of intestinal obstruction. The data supported the hypothesis that CFTR plays an important role in each cell type and may be stimulating
interactions between the intestinal epithelium and smooth muscle that are necessary for the GI tract to function properly. Chapter 3 of this thesis analyzed how a recently identified modifier gene, \textit{SLC26A9}, influenced CF intestinal manifestations. It was determined that reduction of \textsl{Slc26a9} had a detrimental effect and led to increased intestinal obstruction rates in CF mice. In addition, risk alleles of SNPs associated with MI in CF patients correlated with decreased \textit{SLC26A9} mRNA expression in human epithelial cells. While there are many remaining questions, SLC26A9 may be a therapeutic target to alleviate CF intestinal symptoms. Chapter 4 of this thesis analyzed how treatment with a novel guluronate oligomer, OligoG, affected the intestinal symptoms in CF mice. It was determined that drug treatment improved survival rates and GI transit and led to decreased mucus accumulation in the intestine. OligoG is a calcium chelator that it hypothesized to alter the properties of the mucus by removing calcium from the mucins, which allows them to unfold properly. Correctly formed mucins are able to fully expand and move freely along the GI tract, thus allowing the intestine to remain clear of material and preventing intestinal obstruction. This thesis provided novel insights on several factors that contribute to CF intestinal obstruction. However, the data also led to the formation of new questions about CF intestinal manifestations and additional experiments required for each of the chapters in the thesis are discussed below.

5.2. \textit{Cftr} in the Intestinal Smooth Muscle

The work analyzing the role of \textit{Cftr} in the smooth muscle has provided some valuable evidence on how intestinal obstruction develops in CF mouse models. Prior work in our lab showed that absence of \textit{Cftr} in the intestinal epithelium was necessary for
intestinal obstruction to develop, but was not sufficient to be the sole cause as the rate of obstruction was less than that of CF null mice (120). I then hypothesized that absence of Cftr in another cell type may be contributing to the development of CF-like intestinal obstruction. The work in this thesis suggested the absence of Cftr in the intestinal epithelium and smooth muscle together was necessary for the high incidence of intestinal obstruction. My data showed that absence of Cftr in the intestinal epithelium and smooth muscle led to an increased rate of intestinal obstruction, slowed GI motility, elevated mucus accumulation in the intestine, elongated width of intestinal smooth muscle, and abnormal maximal contraction in the intestinal smooth muscle in response to high potassium stimulation. All of the intestinal CF manifestations that occur in the mouse model lacking Cftr in the intestinal epithelium and smooth muscle are similar to those manifestations seen in the CF null mouse model.

I hypothesize that absence of Cftr in each of the tissues plays its own part in contributing to the development of intestinal obstruction. The intestinal epithelium is responsible for secreting prostaglandins, which work to stimulate contraction or relaxation of the muscle. It has been shown in CF mice that the levels of prostaglandins that control contraction and relaxation are both elevated (148). An elevation of both types of prostaglandins may impair the receptors and prevent accurate signaling to downstream activators that contract or relax the muscle. The intestinal epithelium also stimulates the influx of calcium through sodium/calcium channels or voltage-dependent calcium channels and since smooth muscle contraction requires calcium influx, an alteration in calcium transport would hinder smooth muscle function (286). In addition, lack of Cftr in the intestinal epithelium leads to dehydration due to impaired chloride ion transport and
the accumulation of thick, sticky mucus in the lumen. All of these factors together contribute to intestinal obstruction; however absence of \( C_{ftr} \) in the intestinal epithelium alone is not sufficient to recapitulate the CF null mouse model obstruction rates. My data suggest that \( C_{ftr} \) in the smooth muscle also contributes to the incidence of intestinal obstruction. The smooth muscle stimulates the influx of calcium from voltage-dependent calcium channels. Potassium channels inhibit or negatively regulate the influx of calcium and receptor-operated and stretch-operated channels positively regulate calcium influx (286). The net result of calcium influx is muscle contraction and alterations in calcium movement will impair smooth muscle function. I hypothesize that the absence of \( C_{ftr} \) in the smooth muscle leads to altered calcium transport in the cells and impairment of muscle function. My data suggest that all of these components contribute to the development of CF intestinal obstruction. It may be possible to target one or several of the aberrant factors and correct the abnormality, which may prevent the development of intestinal obstruction.

Smooth muscle contraction and relaxation is controlled by a complex network of interactions between many different components and several parts of the pathway have been shown to be altered in CF (286,287). Any alterations in smooth muscle contraction and relaxation signaling may lead to the occurrence of intestinal obstruction.

Calcium is a major component of smooth muscle function and abnormal calcium levels in the cells will lead to improper muscle contraction and relaxation. An influx of calcium is required for the smooth muscle to contract and an efflux of calcium leads to relaxation of the muscle (288). Most of the calcium required for muscle contraction enters the cell via voltage-dependent calcium channels (VDCC) that are regulated in part
by potassium channels, sodium/calcium channels, and G protein coupled receptors (286). Chloride ions are known to assist in calcium ion transport (137,242,286,289), therefore it was hypothesized that CFTR may play a role in calcium mobilization and smooth muscle function. Calcium transport and smooth muscle tone have been analyzed in patients and CF animal models and several defects have been identified. Calcium regulation has been found to be altered in CF patient nasal and airway epithelial cells (243,244). It has been determined that the vascular smooth muscle tone of CF piglets and rats is altered as a result of impaired calcium regulation (137,235). Recently, it has been found that basal calcium levels in pig airway smooth muscle cells were similar between CF and wildtype, however cholinergic simulations delayed the calcium reuptake in the CF cells (229). Taken together these data, and data from Chapter 2 of this thesis, suggest that CFTR does regulate calcium handling and plays a role in smooth muscle function. Additional analyses to monitor calcium mobilization in intestinal smooth muscle cells will provide further evidence to examine this hypothesis.

The sarcoplasmic reticulum (SR) also plays a role in calcium mobilization in the smooth muscle cell and alterations in SR function will create calcium imbalances that will alter the function of the smooth muscle. The SR sequesters calcium from the cytoplasm to create an efflux of calcium and simulate smooth muscle relaxation. It is also able to release the sequestered calcium back into the cytoplasm to stimulate contraction of the muscle. It has been hypothesized that CFTR is present in the SR and alterations in CFTR here may lead to abnormal calcium sequestering or release. It has been shown that CFTR localizes to the SR of airway smooth muscle cells in pigs and of skeletal muscle in humans and mice (141,229,290). One group determined that calcium reuptake after
cholinergic stimulation was delayed suggesting that a SR alteration may be the cause of increased contractility observed in airway smooth muscle in CF (229). Further research is required to determine if CFTR is present and altering calcium mobilization in the intestinal smooth muscle.

As stated previously, the pathways resulting in smooth muscle contraction and relaxation consist of a complex network of players and alterations in expression levels of any of the components may hinder muscle functions. Smooth muscle contraction occurs when the intracellular calcium binds with calmodulin to form a complex that activates the myosin light chain kinase (MLCK) (286). The activated MLCK works to phosphorylate the 20 kDa light chain of myosin (MLC20), which triggers muscle contraction (286). It has been shown that baseline MLCK phosphorylation levels were increased in CF pig airway smooth muscle cells (229) and an increase in MLCK phosphorylation would lead to increased muscle contraction (286). For the smooth muscle to relax, the intracellular calcium must be removed or sequestered by the SR, which prevents the formation of the calcium-calmodulin complex and thwarts MLC20 phosphorylation. It is hypothesized that CFTR alters the pathways that work to dephosphorylate MLC20 or impairs the components that regulate calcium sensitivity in the smooth muscle cells. It has been shown that an upregulation of Rho proteins alters calcium sensitivity in the intestinal and airway smooth muscle and leads to muscle hyperresponsiveness (227,228). Increased levels of RhoA have been shown to be present in the CF epithelium (291) and may also be increased in the intestinal smooth muscle, which would lead to an increase in the pathway that stimulates muscle contraction. Further analyses are required to determine if
abnormal CFTR alters components of the intestinal smooth muscle contraction/relaxation pathway.

Future directions for this project include further analyses to determine how CFTR in the smooth muscle is contributing to the incidence of intestinal obstruction. Since the intestine consists of many different cell types, it is necessary to isolate intestinal smooth muscle cells from the different mouse models so that CFTR may be further analyzed in these cells. CFTR localization in the smooth muscle cells will be analyzed to determine if CFTR localizes to the SR in intestinal smooth muscle, as was seen in the airway smooth muscle and skeletal muscle (141,229,290). Basal and stimulated smooth muscle contractions will also be monitored to determine if differences occur in cells lacking Cftr. It is also necessary to monitor calcium mobilization in the intestinal smooth muscle cells. It would be essential to determine how treatment with extracellular calcium, chloride, and RhoA affects calcium transport and release from the SR. Another future direction for this project is to further analyze muscle contractions utilizing the organ bath technique. Determining how the muscle responds to treatment with prostaglandins or prostaglandin inhibitors would provide information on how the different prostaglandins affect the CF intestine. Similar to the isolated cells, it would be necessary to determine how the addition of calcium, chloride, and RhoA affect intestinal muscle contractions. Monitoring how the absence of CFTR alters components of the intestinal smooth muscle contraction/relaxation pathway would provide information on how intestinal symptoms develop and may also provide a therapeutic target to alleviate the symptoms in patients.

My work has shown that absence of Cftr in multiple tissues, specifically in the intestinal epithelium and smooth muscle, is required in to mimic the intestinal obstruction
rates of the $Cftr$ null mouse model. It is hypothesized that the absence of $Cftr$ in other tissues may also play a role in the occurrence of CF intestinal obstruction, however further research is required to determine the role of $Cftr$ in other intestinal cell types.

Overall, this work suggests that $Cftr$ plays a role in intestinal smooth muscle function; however intestinal obstruction occurs as a result of the absence of $Cftr$ in the intestinal epithelium and smooth muscle. The intestinal smooth contraction/relaxation pathway is complex and several players have been found to be altered in CF. Additional research is required to further analyze the role of $Cftr$ in the intestinal epithelium and smooth muscle and to determine the mechanism for how absence of $Cftr$ in these cells contributes to the development of intestinal symptoms.

5.3. Modifier Effects of $Slc26a9$

My work suggests that $Slc26a9$ is a modifier gene of CF intestinal obstruction in mouse models and SNPs in $SLC26A9$ associated with MI in CF patients correlate with decreased mRNA expression in human epithelial cells. The data show that alterations in $SLC26A9$ have a detrimental effect; however the mechanism behind its modification remains to be elucidated. There are several hypotheses as to how SLC26A9 modifies intestinal obstruction rates in CF. It is hypothesized that $Slc26a9$ may play a role in intestinal cellular proliferation (221) and an increase in proliferation may lead to more material being present in the intestinal lumen and may promote intestinal obstruction. $SLC26A9$ functions as a chloride-bicarbonate exchanger (212,213) and bicarbonate has been shown to play an important role in mucus production (9–11,153,292,293). Alterations in mucus production would create mucus that is viscous and unable to be
easily removed, thus contributing to the accumulation of material present in the lumen and the development of obstruction. SLC26A9 is a chloride channel (212,213) and a decrease of SLC26A9 function, in addition to the absence of CFTR, may lead to a significant deficiency of anions which would enhance CF intestinal symptoms and lead to an increase in intestinal obstruction rates. It is possible that SLC26A9 modifies several factors that contribute to the development of CF intestinal obstruction due to the multiple functions of SLC26A9. Additional functional studies are required to determine how alterations in SLC26A9 alter the incidence of intestinal obstruction in CF.

One way to determine the effects of Slc26a9 is to create a mouse model to directly target Slc26a9. A transgene for Slc26a9 would allow for the conditional inactivation or restoration of Slc26a9 at specific time points so that the developmental effect of the gene may be further analyzed. My data suggest that Slc26a9 is more highly expressed earlier in life, therefore activating Slc26a9 activity at different time points in utero and in the first few days of life may help determine when the gene is required. This information may also be correlated with what is occurring at the developmental stage at the time of Slc26a9 activation and may provide information on what organ system or tissue the gene alters. A mouse model targeting Slc26a9 would also allow for the creation of a model overexpressing Slc26a9. In the CF context, this would help determine if Slc26a9 has therapeutic potential to alleviate CF intestinal symptoms. I hypothesize that an increase of SLC26A9 would lead to increased chloride and bicarbonate transport and more water present in the intestine, thus restoring anions to a functional level and decreasing the incidence of intestinal obstruction. A mouse model targeting Slc26a9 activity would provide the necessary system to test how overexpression affects intestinal
symptoms and in the CF context, this model would provide valuable information on the role of Slc26a9 and how Slc26a9 modifies CF intestinal obstruction.

In addition to determining that Slc26a9 modifies the risk of intestinal obstruction in CF mouse models, my data also led me in a direction that I had not previously anticipated. Chapter 3 discussed the analyses of survival in two different types of CF mouse models, F508del and R117H, which had either wildtype levels of Slc26a9 or a reduction of one copy of Slc26a9. My data showed that, from day 5, a reduction of one copy of Slc26a9 in the mild intestinal CF mouse model, R117H, had a detrimental effect on survival and led to an increase in intestinal obstruction (Figure 3.1.). Chapter 3 analyzed survival from day 5 on because there was a large decrease in survival seen prior to day 5 in the CF mouse models with an absence or reduction of Slc26a9. This early death was determined not to be due to MI. Survival data analyzed from day 0 to day 10 in F508del CF mice with an absence or reduction of Slc26a9 showed death as early as day 1 (Figure 5.1.A). Early death was also seen in the R117H CF mouse models with absent or reduced Slc26a9 (Figure 5.1.B). Since I was seeing early death in the CF mice with altered Slc26a9 and were not seeing a large number of the Cftr-/-;Slc26a9-/+ mice surviving to genotyping and weaning age (~10-20 days), I wanted to determine if these KO mice were dying in utero or right after birth. If death occurred in utero then the reduction of Slc26a9 in the CF context had lethal effects on the embryo, however if the mice were dying right after birth then the mothers may be eating the pups and preventing a necropsy. I analyzed the genotypes of mice at several time-points, 18 days post coitus (dpc), day 0, day 2, and day 4, to determine if Mendelain ratios of the genotypes were observed. I observed the following genotypes: R117H+-/-Slc26a9++/, R117H+-/-
Slc26a9+/-, R117H-Slc26a9+/+, and R117H-Slc26a9+/-). I found no significant differences in the expected and actual genotype ratios; therefore the reduction of Slc26a9 in the R117H CF mice was not embryonic lethal (Table 5.1). I also wanted to determine why low numbers of Slc26a9 KO mice were surviving to weaning age. I performed cesarean sections at 18 dpc and analyzed the pups for two hours after removal from the uterus. Pups were placed on a waterbed (plastic bag filled with water on a 37°C heat block) and breathing was stimulated by rubbing a finger on their chest. The majority of the wildtype mice and mice with a reduction of one copy of Slc26a9 survived for at least two hours after birth (Table 5.2). The majority of the death seen within two hours was in the Slc26a9 KO mice. A previously published paper suggested the reason for the high mortality seen 1-2 days after birth in the Slc26a9 KO mice was “presumably due to pulmonary complications” (221) and my data support this presumption, suggesting that mice dying within 2 hours of birth may have had pulmonary complications. Further analyses are required in order to determine the exact cause of death in these mice.

Further research is required to determine the role of Slc26a9 in these pulmonary complications. Histologic analyses of the lung will help determine if Slc26a9 has a structural effect on the lung. Alterations in organ structure may lead to improper function, such as abnormal expansion upon first breath and impaired breathing. It is also necessary to determine if mice of various ages with complete absence or a reduced amount of Slc26a9 have alterations in lung function. It may be necessary to test lung function via forced oscillation techniques in newborn through adult mice. The conditional mouse model targeting Slc26a9 discussed previously would also be useful to analyze how activating or inactivating Slc26a9 at different stages in development alters lung structure.
and function. These models could also determine if over activation of Slc26a9 early in development prevents the lung complications from occurring at birth. If the adult mice do have altered lung function, these models would help determine if activing, or over expressing, Slc26a9 function in adulthood helps alleviate the lung complications.

Overall, data produced in this thesis provide valuable information on the role of Slc26a9. I have shown that Slc26a9 modifies intestinal obstruction in CF mouse models and SNPs in Slc26a9 are associated with decreased mRNA expression in human epithelial cells. I have also shown that Slc26a9 may play a developmental role and alterations in Slc26a9 may lead to lung complications in Slc26a9 KO mice soon after birth. There are still many questions to be answered and further analyses may help determine if Slc26a9 may be targeted in the future to alleviate intestinal obstruction in CF patients or lung complications seen in other patients.

5.4. Novel Drug Treatments for Intestinal Obstruction in CF

This thesis also analyzed a novel guluronate oligomer, OligoG, and how it affected the CF intestinal tract. OligoG targets mucus accumulation and has calcium binding properties, which aid in normalizing mucus buildup. Therefore it was hypothesized that OligoG treatment may aid in the normalization of CF intestinal mucus (9,10,14,292,294). Mucus rheology is an important biological function and results from the interaction between the components of mucus, mucins and nonmucins (273). It has been shown that guluronic acid oligomers extracted from alginate, such as OligoG, are able to disrupt these interactions and create rheological changes that are associated with altered physiology function (273). Preliminary analyses indicated that OligoG is an
effective treatment to remove mucus from the airways in CF models and OligoG is currently in Phase IIb clinical trials as an inhaled therapy in CF. The goal of this thesis was to determine if OligoG treatment also removed mucus from the CF intestinal tract in mouse models.

My data showed that OligoG treatment in the CF mice had beneficial effects. I found that drug treatment improved survival in the CF mice and decreased the rate of intestinal obstruction. Treatment also improved GI transit and decreased the mucus accumulation in the intestine. Based on previous data, it was hypothesized that OligoG prevented intestinal obstruction by altering the properties of the mucus.

Future directions for this work include analyzing how the drug modifies mucus in the intestine. In the CF intestine the mucus that accumulates in the lumen is thick and sticky and unable to be easily flushed. It is hypothesized that OligoG makes the mucus less viscous, thus allowing it to be easily removed from the intestine and preventing accumulation. It has been shown that G oligomers are able to disrupt complex mucus systems, such as in CF, by weakening the cross linking within mucus (273). Monitoring mucus viscosity before and after drug treatment would allow me to determine if OligoG is able to alter the properties of CF mucus enough to prevent its accumulation in the intestine. It is hypothesized that mucus accumulation in CF may be due to an elevation of mucin gene expression, possibly as a result of increased inflammation (153). Mucin genes have been analyzed in CF and there is contradicting evidence in the literature as to whether mucin gene levels are unaltered or decreased in CF (154,155). However, MUC1 mRNA levels have been shown to be increased in CF due to two splice variants and it is hypothesized that alterations in MUC1 may be contributing to the mucus hypersecretion
seen in CF (154,155,295). The mechanism for how MUC1 may be altering mucus secretion remains to be elucidated. It is possible that OligoG may be interfering with MUC1 and preventing the overproduction of mucus. One way to test this hypothesis is to analyze the levels of MUC1 and total mucins in the GI tract of CF mice before and after treatment with OligoG. It may also be hypothesized that OligoG is causing disruptions in the mucus by acting as a substitute for bicarbonate in the CF intestine. It has been hypothesized that, in a healthy intestine, bicarbonate sequesters calcium from the condensed mucins so they may properly expand and fully disperse, however in the CF intestine the absence of bicarbonate impedes normal mucus formation (9,10,14,16,296). OligoG has calcium chelating properties (297–299) and drug treatment has been shown to aid in dispersing mucus in CF (273,285,298), which lends support to my hypothesis. This hypothesis could be tested by first labeling calcium and the mucins with fluorescent tags and then adding varying concentrations of OligoG to mucus isolated from CF mice and then monitoring if the calcium is sequestered by the drug and if the mucins are able to expand as a result. These experiments will allow me to determine a mechanism for how OligoG is altering mucus in the CF intestine.

I also hypothesize that, in addition to altering the properties of mucus, OligoG may affect other aspects of the CF intestinal environment, such as the bacteria levels and species present in the gut and the inflammatory state of the intestine. Mucus accumulation seen in the GI tract of patients traps bacteria in the lumen, which leads to the development of SIBO (153). In addition, the absence of CFTR has been found to be associated with alterations in the gut microbiome in patients (209). SIBO and intestinal dysbiosis have also been identified in CF mouse models (237,300,301). Analyses of
OligoG have found that the drug has anti-bacterial properties and may play a role in altering intestinal bacteria levels and the gut microbiota (269,282). Antibiotic treatment in control mice altered the bacterial community profile, as well as significantly increased the bacterial diversity in CF mice, thus indicating that antibiotic administration has the ability to change the gut microbiome composition (300). It is necessary to determine how the antibiotic effects of OligoG treatment alter the microbiome in the CF intestine, as healthy intestines require a proper balance of bacteria to function. It is possible that OligoG treatment normalizes the bacteria in the intestine, which would further protect against the development of intestinal obstruction. It is also hypothesized that the gut microbiota, in addition to the mucus accumulation, has the potential to affect inflammation and immune response, therefore aberrant GI microbiota may contribute to the increased inflammatory response seen in the CF intestine (153,301,210,302). OligoG treatment may work to decrease inflammation in the intestine, which in turn decreases the mucus accumulation based on the hypothesis that the pathways that drive mucin over-production and hypersecretion are controlled by inflammation (153). To test this hypothesis I could analyze genes involved in inflammation that have been previously shown to be elevated in the CF intestine, such as mast cell protease 1 and 2 (Mcpt1/2), complement factor 1 (Cfi), and leucine-rich α2 glycoprotein (Lrg) (210). This would allow me to determine if OligoG treatment targets both mucus accumulation and inflammation and if decreased inflammation prevents mucus accumulation. Overall, a more detailed analysis of how OligoG treatment affects the intestinal environment is required to fully understand how the drug is working to decrease the incidence of intestinal obstruction.
In addition to utilizing mouse models to further study the effects of OligoG, other models may be used to determine the mechanism of drug modification. Larger animal models of CF, such as pigs or ferrets, may be used to determine if OligoG treatment alters mucus accumulation and intestinal symptoms as seen in the CF mouse model. The drug could also be used to treat CF patients and determine if treatment in patients alleviates the GI complications of CF. OligoG is currently in clinical trial as an inhaled therapy; however a new study should address how the drug alters intestinal symptoms. It may be necessary to alter the drug dosage depending on the GI phenotype of the patient and patients with more severe intestinal complication may need to receive a higher dose of drug to target their symptoms. Patients would be given a survey to assess their GI complications before and after treatment. The survey should address issues such as number of bowel movements per day, consistency and color of BM, occurrence of constipation or increased flatulence, and overall wellness. In addition, stool samples should be collected and monitored for alterations before and after drug treatment. Samples should be monitored for changes in inflammation and GI microbiome. Biomarkers to test for changes in inflammation include: calprotectin (a marker of neutrophil-driven inflammation), eosinophil protein X (a marker of eosinophil-driven inflammation), and fecal secretory IgA (a marker of gut secretory immunity and barrier function) (303). To monitor if alterations in the GI microbiome occur one can test for decreased levels of metabolic indicators, such as short-chain fatty acids (marker of the health of the GI microbiome) and imbalances in gut commensal bacteria (marker for dysbiosis and general health) (303). Further analyses to determine how OligoG treatment
affects the GI tract are necessary to determine if OligoG may be a therapeutic treatment for the GI complications affecting CF patients.

Another future direction for this work is to determine if other drugs may be used to prevent CF intestinal obstruction. One potential drug candidate, Linzess (linaclotide), has recently been approved by the Food and Drug Administration and has been used to treat constipation in Irritable Bowel Syndrome and Chronic Constipation (304–309). Linzess has been shown to improve intestinal transit and abdominal symptoms in patients. Linzess works to increase cyclic guanosine monophosphate signaling, which activates protein kinase G (PKG) and protein kinase A (PKA) (310–312). Phosphorylation of PKG or PKA activates CFTR, which in turn increases chloride and bicarbonate secretions and leads to hydration of the intestinal lumen. Water present in the lumen softens the stool and allows for easier transit of contents through the intestinal tract. Our lab started preliminary analyses of Linzess at the end of my thesis work and I showed that drug treatment improved intestinal transit in CF mouse models (Figure 5.2.).

I analyzed GI motility rates in control mice and F508del and Null CF mouse models on regular water and with Linzess treatment. The drug treatment in control mice had no significant effect on motility rates (Control=6.1 and Control on Linzess=7.1). F508del and Null CF mouse models had significantly slowed GI motility as compared to control mice (F508del and Null=2.8, p=0.001). Linzess treatment improved the GI transit in both the F508del and Null CF mice (F508del on Linzess=5.7 and Null on Linzess=5.1). Further analyses are required to determine how the Linzess drug is improving GI motility and include analyzing mucus accumulation and its properties, as well as monitoring anion secretion.
Data in this thesis have shown that novel drug therapies may improve CF intestinal manifestations. Further work is necessary to fully understand how these drugs are altering the factors that lead to the development of CF intestinal obstruction to prevent its incidence.

5.5. Conclusion

In conclusion, this thesis provides novel insight into several of the factors that contribute to the development of CF intestinal obstruction. Chapter 2 analyzed the role of \textit{Cftr} in the smooth muscle and my data showed that absence of \textit{Cftr} in the intestinal epithelium and smooth muscle together are necessary to replicate CF-like intestinal obstruction rates. I hypothesize that absence of \textit{Cftr} in the intestinal epithelium leads to dehydration and alterations of prostaglandin production and that absence of \textit{Cftr} in the smooth muscle leads to abnormal calcium handling, which together contribute to the occurrence of intestinal obstruction. The mechanisms that control smooth muscle contraction and relaxation consist of a complex network of multiple components and future directions for this work consists of analyzing several aspects of this pathway. It is hypothesized that absence of \textit{Cftr} in the smooth muscle may alter calcium influx in the intestinal smooth muscle cell, calcium sequestering by the sarcoplasmic reticulum, or the parts that signal relaxation of the muscle. Additional analyses are required to further understand the mechanism for the development of intestinal obstruction. Chapter 3 analyzed a potential modifier gene, \textit{SLC26A9} that was identified via a genome wide association study analyzing MI in CF. \textit{SLC26A9} is an anion channel that functions as a chloride-bicarbonate exchanger, a chloride channel, and a sodium co-transporter. My data
showed that a reduction of one copy of Slc26a9 in R117H CF mice had a detrimental
effect on survival and led to an increase in intestinal obstruction rates. I determined that
reduction or complete absence of Slc26a9 had no effect on GI motility or anion transport
in non-CF mice. In the CF context, reduction of Slc26a9 led to no alterations in gastric
pH or GI motility as compared to CF mice with functional Slc26a9. My data also showed
that Slc26a9 mRNA expression is greater in newborns and expression levels decrease at
21 days and 6-8 weeks of age. In addition, my data show that the majority of mice with
complete absence of Slc26a9 do not survive more than two hours after birth and I
hypothesize this early death to be due to lung complications. Taken together my data
suggest that Slc26a9 may play an important role in development and future directions for
this work are aimed at determining how this gene may be altering early lung function.
Analyses of human epithelial cells show that risk alleles associated with SNPs in Slc26a9
shown to associate with MI in CF correlate with decreased SLC26A9 mRNA expression.
These data support my work analyzing Slc26a9 in mouse models and suggest that
SLC26A9 may prove to be a therapeutic target to alleviate patients of CF intestinal
symptoms. Chapter 4 analyzed a novel guluronate oligomer, OligoG, and tested how drug
treatment altered CF intestinal obstruction. My data found that OligoG treatment in CF
mice led to decreased mucus accumulation, increased intestinal transit, and decreased
intestinal obstruction. The drug has previously been shown to break up mucus, likely due
to its calcium chelation properties. OligoG has also been found to have antibacterial
properties, which may also contribute to the disruption of the mucus. Future directions
for this work include analyzing how OligoG treatment disrupts mucus accumulation and
if drug treatment also alters bacterial levels and composition in the gut and if it alters GI
inflammation. Additional future directions for this work include determining if other drugs may be used to target intestinal obstruction in CF. Our lab has begun testing one potential new drug, Linzess, and plan to continue analyses to determine how the drug is affecting the CF intestine. Overall, this thesis provides insight into the mechanism of development of CF intestinal obstruction, the effects of modifier genes on GI complications, and how novel drug therapies may be used to treat intestinal obstruction. In addition, this work provides the foundation for future studies that will further elucidate the role of \( C\text{ftr} \) in individual cell types, provide more detail on the occurrence of CF intestinal obstruction, and detail a mechanism for how novel drug therapies may be utilized to prevent CF intestinal manifestations.
Figure 5.1. Survival in CF mouse models with reduced or absent Slc26a9. A) Survival in severe intestinal CF mice, F508del, and B) mild intestinal CF mice, R117H, with wildtype, reduced, or absent levels of Slc26a9. A9/+ = reduction of one copy of Slc26a9. A9/A9 = complete absence of Slc26a9.
Table 5.1. Expected and actual genotype ratios in R117H mice with reduction of *Slc26a9*. Mice were analyzed at 18 days post coitus, 0 days, 2 days, and 4 days and their genotypes were determined. The expected and actual genotype ratios were calculated for each age group. The genotypes analyzed were *R117H+/--Slc26a9+/+, R117H+/--Slc26a9+/-*, *R117H-/-Slc26a9+/+*, and *R117H-/-Slc26a9+/-*. The actual genotypes were not significantly different than Mendelian ratios (18 days post coitus - *p* = 0.2632 and days total post-partum - *p* = 0.2723).

<table>
<thead>
<tr>
<th>Age Group</th>
<th># of Litters</th>
<th>Expected Genotype Ratio</th>
<th>Actual Genotype Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 Days Post Coitus</td>
<td>8</td>
<td>1:1:1:1</td>
<td>17:21:15:10</td>
</tr>
<tr>
<td>0 Days</td>
<td>4</td>
<td>1:1:1:1</td>
<td>4:12:6:4</td>
</tr>
<tr>
<td>2 Days</td>
<td>3</td>
<td>1:1:1:1</td>
<td>7:5:9:7</td>
</tr>
<tr>
<td>4 Days</td>
<td>8</td>
<td>1:1:1:1</td>
<td>12:9:11:4</td>
</tr>
</tbody>
</table>
Table 5.2 Slc26a9 KO mice die within 2 hours after cesarean section. Cesarean sections were performed and the survival of mice with wildtype Slc26a9, reduction of one copy of Slc26a9, or Slc26a9 KO was analyzed. The majority of the Slc26a9 KO died within two hours of cesarean section, while mice with wildtype Slc26a9 and a reduction of one copy of Slc26a9 survived. A9/+ = reduction of one copy of Slc26a9. A9/A9 = complete absence of Slc26a9.
Figure 5.2. Linzess treatment improves GI motility in F508del and null CF mouse models. Linzess treatment had no significant effect on control mice. F508del and null CF mouse models on regular water had significantly slowed GI motility as compared to controls. Linzess treatment improved GI motility levels in both the F508del and Null CF mouse models.
(Control=6.1, Control on Linzess=7.1, F508del and Null=2.8, F508del on Linzess=5.7, and Null on Linzess=5.1)


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