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Fetal Programming of Adult Metabolism in Rats

A Dissertation Presented

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Małgorzata Alicja Kokoszka

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Abstract of the Dissertation

Fetal Programming of Adult Metabolism in Rats

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The importance of the prenatal period for adult health is underscored by epidemiological correlations between prematurity and diseases such as coronary heart disease, stroke, and diabetes. To address questions in fetal development and the effect of fetal gene regulation on postnatal phenotype, adenoviral in utero gene therapy was used to transiently alter gene expression during late stages of gestation in the rat.

Several lines of evidence suggest that cystic fibrosis transmembrane conductance regulator (CFTR, the gene defective in cystic fibrosis) plays a role in proper development of the intestine. To analyze the temporal aspects of that requirement, CFTR expression in the intestine of rat fetuses was transiently suppressed using in utero gene therapy. Depending on the gestational age of the fetus, transient reduction of CFTR function resulted in phenotypes ranging from meconium ileus and subsequent death to obesity.

Of particular interest are animals treated during day 17 (E17) of embryonic development. At weaning, the intestines are morphologically normal and appear properly developed. However, older animals have dilated intestines with abnormal histology, suggesting

functional deficiency. Also, these animals display dysregulated production of intestinal hormones responsible for promoting satiety, resulting in increased food intake and excess body fat. Glucose and insulin tolerance tests and clamp studies showed significant peripheral tissue resistance to insulin leading to diabetes in aged animals.

This study provides a clear example of an adult onset disease originating from disruption of fetal development but undetectable in young animals. In addition, it demonstrates that weight gain and insulin resistance in adults can result solely from changes in gene expression during gestation.

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List of Abbreviations

2-DG	^{14}C-2-deoxyglucose
β-gal	β-galactosidase
AAV	Adeno-associated virus
Ad	Adenovirus
AS	Anti-Sense
BMI	Body mass index
CCK	Cholecystokinin
CDC	Centers for Disease Control and Prevention
CF	Cystic fibrosis
CFRD	Cystic fibrosis related diabetes
CFTR	Cystic fibrosis transmembrane conductance regulator
CHD	Coronary Heart Disease
E*xx*	Embryonic development, day *xx*
FABP	Fatty acid binding protein
GCRC	General Clinical Research Center
GFP	Green fluorescent protein
Glp-1	Glucagon-like peptide 1
IACUC	Institutional Animal Care and Use Committee
IP GTT	IP glucose tolerance test
IP	Intraperitoneal
ITT	Insulin tolerance test
microCT	Microcomputed tomography
NHANES	National Health and Nutrition Examination Survey
OGTT	Oral glucose tolerance test
P*xx*	Postnatal day *xx*
SP	Substance-P
T2DM	Type 2 diabetes mellitus
TIUKO	Transient in utero knock out
WAT	White adipose tissue
WHO	World Health Organization

CHAPTER 1

DEVELOPMENTAL REQUIREMENT FOR THE CFTR GENE

SUMMARY

Prematurity is associated with a number of health problems later in life. The role of fetal gene regulation in the development of adult-onset diseases has not been well-studied. In utero gene therapy allows one to manipulate the expression of developmentally important genes during gestation, and to study the effect of those changes on postnatal phenotype. Transient in utero suppression of CFTR expression at a specific time during rat fetal development results in overweight phenotype after birth. Body composition and fat distribution analyses in the overweight animals revealed an increase in adipose tissue in the abdominal region, putting the rats at risk of developing diabetes.

INTRODUCTION

A. Fetal origins of health and disease

Prematurity is a rising public health problem in the United States. The number of premature births has been steadily increasing and is now about 30% higher than just 30 years ago. In 2006, preterm births (<37 of 40 weeks) accounted for 12.8% of all live births in the U.S., with 2% of children born extremely preterm (<32 weeks) [1]. Fertility treatments, leading to a higher number of multiple births, are a known risk factor for preterm birth. Some experts are also proposing that the ever-increasing number of elective caesarian deliveries contributes to this trend, but the cause of most premature births remains unknown [2]. Luckily, the rise in the incidence of prematurity coincided with advances in neonatal care. Children as early as week 23 are now expected to survive, and some cases of survival at week 21 and 22 have been reported. Even though only about 20 years of good quality epidemiological data on the outcomes of prematurity are available, it is already clear that prematurity correlates with adult conditions such as hypertension, coronary heart disease (CHD), stroke, and type 2 diabetes mellitus (T2DM) [3-5].

The theory that adverse in utero environment (of which premature birth can be considered an extreme case) can result in serious health consequences was first proposed by David J. P. Barker [4]. The Barker Hypothesis states that prenatal stress triggers adaptive changes in the fetus that negatively impact adult health. Barker's work is mostly focused on fetal origins of coronary heart disease, especially as related to maternal malnutrition, and the resulting growth retardation of the fetus. Examples of fetal programming of postnatal metabolism also link adult disease to maternal health and nutritional status [6].

Recently introduced is the concept that changes in fetal gene expression in response to in utero environment may lead to incomplete maturation of cells in organogenesis [7]. The theory states that even organs that appear morphologically normal at birth may not be functionally mature - as a result of altered development. The subtle functional deficiencies remain undiagnosed until the patient develops clinical symptoms as an adult. This hypothesis, termed the Peter Pan paradigm (after the fairy-tale character who never matured), is generating increasing interest.

B. In utero gene therapy provides a tool to study fetal development of the lung and gut

To address questions in fetal organogenesis, a method to alter gene expression during late stages of gestation was developed. The technique – termed in utero gene therapy – involves injecting adenoviral (Ad) vectors carrying the gene of interest directly into the amniotic sac of the fetus to target developing organs.

A number of unique features make in utero gene therapy well-suited to study development. Adenoviruses are double-stranded DNA viruses that remain epichromosomal after gaining entry to the host nucleus. This ensures that any resulting phenotype is specific, consistent and reproducible, since non-integrating viruses do not disrupt other genes or activate oncogenes. The effect of transient changes in gene expression can be studied, since all Ad vectors used in gene therapy are replication-defective [8, 9]. Also, both dividing and non-dividing cells can be targeted, as long as they express the coxsackievirus adenovirus receptor (CAR) [10]. Undesirable immune reaction to the vector is a major limitation for Ad-based gene therapy in adults [11]. However, when the rat fetus between day E15 and

E18 (of 22) is treated with low concentrations of adenovirus, successful gene transfer occurs with no adverse immune response [12, 13]. Immunotolerant windows have also been defined in mice, pigs, and non-human primates [14, 15]. Extensive tissue analysis (by staining for the presence of virus-encoded reporter genes and/or PCR for the presence of vector) in all species revealed the epithelium of the developing lung and gut as the main target of this treatment (Table 1). This is consistent with the expression pattern of the CAR receptor which is a tight junction protein widely present in polarized epithelial cells [16]. In utero gene therapy was originally used to overexpress exogenous genes, but was later adapted to reduce the expression of cellular genes with Ad-encoded anti-sense (AS) gene fragments (this method is sometimes referred to as transient in utero knock out - TIUKO) [17].

In utero gene therapy has been successfully applied to study prenatal development in model organisms for over a decade. Studies conducted using this technique contributed to identifying CFTR as a developmentally important gene.

C. CFTR is required for normal development of the intestine

CFTR has been shown to encode a cAMP-dependent chloride channel expressed by the apical surface of epithelial cells. This is consistent with disrupted ion transport in epithelial-lined organs seen in patients with CF. Even though it is now recognized that all cystic fibrosis pathology cannot be explained by ionic imbalance, and abnormal transepithelial Cl^- movement alone is unlikely to cause CF lung disease, the traditional view held by clinicians has been that all CF symptoms could be mitigated by restoring normal CFTR channel function at any point during the life of the patient. Interestingly, a body of evidence suggests that in addition to its contribution to proper ion balance in epithelia of

mature organs, CFTR is also developmentally required. Altered organogenesis in the absence of CFTR is an attractive hypothesis. The role of CFTR in lung development has received some attention, but no rigorous studies have been performed to assess CFTR's role the intestine, even though there is evidence suggesting that it is required for normal intestinal

The cystic fibrosis transmembrane conductance regulator (CFTR) gene has been studied extensively because its mutations are the cause of cystic fibrosis (CF) [18]. CF is a multiorgan disease whose main symptoms include airway obstruction and recurrent pulmonary infections, pancreatic insufficiency, and intestinal abnormalities. It is the most common genetic disorder in Caucasians, affecting 1 in 3,000 live births in the United States. There are currently 30,000 Americans living with the disease, and 1 in 27 white people carry a mutation in the CFTR gene. Although advances in medical care have extended the average life expectancy of CF patients to 37 years (from ~3 years in the 1950's), there is no cure for the disease at this time [19].

CFTR has been shown to encode a cAMP-dependent chloride channel expressed by the apical surface of epithelial cells [20]. This is consistent with the disrupted ion transport in epithelial-lined organs seen in patients with CF. Even though it is now recognized that all cystic fibrosis pathology cannot be explained by ionic imbalance, and abnormal transepithelial Cl^- movement alone is unlikely to cause CF lung disease, the traditional view held by clinicians has been that all CF symptoms could be mitigated by restoring normal CFTR channel function at any point during the life of the patient. Interestingly, a body of evidence suggests that in addition to its contribution to proper ion balance in epithelia of mature organs, CFTR is also developmentally required. Altered organogenesis in the absence of CFTR is an attractive hypothesis. The role of CFTR in lung development has

received some attention, but no rigorous studies have been performed to assess CFTR's role in the intestine, even though there is evidence suggesting that it is required for normal intestinal organogenesis.

Intestinal abnormalities are a known symptom in infants with cystic fibrosis. Children born with meconium ileus (a blockage of the small intestine indicative of prenatal malfunction) are almost invariably diagnosed with CF.

Similarly, the cystic fibrosis mouse bearing the CFTR S489X mutation suffers from intestinal obstruction and perforation, resulting in lethality around the time of weaning (3 weeks of age) [21]. In mice, neonatal lethality occurs specifically due to the lack of CFTR in the intestine, because it can be rescued by providing human CFTR as a transgene under the control of rat intestinal fatty acid binding protein (FABP) promoter [22, 23]. The mouse is born with healthy intestines (without meconium), consistent with FABP expression in the intestinal epithelium during gestation (the gene then stays on throughout the life of the animal).

Another experiment that strongly points to the requirement for CFTR during development is the rescue of CF mice by in utero gene therapy [15]. Transiently providing the gene prenatally improves survival of the CF S489X mouse beyond weaning, again strengthening the case for CFTR's role in intestinal organogenesis. It should be noted that the success rates of the therapy vary with genetic background and experimental procedure. Another group working with a different mouse strain and applying a modified protocol (e.g. Caesarian section instead of vaginal delivery and higher concentrations of virus among other modifications), achieved a lesser improvement in survival rates when attempting to rescue the CF mouse by gene therapy [24]. The altered experimental conditions were associated

with an increase in overall mortality rates (even in the control group). That combined with a small sample size resulted in an improvement in survival that was not statistically significant.

Taken together, these observations establish the role for CFTR in the maturation of fetal intestinal epithelium and pose the question of the precise timing of this requirement - and of the consequences of CFTR's absence during specific stages of gestation for adult phenotype. A study was designed to assess the temporal aspect of CFTR's role by transiently reducing its expression (by means of in utero gene therapy) at various stages of development.

MATERIALS AND METHODS

Animals. Time-pregnant Sprague-Dawley female rats were purchased from Charles River Laboratories. Upon arrival, they were housed in individual cages and handled according to the guidelines of the Institutional Animal Care and Use Committee (IACUC). Females were allowed at least 48hrs to recover from transport before surgery. In weight gain and body composition analyses, female rats were removed from the study at one week of age. Litter sizes were equalized to 4 male pups per nursing female. At weaning, the males were individually housed and their weight increase was followed into adulthood.

In Utero Gene Therapy. Pregnant rat females with litters at E15, E16 and E17 were used (counting the day following mating as E1). In utero gene therapy method to alter gene expression in the fetus was previously optimized [12]. Briefly, laparotomy was performed using sterile surgical technique under Isoflurane anesthesia (5% induction, 2% maintenance). The uterine horns were exposed one at a time and the individual amniotic sacs were injected with virus at 10^9 pfu/mL in estimated 10% of amniotic fluid volume (~0.05mL) of DMEM. The uterine incision was closed and the females were allowed to deliver naturally.

Recombinant adenoviruses. Adenovirus encoding green fluorescent protein (AdCMVgfp) was provided by Dr. J. Kolls (LSHHSC, New Orleans, LA). A 920 bp fragment of human CFTR cDNA (exons 1–6, 88% identity to rat) was used to generate AdCMVAScftr (ATCC 61123) [17, 18]. 293 packaging cells were used to produce the virus [25]. All viruses were CsCl or HPLC purified.

Microcomputed Tomography. Animals from each experimental treatment group were randomly selected for analysis. The rats were anesthetized with Isoflurane (1.5%) and scanned in VivaCT 75 scanner (SCANCO Medical, Switzerland). The abdominal region, from the sacrum through the vertebrae immediately below the ribs, was designated as the volume of interest. The highest available current (133μA) and the lowest voltage setting (45KV) were chosen to optimize fat contrast. Resolution of 156μm was found to be sufficient to delineate muscle and fat tissues. Image analysis was performed by the General Clinical Research Center at Stony Brook University. Adipose tissue volume was quantified based on voxel densities as described previously [26, 27].

Statistics. Results are presented as mean ±SEM. Significance levels were calculated using t-test or 2-way ANOVA, as appropriate. All computations were performed with GraphPad Prism software. $p<0.05$ was considered significant.

RESULTS

A. Reduction of CFTR levels in the developing fetus results in a range of phenotypes.

Adenovirus carrying a fragment of the CFTR gene in the anti-sense (AS) direction was used to transiently reduce CFTR expression in utero. Control animals received virus

encoding GFP. Following CFTR suppression, the resulting postnatal phenotype was carefully analyzed with special attention given to potential intestinal abnormalities. Several phenotypes were found.

Animals treated the earliest (E15) displayed the most severe symptoms: abnormal intestinal morphology, a few cases of meconium ileus, and lethality around the time of weaning (Fig. 1). This very intriguing phenotype is now the subject of a separate study.

CFTR suppression at E16 and E17 yielded animals that were viable (Fig. 1), with no intestinal blockage and no obvious structural abnormalities in the intestine. Strikingly, E17 animals consistently showed a significant weight increase over controls, suggesting metabolic imbalance and providing the rationale to focus the study on that group.

B. Suppression of CFTR at E17 results in weight gain and abdominal adiposity

It is standard practice to perform all metabolic studies on male animals only, since hormonal shifts of the female estrous cycle make such experiments difficult to control. For that reason, even though at weaning both males and females displayed the same trend in weight difference, females were excluded from the study.

In males, a 10-15% difference in body mass was found that persisted throughout the life of the animal (Fig. 2). This phenotype was subsequently reproduced in three independent gene therapy experiments.

The weight difference between the two groups prompted microcomputed tomography (microCT) analysis to determine the exact body composition of the animals, particularly of the abdominal region, since abdominal fat is a known risk factor for developing diabetes and other obesity related diseases. Adipose tissue volume in the abdominal area (from the

sacrum to the ribs) was significantly higher in AS rats when normalized to total volume and compared to age-matched controls (Table 2). Notably, in younger animals a comparable difference was seen in both visceral and subcutaneous fat volume, but in the aged population there was a larger increase in visceral fat in AS CFTR rats (Fig. 3).

According to the guidelines of the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO), clinical obesity is defined as a body mass index (BMI=weight/height^2) of 30kg/m^2 or more, and patients with BMI=25-30 are diagnosed as overweight. No equivalent scale has been developed for rats. However, the 10-15% increase in body weight in AS rats compared to controls corresponds to clinical overweight in human patients (the BMI difference between normal weight and obesity is associated with a 20% change in body mass). In addition, increased visceral adiposity is a risk factor for insulin resistance and diabetes in both humans and rats [28, 29].

DISCUSSION

Neonatal manifestations of CF, as well as the results of various scientific studies, indicate the developmental requirement for the CFTR gene. This study demonstrated that when CFTR's levels are reduced during gestation, fetal development is permanently altered resulting in a postnatal phenotype, even when CFTR expression is restored after birth.

CFTR's levels were reduced using a vector carrying a fragment of the gene in the anti-sense direction. The specificity and effectiveness of this method has been demonstrated in the pulmonary epithelium [17]. Attempts to detect the expression of CFTR in the intestine have been unsuccessful, using a variety of commercially available antibodies. Although

there have been reports of CFTR expression in the intestine demonstrated by in situ hybridization, CFTR can most reliably be detected functionally by measuring the potential difference across the intestinal epithelium ex-vivo [15]. The method cannot be applied in a living animal (especially not in the fetus), and it requires expensive equipment and considerable expertise in electrophysiology. For that reason, the effectiveness of the AS fragment for suppression of CFTR levels in the developing intestine was assumed based on the results in the fetal lung, and was not measured directly.

The observed phenotypes following suppression of CFTR are specific to the target gene and not related to a possible cellular reaction to AS RNA, because when an AS fragment was used to suppress the expression of c-myc in the developing lung and gut, a different set of phenotypes resulted [30].

The fact that the postnatal phenotype was dependent on the gestational age of the fetus at the time of gene therapy demonstrates that CFTR is continuously required for multiple developmental processes between E15 and E17. The phenotypes are distinct and at least partially non-overlapping, consistent with the transient nature of Ad-mediated gene transfer.

In utero gene therapy targets both the lung and gut of the developing fetus, so technically changes in gene expression in either organ could be responsible for the significant weight gain observed in the E17 AS population. No known link exists between altered lung morphogenesis and metabolism, other than the effects of lower physical activity resulting from decreased lung function. No changes in the level of physical activity between the AS and control population were detected. Also, the lungs of the AS animals were healthy enough to support weight gain. The intestine, on the other hand, is an endocrine organ with a

known role in the regulation of energy balance. Therefore, the overweight phenotype after birth was attributed to the changes resulting from reduction of CFTR levels in the intestine and not in the lung.

These initial observations of the effect of reduction in CFTR levels in the intestine at E17 reveal that adult metabolism can be influences by events of fetal life. In addition, they define the organ and the developmental stage when the programming occurs. In an attempt to understand the consequences of altered intestinal development, an inquiry was initiated into the causes of weight gain and the extent of changes in metabolic profile of the AS animals.

CHAPTER 2

METABOLIC IMBALANCE IN AS CFTR RATS

SUMMARY

Significant postnatal weight gain and increased intra-abdominal fat deposits resulted from transient reduction of CFTR levels in the intestine of the rat fetus, putting the animals at risk of developing diabetes. Therefore, the metabolic profiles of the overweight and control rats were examined. Marked peripheral tissue insulin resistance in younger animals developed into clinical diabetes in aged rats. This observation provides a paradigm for fetal origins of adult weight gain and diabetes that cannot be attributed to poor maternal health or nutrition, but rather to changes in gene expression in a specific fetal organ.

INTRODUCTION

A. **Obesity epidemic**

Metabolic disorders that result in weight gain are of considerable scientific and social interest. Over 1 billion people in the world are overweight, equal to the number of those suffering from hunger [31]. Obesity is now considered an epidemic by the World Health Organization [32]. In the United States, the health and nutritional status of the population is most accurately assessed by National Health and Nutrition Examination Survey (NHANES) which combines detailed interviews with patients and data collected by physicians during office visits. According to the survey, 32% of adults in the US are obese, and an additional 35% are overweight [33]. Overweight is more common among men, but more women are obese. Both conditions are more common among minorities and there is an inverse correlation with education level and socioeconomic status. Nevertheless, since 1970 when reliable data collection was initiated by NHANES, the incidence of overweight and obesity has steadily increased in both genders, all age groups, and individuals of all racial and educational backgrounds. Alarmingly, in that time the prevalence of obesity has more than doubled - from 13.3 to 32.1% - and the rates are still increasing.

B. Possible causes of obesity

Overweight results from a chronic imbalance between caloric intake and energy expenditure. Epidemiological correlations with geography, education, and socioeconomic status suggest that high-fat diets, sedentary lifestyle, and inadequate adjustment of calorie intake to decreased demand for physical activity in modern society lie at the root of the

obesity epidemic in the developed world [34]. However, within each risk group, extreme cases of underweight and overweight can be found, implying the existence of other predisposing factors.

Much scientific effort has been spent trying to identify genetic mutations that may cause energy imbalance, with the aim of developing better prevention strategies and effective pharmacologic treatments. Spontaneous and engineered mutations resulting in obesity and other forms of energy imbalance in mice have greatly contributed to the understanding of the molecular mechanisms of intrinsic regulation of body metabolism, but are only marginally relevant to the etiology of obesity in humans [35]. For example, the cloning of the mutated locus of the famous severely obese *ob/ob* mouse lead to the discovery of leptin, a hormone released by adipocytes which plays a major role in the inhibition of food intake [36]. Only two families in the United States have been diagnosed with a mutation in the leptin gene. One additional family carries a mutation in the leptin receptor [37-39]. It is clear that leptin deficiency and other genetic syndromes causing obesity in humans are very rare. Instead, genetic susceptibility to obesity is generally interpreted as the presence of modifier genes that cause more minor shifts in energy homeostasis which, in combination with demographic and socioeconomic factors, can lead to excessive weight gain in genetically susceptible individuals [40].

C. **Health consequences of obesity**

Ideal weight is that weight allowing one to achieve maximum life expectancy. In fact, healthy weight was defined based on total mortality rates [32]. Overweight, then, by

definition, increases the risk of premature death. In the United States, obesity-related diseases are associated with 112,000 preventable deaths per year [40].

It is generally assumed that chronic conditions most commonly associated with obesity are those resulting from the cardiovascular burden of overweight. Indeed, the incidence of hypertension and coronary heart disease correlates closely with increasing BMI in both genders [41]. However, the most striking epidemiological correlation exists between excess body fat and the risk of developing type 2 diabetes mellitus. The relative risk of CHD and hypertension rises sharply in overweight individuals (BMI>25), but shows only a modest increase across the normal weight range. The risk of T2DM, on the other hand, goes up 5-fold between BMI 21 and 25. More people are overweight than severely obese, so health problems that correlate strongly with a wider range of BMI values significantly contribute to morbidity and mortality. In the case of T2DM, the risk of developing the condition increases over a BMI interval that is otherwise considered healthy weight, making diabetes a potential concern for individuals with very little excess body fat.

Rats treated with AS CFTR at day 17 of gestation gain weight after birth and are therefore at risk of developing obesity-related diseases including diabetes. Visceral fat deposits observed in the older population further increase the relative risk of T2DM [28, 29]. A series of experiments was performed to determine whether the phenotype of these rats resembled the weight gain and progression of T2DM in humans.

MATERIALS AND METHODS

Animals. Experimental animals were generated essentially as in Chapter 1. AdCMVascftr

was used to transiently suppress CFTR expression in utero in the rat fetus at E17. Control animals received the reporter gene AdCMVegfp. Again, females were removed, litters were equalized and only males were used in all metabolic studies.

Calorimetry. Indirect calorimetry was performed using the Oxymax 8-cage system combined with measurements of physical activity by beam breaking (Columbus Instruments). Animals were allowed to acclimatize to the chambers for 16hrs, and measurements were taken for 48hrs during the light cycle (fasting period) and the dark cycle (feeding period). Data were analyzed as the average of 12hr measurements for each rat during either the light or dark cycle. All analysis was performed by the GCRC at Stony Brook University.

Food Intake. Three-month-old rats were housed individually and ad libitum food intake was measured for 15 days.

Glucose and Insulin Tolerance Tests. Animals were fasted either overnight or for 6hrs, as noted for each experiment. Intraperitoneal (IP) injections of 50% dextrose solution or human recombinant insulin (Novolin, short-acting) in isotonic saline were performed with a hypodermic needle. Oral gavage needle was used to deliver dextrose solution to the stomach of conscious animals. Blood was collected from the tail and glucose levels were measured with a glucometer (Precision Xtra, MediSense) at indicated time points.

Plasma Insulin. Tail vein blood was collected into heparin-coated tubes. Plasma was immediately prepared by spinning the samples in a refrigerated microcentrifuge at 3,500rpm for 5min., and stored at -20°C. Endogenous insulin levels were assayed with an ELISA kit (CrystalChem) according to manufacturer's instructions.

Hyperinsulinemic-Euglycemic Clamp. Indwelling catheters were surgically inserted into the carotid artery and the right jugular vein of rats under standard ketamine/xylazine anesthesia. The animals were allowed 2-3 days to recover from the surgery and were fasted overnight prior to clamp analysis. All clamp infusions into the jugular vein were performed using microdialysis pumps (Harvard Apparatus and CMA Microdialysis). [3-^{3}H]-glucose was continuously infused for 2 hr prior to the start of the clamp. A 2 hr euglycemic clamp was then conducted with primed continuous infusion of human insulin (2.5 mU/kg/min.). A 20% glucose solution was infused at variable rates to maintain glucose at basal concentration (110 mg/dl). Continuous infusion of [3-^{3}H]-glucose (0.1 mCi/minute, Perkin Elmer Life and Analytical Sciences) was given throughout the clamp. Arterial blood samples were collected every 10min. for blood glucose measurements. Forty-five min. before the end of the clamp, 2-deoxy-D-[1-^{14}C]-glucose was administrated as a bolus (10 mCi). Blood samples were taken at 5–10 min intervals from the artery until the end of the clamp to measure plasma glucose, plasma [3-^{3}H]-glucose, $^{3}H_2O$, 2-deoxy-D-[1-^{14}C]-glucose, and insulin. At the end of the clamp, animals were euthanized with sodium pentobarbital overdose, tissues were dissected, immediately flash-frozen in liquid nitrogen, and stored at -80°C until analysis. Tissues were homogenized and passed through an ion-exchange column (BioRad) before radioisotope counting.

RESULTS

A. Hyperphagia in AS animals.

Weight gain could result from higher food intake, lower level of physical activity, Weight gain can result from higher food intake, lower level of physical activity, altered

capacity to oxidize fat or carbohydrates as a source of energy, or a combination of these factors. Average daily food consumption was found to be higher in the AS rats (Fig. 4). Indirect calorimetry measurements were performed to assess the contribution of factors other than increased caloric intake to the overweight phenotype. Physical activity was recorded with an infrared beam monitoring device. Oxygen consumption (VO2) was calculated in both fed and fasted states based on gaseous composition of air exhaled by the rats. No significant difference in any of the parameters was seen between the two groups (n=4 AS CFTR, n=4 control), suggesting that altered feeding behavior alone was the cause of weight gain in the AS group (Fig. 5 and data not shown).

Constant excessive caloric consumption throughout the life of an animal (patient) invariably leads to weight gain and metabolic imbalance, and in some cases, type 2 diabetes mellitus. In order to check if the AS rats were developing diabetes, basic metabolic parameters of the overweight population were examined.

B. Normal glucose tolerance

The simplest clinical test that is recommended for patients with a suspected metabolic condition is the glucose tolerance test. Blood sugar levels at fasting and 2hr (post administration of glucose) time points are especially important. Elevated blood glucose at those times is diagnostic of the pre-diabetic state or diabetes (Table 3).

Animals at ages ranging from 90 days to 5 months were subjected to oral and IP glucose tolerance tests (OGTT and IP GTT). While the fasting blood sugar was slightly elevated in the AS population, the overall glucose tolerance was not impaired (Fig. 6).

Numerous clinical studies show that insulin resistance can precede measurable glucose intolerance in the development of T2DM. In fact, chronic hyperinsulinemia and the resulting insulin insensitivity are typical conditions leading up to clinical diabetes.

C. Hyperinsulinemia and peripheral insulin resistance

Endogenous levels of plasma insulin in AS and control rats during an IP GTT were assayed (Fig. 7). As predicted, insulin levels were elevated in the AS population, implying decreased peripheral sensitivity to insulin (animals in both groups were challenged with the same dose of glucose, and the efficiency of glucose uptake by tissues was comparable, as reflected by similar blood glucose levels).

To verify peripheral tissue resistance to insulin in AS rats, an IP insulin tolerance test (ITT) was performed. Consistent with the previous observations, insulin response, i.e. the ability of peripheral tissues to uptake glucose lowering its levels in the blood, was markedly decreased in AS animals when compared to age-matched controls (Fig. 8).

Changes in levels of glucose and insulin in the blood are interrelated. The existence of a two-way feedback loop ensures that insulin (pancreatic output) rises in response to high blood glucose, and tissue glucose uptake increases (blood sugar drops) in response to insulin. These processes occur simultaneously, so insulin levels are constantly changing. For that reason, it is not possible to accurately measure peripheral tissue response with a simple ITT, since pancreatic insulin production (and therefore total dose of insulin) during the experiment cannot be controlled. To get around that problem, a technique that allows one to eliminate the contribution of pancreas was developed and has been the gold standard for quantifying tissue sensitivity to insulin for 30 years [42]. Primed-continuous infusion of high (5-6X

endogenous) level of insulin shuts down pancreatic production for the duration of the experiment. To avoid severe hypoglycemia, a variable glucose infusion is given, as needed to maintain blood glucose at basal level. Because glucose is 'clamped' at a constant level and insulin is raised acutely, the technique is termed 'hyperinsulinemic-euglycemic clamp.' Under the steady-state conditions of euglycemia, the rate of glucose infusion becomes a measure of peripheral tissue response to a defined dose of insulin (tissue uptake is balanced by regulated glucose infusion).

AS CFTR rats and age-matched controls were subjected to hyperinsulinemic-euglycemic clamp. The glucose infusion rate (measured by the settings on the infusion pump that are manually adjusted to maintain euglycemia) was reduced by half in the AS animals, reflecting the decreased ability of peripheral tissues to respond to insulin that was first indicated by ITT (Fig. 9A).

To more accurately calculate whole body glucose metabolism, trace amounts of [3-^{3}H]-glucose were included in the infusion [43]. Upon entry into metabolic pathways, [3-^{3}H]-glucose loses its tritium atom completely to water. Therefore, appearance of $^{3}H_2O$ in plasma indicates the rate of whole-body glycolysis. In concert with all earlier findings, insulin-stimulated whole body glucose turnover was decreased in AS rats, again indicating lower sensitivity to insulin (Fig. 9B).

The response to insulin in specific target tissues can be measured with ^{14}C-2-deoxyglucose (2-DG), a non-metabolizable analog of glucose that can be taken up by tissues but does not enter any metabolic pathways [44]. When 2-DG is injected as a bolus towards the end of the clamp, ^{14}C content in tissues harvested immediately after the experiment is a direct index of glucose transport, i.e. insulin action in specific tissues. White adipose tissue

(WAT) and skeletal muscle (gastrocnemius and soleus) were analyzed. Similar level of radioactivity in WAT of both control and AS groups indicated normal response to insulin (Fig. 10). The soleus – red, slow twitch oxidative muscle (made up of Type I fibers), the most sensitive to insulin – also showed no defect in glucose uptake (Fig. 11A). However, the gastrocnemius – mixed Type I and Type II muscle, made up of both red and white (fast twitch glycolytic) fibers – showed reduced insulin sensitivity (Fig. 11B). This is analogous to what is observed in (pre)diabetic patients where Type II muscles can become insulin resistant while Type I fibers retain their ability to respond to insulin normally.

In addition to skeletal muscle and WAT, liver is another major target of insulin action. During periods of fasting, the liver releases glucose into the bloodstream, preventing hypoglycemia. In the fed state, insulin suppresses glucose production by the liver. Hepatic glucose output prior to clamp (basal fasting level) as well as the degree of its suppression by insulin during clamp were determined by subtracting the steady-state glucose infusion rate from the total blood glucose (that also has a liver-derived component, Fig. 12A). Liver glucose production was found to be properly suppressed by insulin in the AS CFTR animals (Fig. 12B). High fasting blood glucose in the absence of overall glucose intolerance can also indicate impaired liver function. Fasting blood sugar levels in AS rats were only slightly higher than control in all GTT and ITT experiments, indicating normal liver function consistent with the results of the clamp. Collectively, these studies identified Type II skeletal muscle as the major tissue responsible for insulin resistance in AS animals.

D. Diabetes in aged animals

Because it is typical for untreated metabolic conditions to worsen over time, IP GTT was repeated in aged rats. At 15 months, the AS rats' fasting blood sugar was significantly

higher than normal and their ability to clear blood glucose post challenge was profoundly reduced. A high degree of variability was observed in the AS population, with one rat so severely diabetic its fasting blood sugar was three times higher than control and its blood glucose during IP GTT was outside the range of the glucometer (Fig. 13). Even when that animal was excluded from the analysis, the AS population appeared diabetic based on current guidelines for diabetes diagnosis (Table 3).

DISCUSSION

Type 2 diabetes mellitus (formerly known as non-insulin dependent) is an adult-onset progressive imbalance between insulin demand and insulin production, resulting in high blood sugar. According to the CDC, T2DM accounts for 90 to 95 newly diagnosed cases of diabetes in the United States. An estimated 23.6 million Americans are living with the disease, including 5.7 million undiagnosed cases. With 233,000 deaths per year, diminished quality of life, and a direct annual cost of $116 billion, T2DM present a major economic and health care challenge.

Type 2 diabetes is an acquired progressive disorder that usually starts with frequently elevated blood sugar levels, typically resulting from increased food intake. The increased demand to clear blood glucose leads to increased insulin production and abnormally high levels of insulin in the blood. The body eventually adapts to the chronically elevated insulin levels, and peripheral tissues do not respond to the hormone as robustly. The progressive resistance to insulin that develops as a result of hyperinsulinemia is a hallmark of T2DM. Pancreatic insulin production increases together with decreasing tissue sensitivity, until high

degree of tissue resistance, sometimes accompanied by loss of β cell function, leads to relative insulin deficiency and dysregulated blood glucose (Table 3).

Preexisting liver conditions can contribute to the progress of T2DM, since liver is responsible for maintaining blood glucose at the basal level during periods of fasting. However, overweight is the single most important risk factor for the development of T2DM [41]. The incidence of diabetes also correlates with adverse conditions during fetal development, including maternal obesity or diabetes and low birth weight.

Rats in which the expression of CFTR was transiently reduced at E17 develop a metabolic phenotype as adults. The progression of the phenotype closely parallels the development of T2DM in humans. In younger animals, resistance to insulin develops in peripheral tissues, but blood glucose is properly controlled due to upregulated insulin production. Aged AS rats develop clinical diabetes, as is seen in human patients who do not seek treatment for impaired glucose tolerance.

The results of this study establish a novel principle in fetal programming of adult metabolism. No differences in maternal or environmental factors exist between the AS and control groups. The adult phenotype results solely from changes in gene expression in the developing fetus. Understanding of the nature of these changes may provide a clue to what developmental processes must take place for full functional maturation of fetal organs, ensuring adult health.

CHAPTER 3

CHANGES IN THE INTESTINE FOLLOWING DEVELOPMENTAL INSULT

SUMMARY

Transient reduction of CFTR expression in the intestine of the rat fetus resulted in hyperphagia and weight gain that lead to insulin resistance and diabetes in older rats. In this study, production of substances involved in regulation of satiety was examined and was found to be dysregulated, providing an explanation for the increased appetite. Microarray analysis performed following CFTR suppression showed altered gene expression in the intestine both before and after birth, suggesting that development of the intestinal epithelium had diverged from normal. Even though no obvious morphological changes were noticed in the intestines of younger animals, aged rats had distended bowels, suggesting functional deficiency.

INTRODUCTION

A. The intestine as an endocrine organ

The endocrine function of the intestine was already recognized over 100 years ago. In fact, secretin - produced by the cells of the of the upper small intestine - was the first substance to be called a hormone, a term created to describe its effect on the release of bicarbonate by the exocrine pancreas [45]. Around the same time, the existence of gut secretions that could act to stimulate the endocrine pancreas [46] and even potentially aid the treatment of diabetes mellitus [47] was suggested. In the 1930's, the term 'incretin' was coined to describe the blood glucose-lowering effect of gastrointestinal-derived factors [48]. Cholecystokinin (CCK) was the first intestinal hormone that was demonstrated to be involved in the control of satiety [49]. CCK is also widely expressed in the CNS where it functions as a neurotransmitter [50]. It is not unusual for peptides produced by the gut to have a distinct function in the nervous system. For example, substance P (SP) is involved in pain sensation, but in the intestine it increases contractility of smooth muscle cells, aiding peristalsis. Even though the term hormone was originally created to distinguish secretin from signals released by the nervous system, now hormones and neuropeptides produced by either epithelial cells or by nerve endings in the gut are considered in one category and are collectively referred to as 'neuroendocrine' substances. Over 20 intestinal hormones and neuropeptides are now known. All aspects of gut function: gastrointestinal motility, release of digestive substances, enhancing of the production of insulin, and control of appetite – are subject to neuroendocrine regulation.

B. Diversity of intestinal cell types

The epithelial lining of the intestine (Fig. 14) is composed mostly of enterocytes – columnar cells with apical microvilli located in the upper portion of the villus. Enterocytes' morphology greatly increases the absorptive surface and their hydrolytic capability the digestive process by degrading nutrients. The mucus-secreting goblet cells are scattered throughout the villus from the crypt to the tip, and represent about 5% of the intestinal epithelial cells. The Paneth cells are columnal cells with cytoplasmic granules that function in antimicrobial defense. They are located at the very bottom of the crypt and are present in smaller numbers, about 10 per crypt. Finally, intestinal endocrine cells make up about 1% of the epithelium. Remarkably, the hormone-releasing cells of the intestine comprise the largest and most complex endocrine organ in the body [51].

All intestinal epithelial lining is composed of the same four basic cell types, but its morphology changes along the length of the intestine. Also, like any epithelial layer, the intestinal lining undergoes constant renewal, so the distribution of different cell types is constantly reestablished, even within a given region (defined by the distance from the stomach). Stem cells located in the crypts give rise to undifferentiated progenitors which then move up the villus as they complete the maturation process. The turnover of the epithelial cells is about three days, except for the Paneth cells which have a lifespan of about 20 days. Cells at all differentiation stages are present at any given time, contributing to the complexity of the epithelium

The most diverse of the mature epithelial cells are the hormone-releasing cells. Different populations of endocrine cells are not distinguishable histologically, but they are defined as separate based on the expression of a marker, e.g. L cells express GLP-1, GIP is

made by K cells, etc. Enteroendocrine cells often express more than one marker at a time, so more and more cell types are named all the time [51, 52], resembling the situation in the immune system where the discovery of one new protein can lead to the identification of several lymphocyte subpopulations. As a consequence, the exact pattern of expression of molecular markers – and thus the distribution of different cell types - is less well-defined than in other endocrine organs.

MATERIALS AND METHODS

Animals. CFTR levels in the intestine of E17 rats were reduced as in Chapter 1and 2. For fetal and early postnatal tissue analysis, both male and female animals were used. At 1 week of age, female rats were excluded from the study as described in Chapter 1. For neuroendocrine studies, all rats were fasted overnight, and half of the animals were gavaged with 5mL of baby formula in 50% dextrose solution – to simulate fed state.

Immunofluorescence. The initial segment of the proximal intestine (1-2cm) was dissected and fixed in 4% paraformaldehyde for 24hrs at room temp. The samples were washed 3X with PBS and stored in 30% sucrose/PBS solution at 4°C. Fixed tissues were embedded in OCT and 8μm frozen sections were prepared. All 1° antibodies were from SantaCruz Biotechnology. Fluorescent 2° antibodies AlexaFluor-488, -568 and -647, DAPI, and phalloidin-488 were purchased from Invitrogen.

RNA isolation. Qiagen RNeasy kit was used to prepare RNA from the initial 1cm of proximal intestine of E17 AS CFTR and control rats at E21 and P3.

Microarray Analysis. Analysis was performed by the genomics facility at Stony Brook University using Affymetrics Rat Genome 230 2.0 chips.

Bioinformatics. Microarray results were analyzed using GeneSifter software. The significance threshold was set very high ($p<0.001$) to reduce false discovery rate.

RESULTS

A. Dysregulated appetite in AS CFTR rats

Production of several intestinal hormones and neuropeptides that normally contribute to the regulation of appetite was examined in overweight rats following Ad-based suppression of CFTR levels in fetal intestine at E17. The initial segment of the proximal intestine (duodenum) was chosen for this analysis. Enteroendocrine cells are distributed throughout the small and large intestine, but the duodenum has by far the highest density of hormone-releasing cells [51]. Moreover, expression of the CAR receptor which mediates adenoviral entry into the cell decreases along the length of the intestine [53]. Also, clinical outcomes of bariatric surgery show that excluding the proximal intestine in addition to the stomach results in higher weight loss than bypassing the stomach alone [54], stressing the contribution of the duodenum to the control of energy balance.

CCK is the prototypical satiety hormone produced by the proximal intestine that acts to inhibit food intake after a meal [49, 55]. Pre- and postprandial levels of CCK were examined in the duodenum of AS and control rats (Fig. 15). Proper induction of CCK was observed in the control population. However, the expression of CCK was elevated in fasted AS rats and did not change in response to food. Instead, it remained at a level intermediate

between pre- and post-meal in the controls. Constant intermediate levels of CCK, independent of food intake, suggest that that level of CCK-regulated control of satiety is missing from the overweight population.

A similar lack of food-stimulated induction was observed for substance P (SP, Fig. 16). Normally, SP is released in response to food by both nerve endings and epithelial cells of the small intestine [56]. It causes smooth muscle contractions to promote gut peristalsis – and in at that manner indirectly contributes to the control of satiety. Again, the levels of SP were elevated in fasted AS animals, and did not change in response to food intake.

Among the intestinal hormones that can enhance glucose-induced release of insulin (incretins), glucagon-like peptide 1 (Glp-1) is the most potent. Disappointingly, analysis of pre- and postprandial levels of Glp-1 in AS and control rats gave inconclusive results (data not shown). The expression of DPP IV, the protease that breaks down Glp-1, was found to be normal (data not shown).

B. Analysis of marker gene expression following suppression of CFTR

The mechanisms by which events following CFTR suppression in the fetal intestine can be 'remembered' and passed on to produce a shift in the metabolic profile of adult animals are unknown. To check whether the process was accompanied by a transcriptional response, we performed gene expression microarray analysis in the intestine of the fetus and newborn rat. E21 was chosen to collect fetal tissue and postnatal day 3 (P3) was used for newborns. As in the analysis of production of neuroendocrine substances regulating satiety, only the initial segment of the duodenum was used. Arrays were performed in triplicate, with each sample consisting of intestines pooled from 3 animals, so a total of 9 rats were

analyzed at each condition. The results were then averaged within each group, and the transcriptional profile of E17 AS CFTR animals was compared to controls at the same stage of development. Entire cross sections of the proximal intestine were used, so only drastic gene expression changes would be identified in this analysis, since the samples were a mixture of epithelial cells, smooth muscle, and cells of the enteric nervous system (Table 4).

The altered expression of the annotated gene that showed the greatest difference at each developmental stage (and to which an antibody was commercially available) was confirmed by immunofluorescence. As predicted based on low mRNA levels, expression of the protein claudin-6 was drastically reduced in the AS fetus (Fig. 17). Claudin-6 is a tight junction protein known to be expressed by enterocytes which form lateral junctions with neighboring cells. The absence of claudin-6 following AS treatment indicated that the normal pattern of gene expression was disturbed by reduced levels of CFTR. At P3, galanin receptor 2 protein showed upregulated expression, again, as predicted based on mRNA levels. The pattern persisted at weaning, demonstrating that changes in gene expression induces by transient suppression of CFTR could be long-lasting (Fig. 18). The levels of calcitonin gene related peptide (CGRP) and chromogranin A, two widely-used intestinal epithelial markers, were unchanged in the AS group at either E21 or P3 (data not shown).

C. Adult-onset functional deficiency of the intestine

No obvious morphological abnormalities were noticed in the duodenum of E21, P3, 3-week, 3-month, and 5-month old AS CFTR rats. However, older animals (12-18 months) had severely distended bowels (Fig. 19). Seven out of sixteen aged AS rats, and none of the age-matched controls, showed these symptoms. Both small and large intestine were affected.

Understanding of this phenotype would first require careful histological analysis of sections of the entire length of the small and large intestines. Such analysis is beyond the scope of this study which only focused on regulation of metabolism by the duodenum.

DISCUSSION

Both CCK and SP are normally released in response to the presence of food in the intestine. The production of both substances is uncoupled from food intake in adult rats as a result of AS CFTR treatment at E17. This demonstrates that the control of satiety is dysregulated in the AS rats, but the exact role of these substances in the development of hyperphagia is difficult to interpret. Many more neuroendocrine factors are involved in the control of appetite, and the effects of changes in their levels are not always additive [57]. Also, both CCK and SP are constantly present in the duodenum of the AS animals at levels intermediate between the fasted and fed states in control rats. The effect of such pattern of CCK and SP expression on food intake has not been tested directly in another context.

CCK is known to inhibit food intake. However, administration of exogenous CCK in rats results in decreased meal size but increased meal frequency, with unknown effect on total food consumption [58]. Moreover, the effect of CCK is either species-specific or subject to modifier genes. Rats lacking functional CCK_A receptors are diabetic, hyperphagic and obese but the absence of the receptor has no effect on food intake or body weight in mice [59]. SP causes smooth muscle contractions and stimulates intestinal peristalsis. The effect of SP on body weight has not been studied extensively. However, administration of a pharmacological inhibitor of SP action prevented weight gain in mice, suggesting that high

levels of SP could play a role in the development of obesity [60]. As in the case of CCK, more studies are needed to understand the relative contribution of SP to hyperphagia in AS CFTR rats. The final factor that complicates the interpretation of this phenotype is that the levels of neuroendocrine substances present at any given time are interrelated. For example, SP can be released in response to CCK [61], making it even harder to differentiate between the primary (causal) and secondary changes and their contributions to the dysregulated appetite.

The analysis of neuroendocrine signals involved in the control of appetite that may cause weight gain in the AS rats is very preliminary. However, the altered levels of CCK and SP demonstrate a differentiation defect of the endocrine cells of the epithelial lining of the duodenum. In addition, microarray analysis identified two marker proteins whose expression was drastically altered following CFTR reduction. Moreover, older AS animals suffer from intestinal dilation, indicating severe functional deficiency of the organ. Taken together, these findings provide an example of an adult-onset metabolic imbalance that results from altered development of the fetal intestine and that is not detectible in younger animals.

The adenoviral in utero gene therapy method that was used to suppress the expression of CFTR at E17 targets both the lung and intestine of the developing rat fetus (Table 1), since the AS CFTR fragment is under the control of the ubiquitously expressed CMV promoter. Pre- and postnatal gene expression changes following AS in utero gene therapy and the resulting impaired function of the adult organ provide a clear link between the fetal intestine as the primary site of insult and the metabolic phenotype after birth. The overweight phenotype is therefore likely to be the result of CFTR's reduction in the fetal intestine.

CHAPTER 4

FUTURE DIRECTIONS AND CLINICAL IMPLICATIONS

SUMMARY

Reduction of CFTR levels in the intestine of the developing rat fetus causes divergence from normal development, as demonstrated by altered expression of epithelial cell markers, misregulated appetite leading to weight gain and diabetes, and functional deficiency of the intestines of aged animals. The most intriguing question posed by these results is the molecular nature of changes in the intestinal epithelium that originally result from CFTR suppression and then permanently regulate cellular function. Moreover, the findings of this study have potential implications for the treatment of CF and neonatal care of premature infants.

A. Possible mechanisms

All animals used in this study were inbred (genetically identical), and the developmental insult was mediated by a non-integrating adenovirus that should not disrupt the cellular genome. Therefore, the altered the development of the intestine of AS rats must be mediated by epigenetic changes.

Microarray analysis of gene expression immediately following CFTR suppression did not identify up- or down regulation of the expression of transcription factors or any other proteins that could then potentially direct the course of development of the intestinal epithelium. Histone and chromatin modifications are a well-established epigenetic mechanism capable of producing long-term changes in gene expression. However, this study did not identify any candidate genes that could be screened for the presence of such modifications, and the cost and effort of a whole-genome scan could not be justified without any preliminary data on the molecular basis of the changes in AS animals.

One possible mechanism underlying altered development in the AS rats could be changes in the physical pressure exerted on the epithelium by the amniotic fluid. The idea that physical stretch could induce cell differentiation during organogenesis was originally proposed in the 1920's to explain the development of the fetal lung. Since then, the process of translating physical forces into a biochemical signal, or mechanotrasduction, has been demonstrated to play a role in development, in sensory cells, and in regulation of smooth muscle activity in the vasculature [62].

The role of CFTR during development is best studied in the lung, since pulmonary obstruction and recurrent infections are the leading cause of death in CF patients. Overexpression of CFTR in the mouse lung around E16 induces the expression of smooth

muscle contraction related proteins, as demonstrated by microarray analysis of the organ at birth. Upregulation of CFTR also increased amniotic fluid flow into the organ. These results suggest that CFTR controls a developmental cascade leading to smooth muscle contractions in the fetal lung [17].

An analogous situation could exist in the intestine where altered levels of CFTR expression could affect differentiation of the epithelial layer by regulating smooth muscle contractions and causing a change in the net amniotic fluid flow. This hypothesis could be tested measuring the movement of fluorescent beads in response to changes in CFTR expression, as was done in the developing mouse lung.

B. Lessons for Cystic Fibrosis

The diabetic AS CFTR rats may be a useful model for studying cystic fibrosis related diabetes (CFRD). Diabetes mellitus was first recognized as a rare complication of cystic fibrosis in the 1950's [63]. As better medical treatments extended the average life expectancy to almost 40 years, cystic fibrosis related diabetes has become an increasing clinical concern. Some studies report that as much as 75% patients over 30 develop diabetes [64].

Rats in which CFTR is suppressed during day 17 of gestation develop diabetes as adults and the condition results from functional deficiency of the intestine. Unlike in other animals models of CF, the specific role of functional deficiency of the intestinal epithelium (and not other organs) in the development of CFRD could be studied with the AS technology. This could be because the AS CFTR rats are genetically normal, so a typical pattern of CFTR expression is expected in all adult tissues, and the only organs involved are the fetal lung and

intestine. In cystic fibrosis, CFTR is absent from all tissues at all times, so all epithelial-lined organs are affected and their relative contribution to the generation of the disease can be difficult to dissect. CFRD is associated with reduction in the β cell mass in the pancreas, and there is evidence that islet dysfunction may contribute to the development of the condition [65, 66]. In AS CFTR animals, the pancreas retains its capacity to (over)produce insulin in adults, and is unlikely to contribute to the progression of diabetes in these rats.

In addition, the findings of this study are potentially significant for the development of effective gene therapy treatment for CF patients. The developmental requirement for CFTR should be considered when choosing the timing for CF gene therapy.

Cystic fibrosis remains an attractive target for gene therapy for several reasons. Current therapeutic approaches greatly improve the health of individuals with CF, but do not offer a cure for the condition – patients often require professional care every single day. If successful, gene therapy could offer a permanent solution and limit office visits for CF patients to periodic check-ups. Also, CF is a monogenic disease, and even though modifier genes undoubtedly exist, they usually cannot cause the disease in the absence of inactivating CFTR mutations. Providing just one good copy of CFTR should be sufficient, since humans and animals heterozygous for CFTR mutations are generally asymptomatic. Moreover, the surface epithelia of organs affected in CF are easily accessible for viral delivery.

In utero gene therapy has been very successful in model animals including primates. However, performing the procedure in human patients would present serious obstacles. Over 750 disease causing mutations in CFTR have been identified [67, 68]. The deletion of phenylalanine at position 508 (CFTR ΔF508) accounts for 70% of all cases of CF, and about 90 other mutations make up another 20%. Testing pregnant couples for their carrier status

would be expensive and time-consuming. Also, this study demonstrates that CFTR plays a role during development, but does not dispute the requirement for CFTR in the adult organ, so that possibility has to be considered. Vectors for permanent expression of the transferred gene are available. The adeno-associated virus (AAV) has been adapted for gene therapy and does not cause an immune reaction. Still, AAV integrates into the host genome, presenting a long-term risk since it may disrupt cellular genes. Given these concerns, and how little attention the developmental requirement for CFTR receives from clinicians - there is currently not much incentive to consider prenatal gene therapy for CF in humans.

An excellent laboratory experiment that could provide rationale to consider a pilot clinical study could be performed in mice, taking advantage of the many tools available to manipulate the mouse genome. Several mouse models of cystic fibrosis have been created, including alleles CFTR ΔF508 and S489X – corresponding to the two most common mutations in humans [21, 69]. Either one of these could be crossed to a strain carrying an inducible (e.g. by tetracycline) CFTR transgene. CFTR could then be turned on in the knock-out background at different points in pre- and postnatal life, and the effect on CFTR expression on the CF phenotype could be examined.

C. Implications for neonatal care

Day 17 of embryonic development in the rat corresponds to Carnegie stage 23, or day 58, of human embryonic development [70]. Delivery at that time is considered a miscarriage, not a preterm birth, and no therapeutic interventions exist to assist the development of the fetus at such an early stage. However, the Carnegie staging system was created to describe the apparent maturity of the human embryo based on various external

physical features and does not provide any information about organogenesis. The E17 rat lung, for example, resembles the human organ at week 23-24 of gestation. The degree of maturity of the E 17 intestinal epithelium in relation to human remains to be established, but if it is at a similar stage of development as the lung, then the findings of this study could have potential implications for neonatal care.

The developing lung and gut of the premature infant face a similar challenge at birth. Both are non-functional in utero (the fetus does not rely on its own organs for gas exchange or nutrition) and at birth, both have to make the transition to non-aqueous environment. Previous studies demonstrated the role of CFTR in stretch-induced differentiation of the lung epithelium. This study strengthens the evidence that CFTR is developmentally required in the intestine, although it does not provide any insight into the molecular mechanism of CFTR's function. Still, it is a feasible hypothesis that the level of CFTR in the intestine affects the net flow of the amniotic fluid into the organ. Once that hypothesis is verified, therapeutic approaches simulating amniotic fluid presence in the intestine could be designed – and hopefully result in the enhancement of maturation of the intestinal epithelium.

The potential clinical applications of the results of this study require extensive laboratory and animal experiments before they can be tested in humans. The most valuable lesson to be learned may be that even subtle changes in gene expression during gestation can have drastic consequences for the adult phenotype. It is clear that the intestine is a key organ in programming of adult metabolism. This is the first study that demonstrates that altered development of the intestinal epithelium can result in increased appetite, weight gain, insulin resistance, and diabetes.

Table 1. Target organs of in utero gene therapy. Expression of reporter genes delivered by in utero gene therapy was analyzed. Tissues from rhesus monkey fetuses treated with adenovirus encoding both luciferase and β-galactosidase (β-gal) reporter genes were analyzed 10 days post infection. Typical results found in independent enzyme and immunohistochemistry examinations are presented. Enzyme assays were graded based on enzyme units/mg protein on the following scale: (+) $< 10^3$, (++) 10^3-10^4, (+++) 10^4-10^5, (++++) $>10^5$. The presence of infecting adenovirus was detected by PCR [14]. In mice and rats, tissues from fetuses treated with adenovirus carrying green fluorescent protein (GFP) were analyzed 48hrs post treatment (JC Cohen, unpublished data). ND, no data.

	Primate (Rhesus macaque)			Rodent (Mouse and Rat)
Tissue	Transgene enzyme assay	Immunoassay	PCR	Immuno-fluorescence
Lung	++++	Yes	Yes	Yes
Intestines	++++	Yes	Yes	Yes
Esophagus	++++	Yes	Yes	ND
Trachea	++++	Yes	Yes	ND
Stomach	-	No	No	No
Colon	+	No	Yes	Yes
Liver	-	No	No	No
Spleen	+	No	No	No
Kidney	++	Yes	No	No
Bladder	-	No	No	No
Gonads	-	No	No	ND
Pancreas	ND	ND	ND	No
Skin	++++	Yes	Yes	No

Table 2. Increased abdominal adiposity in AS CFTR rats. Micro CT images of the abdominal region were used to quantify total abdominal fat as described in Methods. AS treatment resulted in increased abdominal fat in both young and aged rats. p=0.04; the % difference in the aged group is an underestimate since the most overweight animals in that population were excluded from the analysis because their size exceeded the capacity of the scanner.

Age	Treatment	Fat volume/total volume	Sample size	**% difference**
3 months	AS CFTR	33.73 ± 2.66	n=3	**20%**
	control	26.85 ± 4.85	n=2	
15 months	AS CFTR	52.10 ± 1.29	n=3	**10%**
	control	46.73 ± 1.57	n=3	

Table 3. Current clinical guidelines for the diagnosis of impaired glucose tolerance and diabetes (WHO). Elevated blood sugar level 2 hours post administration of glucose indicates relative insulin insufficiency, often caused by peripheral insulin resistance.

	Normal	Impaired	Diabetic
Fasting blood sugar	< 100 mg/dL	100-125 mg/dL	>125 mg/dL
Blood sugar 2hrs post OGTT (mg/dL)	< 140 mg/dL	140-199 mg/dL	>199 mg/dL

Table 4. Transcriptional response following CFTR suppression. To identify potential marker genes whose expression might be changed as a result of in utero CFTR reduction, microarray analysis was performed. The duodenum of E17 AS CFTR treated and control fetuses (at E21) and pups (at P3) was examined. The transcriptional profiles were compared using GeneSifter software, with p value set to <.001 to reduce false gene discovery rate. Annotated genes are in bold. Highlighted in red are the genes whose altered expression was confirmed by immunofluorescence (Fig. 17-18). *Fold change represents relative ratio of control to AS mRNA levels.

FETUS	
Fold change*	Description
6.703	**Claudin 6**
5.27	Similar to expressed sequence AW413625 (predicted)
4.123	Exosome component 3 (predicted)
4.09	**Glutathione S-transferase, alpha 4**
1.87	Transcribed locus, strongly similar to XP_238433.2
1.849	Transcribed locus
1.819	Similar to RIKEN cDNA 5133401N09
1.801	Hypothetical LOC300751 (predicted)
0.584	**UDP-GalNAc transferase 10**
0.496	**Mouse zinc finger protein 14-like**
0.429	**ADP-ribosyltransferase 3**
0.214	Transcribed locus
PUP	
Fold change*	Description
11.434	Transcribed locus
1.821	**BCS1-like**
1.668	**Similar to ecotropic viral integration site 2A**
1.591	Rattus norvegicus cDNA clone UI-R-E1-gb-f-11-0-UI
1.528	Rattus norvegicus cDNA clone UI-R-CA0-axm-f-03-0-UI
1.525	Transcribed locus
0.664	Transcribed locus
0.637	**Nuclear receptor subfamily 1, group D, member 2**
0.618	**Galanin receptor 2**
0.508	**Potassium voltage-gated channel, shaker-related subfamily, beta member 1**
0.462	Rattus norvegicus cDNA clone UI-R-CA1-biz-d-23-0-UI
0.372	Rattus norvegicus cDNA clone UI-R-C1-kx-g-01-0-UI

Figure 1. Survival of neonatal rats following in utero reduction of CFTR levels. Rats were treated with Ad.CMVascftr at E15, E16, or E17 as described in Methods. Control rats were injected with Ad.CMVegfp. Four pregnant females (10-15 fetuses each) were included in each group and percent survival was calculated comparing the number of fetuses at the time of gene therapy to the number of viable animals at weaning (22 days). * indicates statistically significant ($p<0.05$) decrease in survival in animals treated at E15, compared to all other treatment groups.

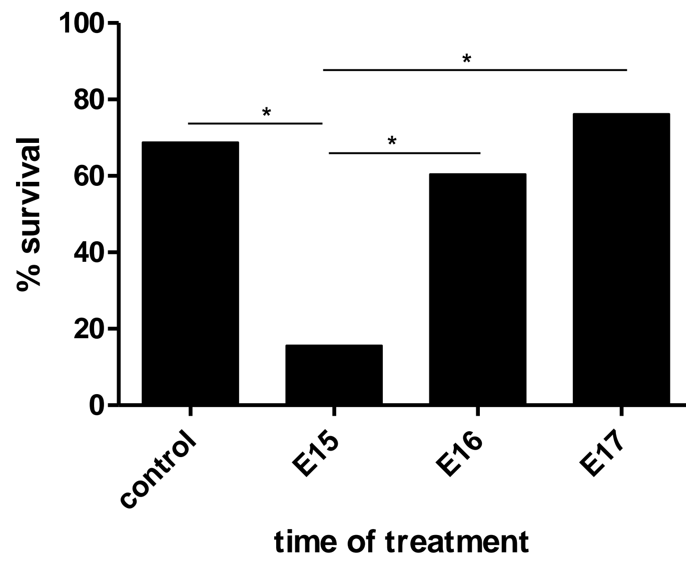
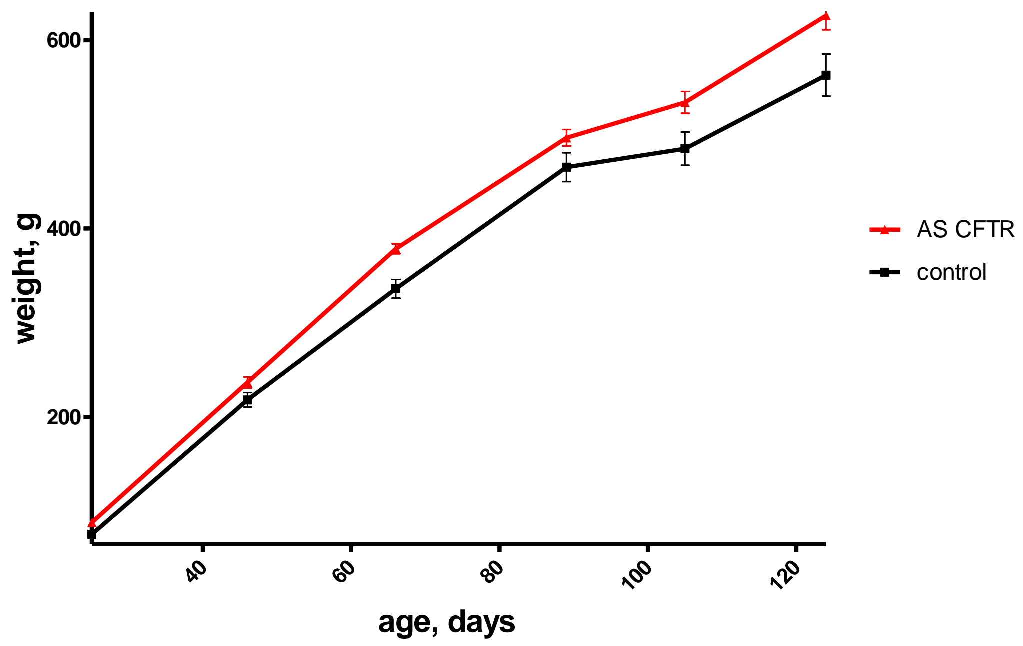

100
80
60
40
20
0
% survival
*
*
*
control
E15
E16
E17
time of treatment

Figure 2. Growth curve of E 17 AS CFTR rats. Following AS CFTR treatment at E17, male rats were weighed periodically between weaning (3 weeks) and 4 months of age (n=10 AS CFTR, n=8 control). AS animals show a significant weight increase over GFP treated controls ($p<0.0001$).

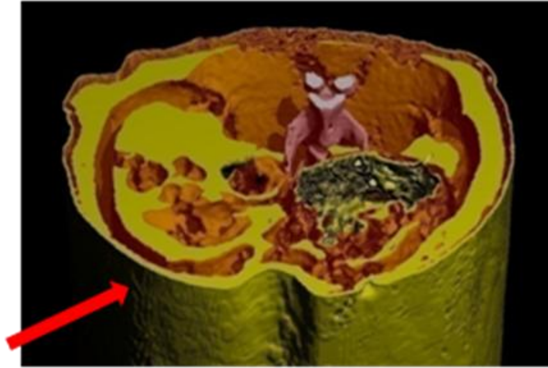


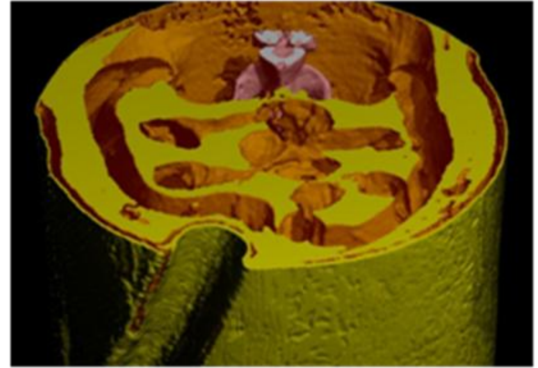
600
400
200
weight, g
40
60
80
100
120
age, days
AS CFTR
control

Figure 3. Altered body composition in AS CFTR rats. Sequential microCT images were used to reconstruct the abdominal region of control and AS rats. Adipose tissue is in yellow; arrows are pointing to subcutaneous fat in A and to visceral fat in B. **A.** Similar difference in both visceral and subcutaneous fat volume in young animals (3months). **B.** Marked increase in visceral fat in aged rats (>1yr old).

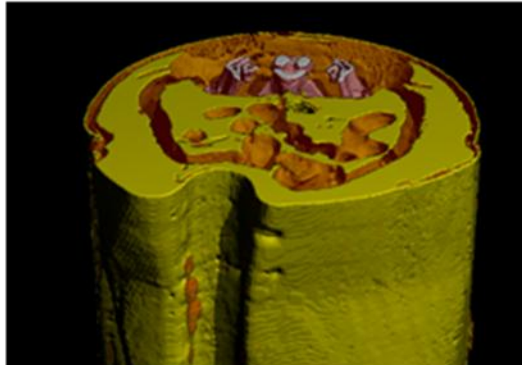
CONTROL **AS CFTR**

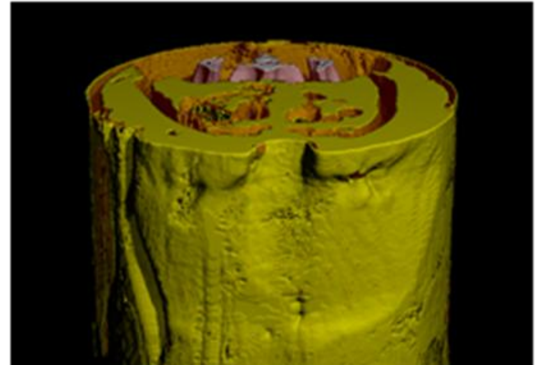
A.

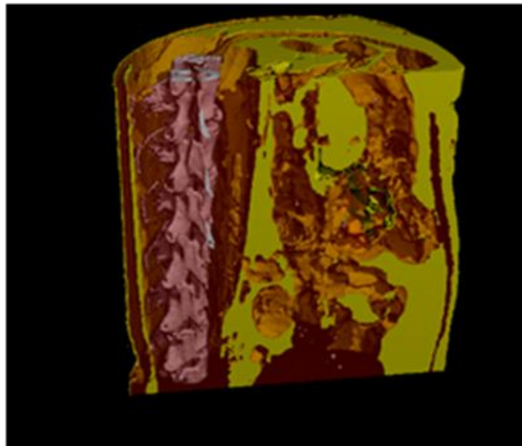




B.







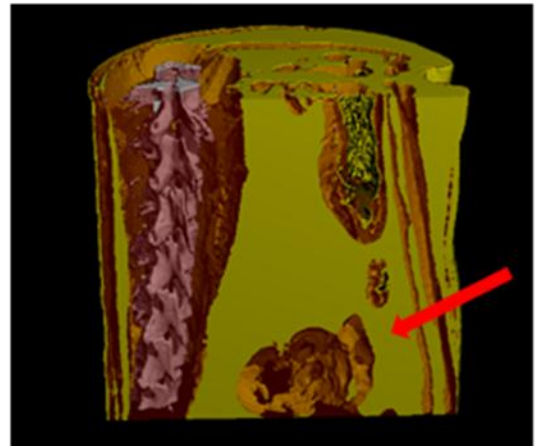


Figure 4. Hyperphagia in AS CFTR rats. Individual ad libidum food intake in 3-month-old males was measured daily for 15 days (n=8 AS CFTR, n=8 control); p=0.04

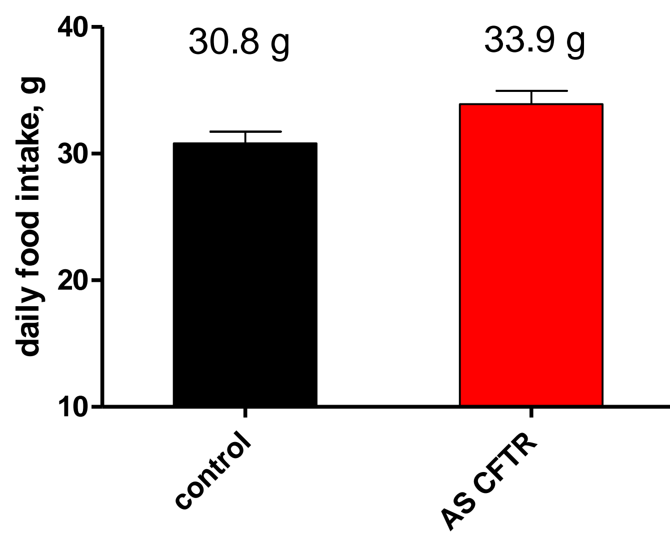

30.8 g
33.9 g
40
30
20
10
daily food intake, g
control
AS CFTR

Figure 5. Oxygen consumption in AS and control animals. Calorimetry measurements were performed in 1-month-old males as described in Methods. Although the average consumption (VO2) in AS-treated animals tends to be lower in both fed and fasted states, the decrease failed to reach the level of statistical significance. $p=0.22$ (n=4 AS CFTR, n=4 control)

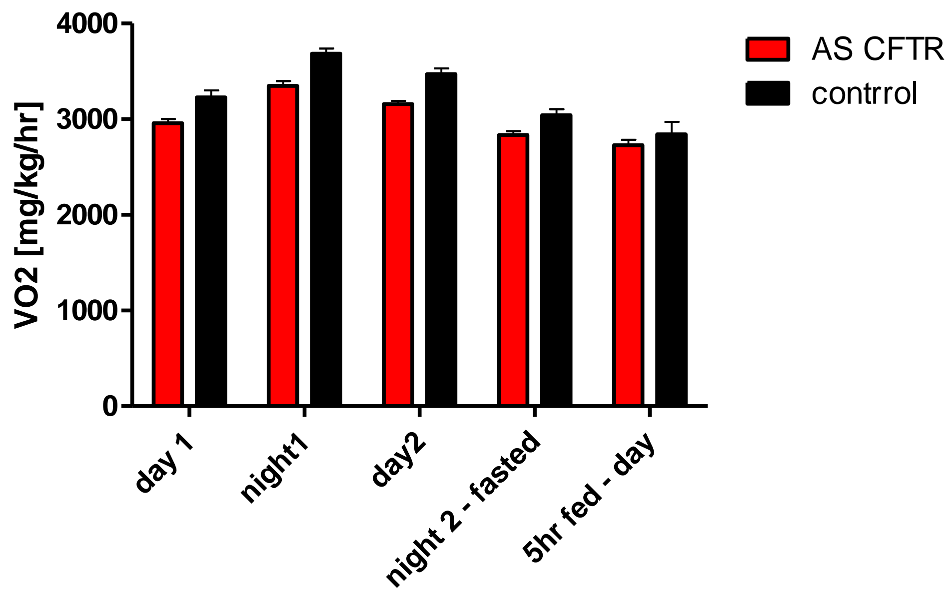
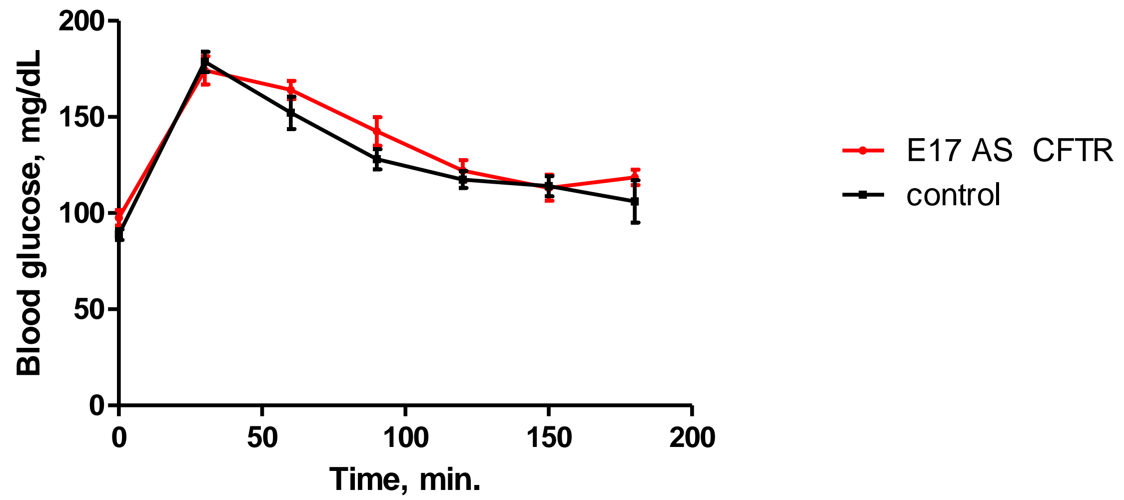

4000
3000
2000
1000
0
VO2 [mg/kg/hr]
day 1
night1
day2
night 2 - fasted
5hr fed - day
AS CFTR
contrrol

Figure 6. Normal glucose tolerance in young AS rats. 3-month old animals were fasted overnight prior to the experiment and challenged with 2g/kg of dextrose (n=8 AS CFTR, n=9 control). **A.** Oral GTT, p=0.67; **B.** IP GTT, p=.87. Similar results were found in animals aged 90 days to 5 months (data not shown).


A.
200
150
100
50
0
Blood glucose, mg/dL
0
50
100
150
200
Time, min.
E17 AS CFTR
control

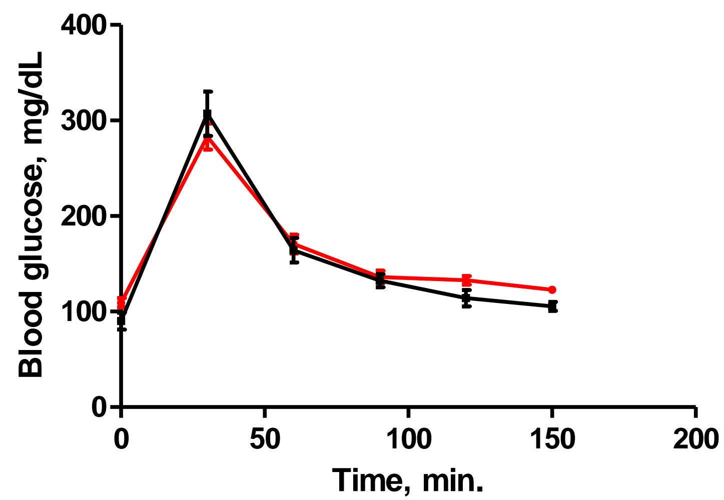
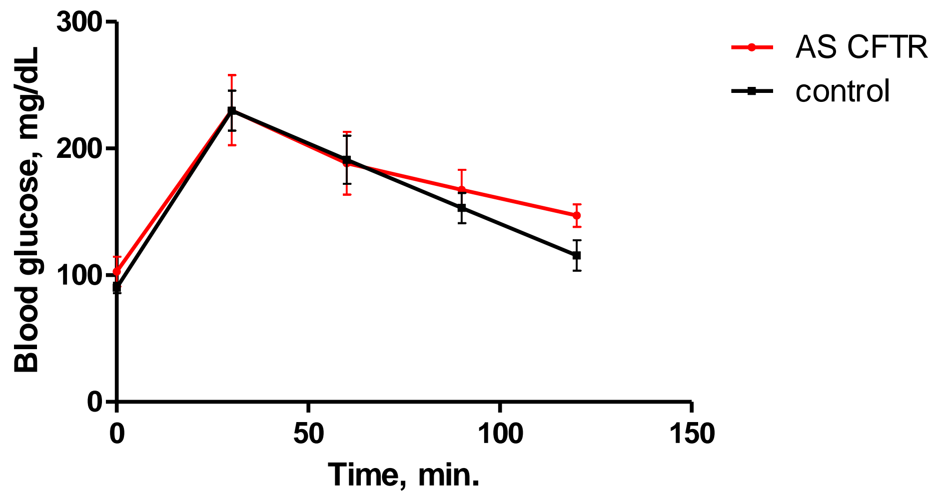

B.
400
300
200
100
0
Blood glucose, mg/dL
0
50
100
150
200
Time, min.

Figure 7. Endogenous insulin production during glucose challenge. Five-month-old animals were fasted overnight and subjected to 2g/kg IP glucose tolerance test (n=9 AS CFTR, n=9 control) **A.** Normal clearance of blood glucose, p=0.37 **B.** Hyperinsulinemia in AS rats during IP GTT, p=0.015; the corresponding blood sugar levels are presented in panel A.


A.
300
200
100
0
0
50
100
150
Blood glucose, mg/dL
Time, min.
AS CFTR
control

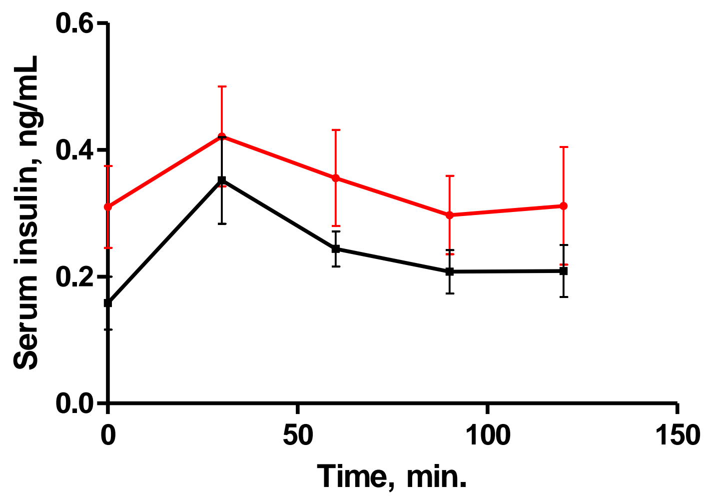
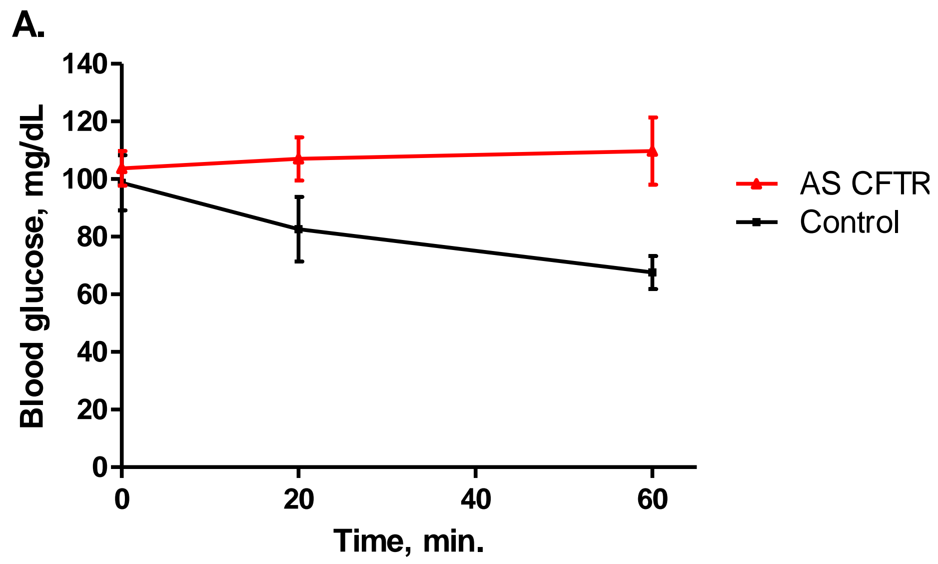

B.
0.6
0.4
0.2
0.0
0
50
100
150
Serum insulin, ng/mL
Time, min.

Figure 8. Resistance to insulin in AS CFTR rats. Following an overnight fast, animals (5 months old) were injected with indicated doses of insulin and blood glucose was monitored for 1hr. **A.** ITT at 0.5U/kg insulin, n=7 AS CFTR, n=7 control, p=0.04. **B.** ITT at 1U/kg insulin


A.
140
120
100
80
60
40
20
0
Blood glucose, mg/dL
0
20
40
60
Time, min.
AS CFTR
Control

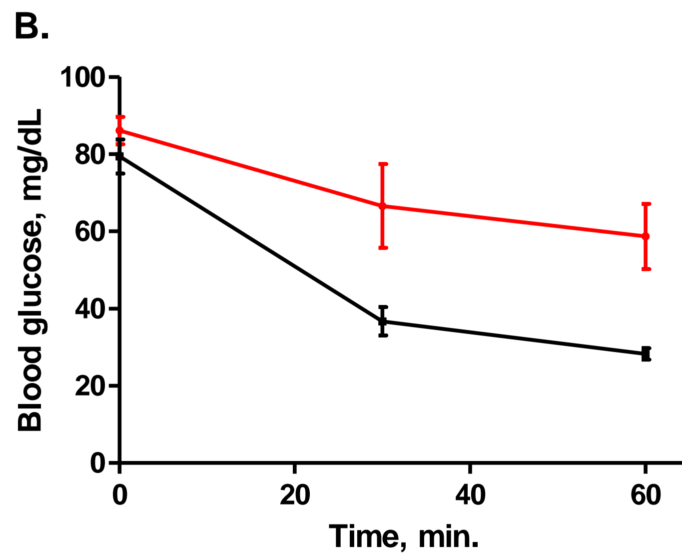
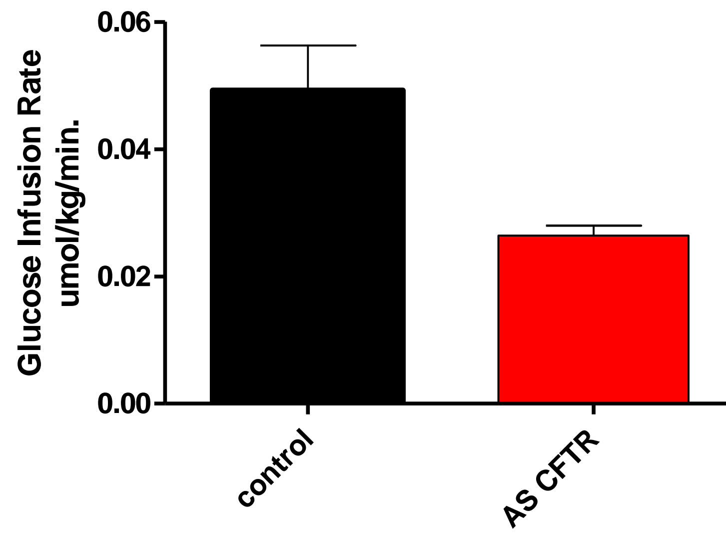

B.
100
80
60
40
20
0
Blood glucose, mg/dL
0
20
40
60
Time, min.

Figure 9. Peripheral resistance to exogenous insulin in AS rats. Hyperinulinemic-euglycemic clamps were performed on fasted 5-month-old animals (n=5 AS CFTR, n=5 control) as described in methods. Both panels indicate the amount of glucose cleared from the blood and taken up by tissues in response to a fixed level of insulin. **A.** Glucose infusion rate during clamp, measured by the settings on the infusion pump that are manually adjusted to maintain euglycemia, $p=0.01$. **B.** Whole body glucose uptake during clamp, as indicated by the rate of appearance of ^3H_2O in plasma. Trace amounts of tritiated glucose were included in the infusion. In glycolysis, the tritium is lost to water, providing a measure of whole body glucose metabolism. $p=0.03$.

A.



B.

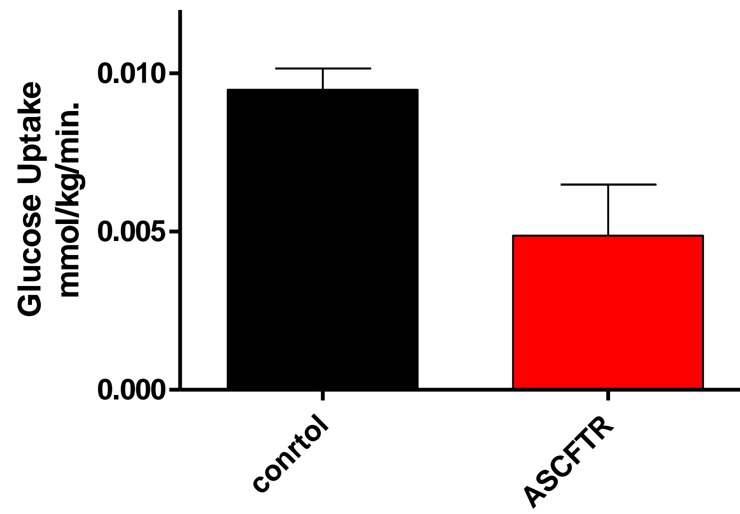


Figure 10. Normal response to insulin in adipose tissue. ^{14}C-labeled non-metabolizable analog of glucose was injected towards the end of hyperinsulinemic-euglycemic clamps in Fig. 9. ^{14}C content in abdominal fat pad was measured, reflecting total glucose uptake by adipose tissue in response to a fixed insulin dose (n=5 AS CFTR, n=5 control), p=0.59

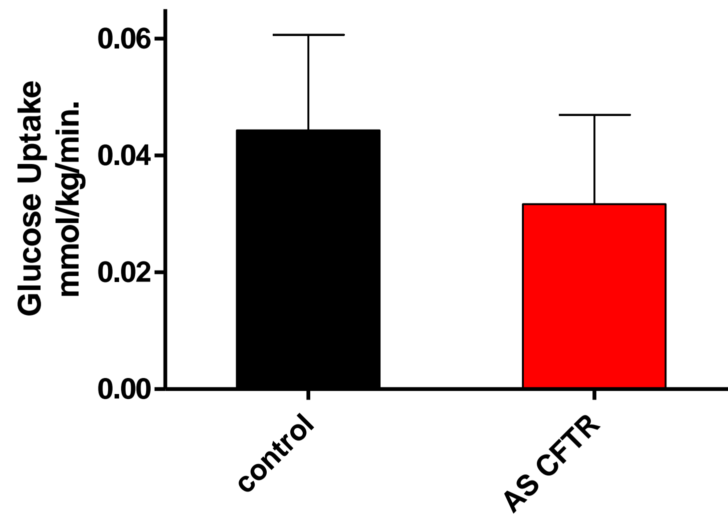
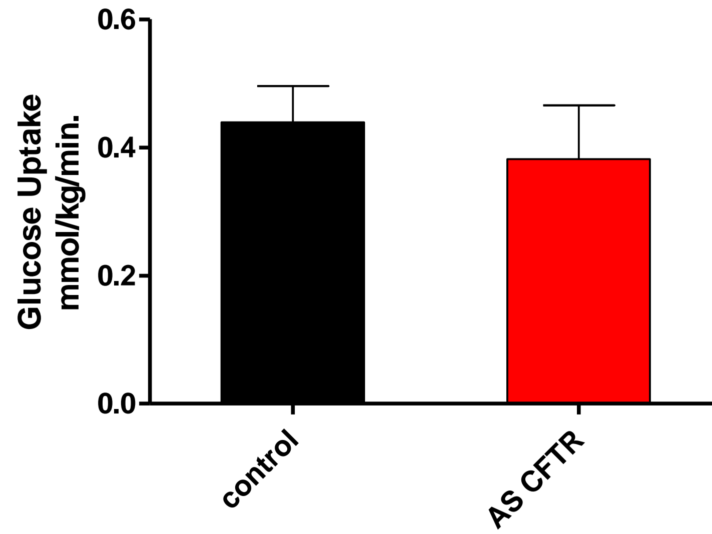

Glucose Uptake
mmol/kg/min.
0.06
0.04
0.02
0.00
control
AS CFTR

Figure 11. Differential sensitivity to insulin in skeletal muscle. ^{14}C-labeled non-metabolizable analog of glucose was injected towards the end of hyperinsulinemic-euglycemic clamps in Fig. 9. ^{14}C content in skeletal muscle was measured, reflecting total glucose uptake by the tissue in response to a fixed insulin dose (n=5 AS CFTR, n=5 control). **A.** Glucose uptake during clamp in Soleus (Type I) muscle, $p=0.29$. **B.** Glucose uptake during clamp in Gastrocnemius (mixed Type I and Type II) muscle, $p<0.05$.

A.



B.

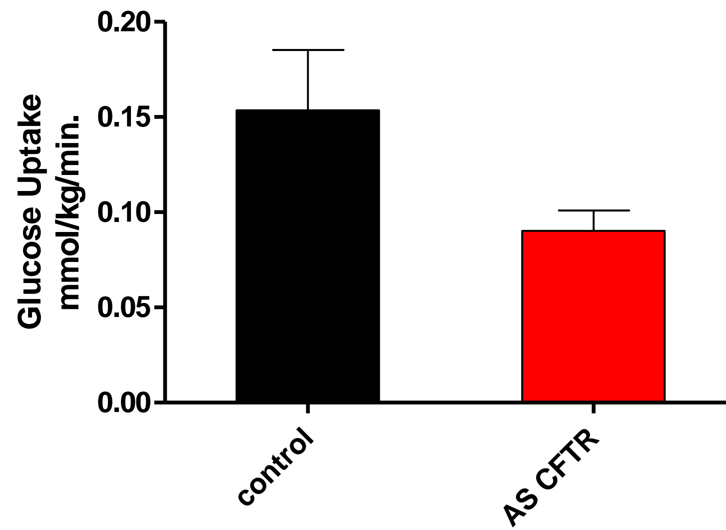
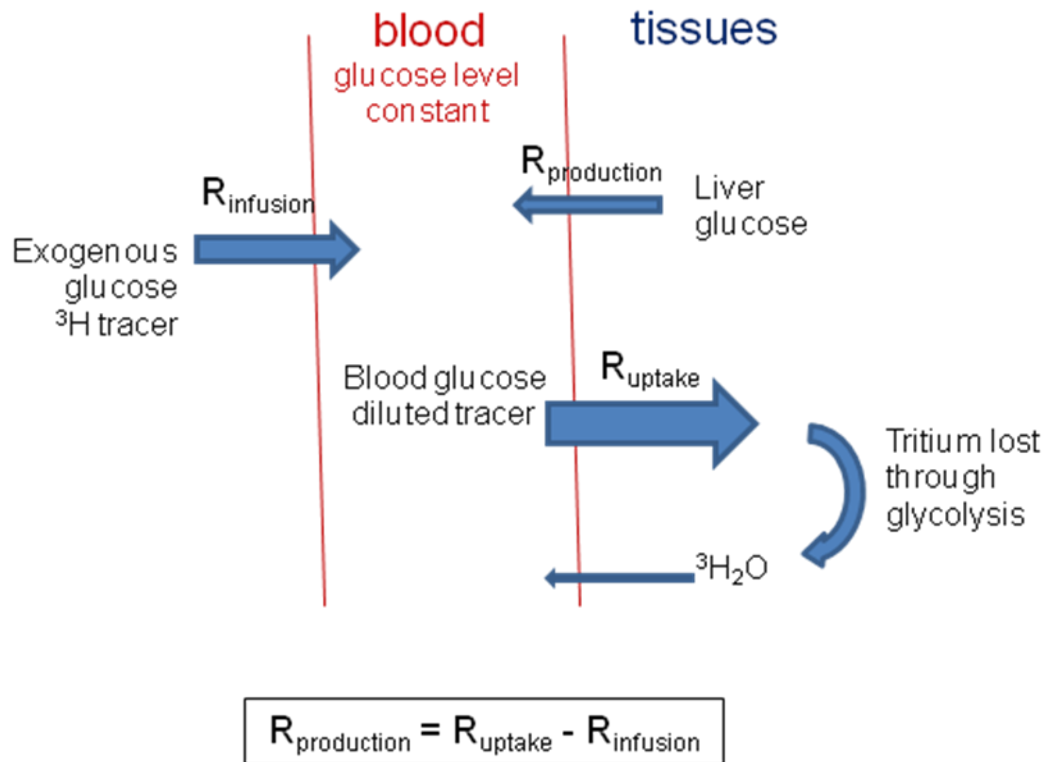


Figure 12. Hepatic glucose output before and after clamp. Trace amounts of [3-^{3}H]-glucose were infused prior to the start of clamps in Fig. 9 to enable the determination of whole body glucose uptake and hepatic glucose output. **A.** Fate of glucose molecules and of the radioactive tracer during clamps. Under steady-state conditions of euglycemia, the total blood glucose pool is made up of the exogenously infused glucose, as well as a small fraction of glucose released by the liver. Hepatic glucose output was calculated by subtracting the steady-state glucose infusion rate from whole body glucose uptake. Whole body glucose uptake was determined based on the rate of appearance of $^{3}H_2O$ in plasma, since the transfer of tritium from glucose to water is an indication of radioactive glucose uptake and metabolism (see also Fig. 9B). **B.** Hepatic glucose uptake before clamps (fasted, the liver is responsible for maintaining basal blood glucose) and during clamps (suppressed by the acutely raised insulin level); $p=0.9$ (n=5 AS CFTR, n=5 control)

A.



B.

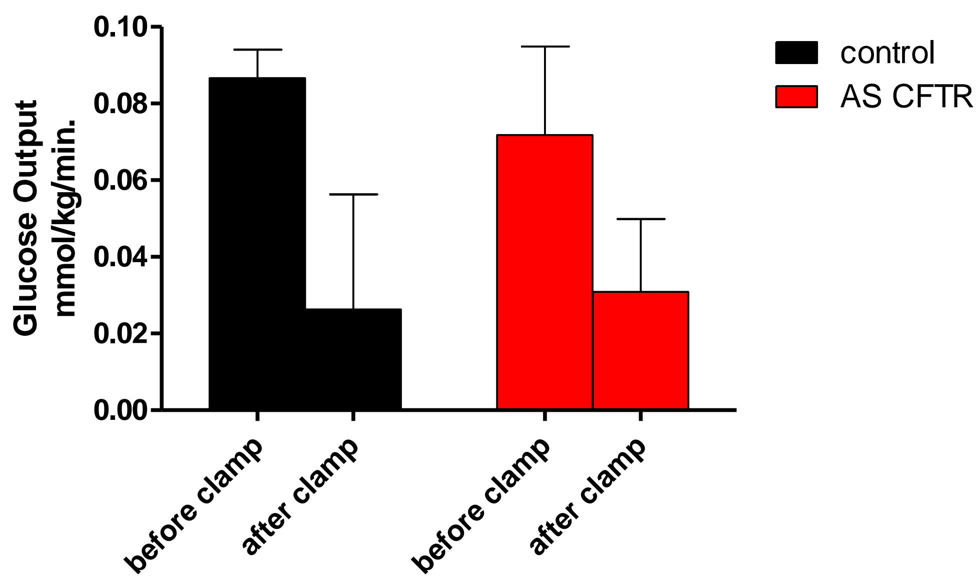
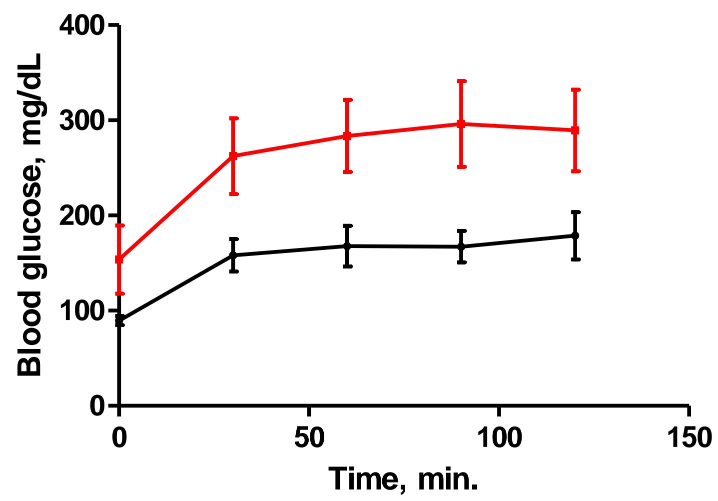


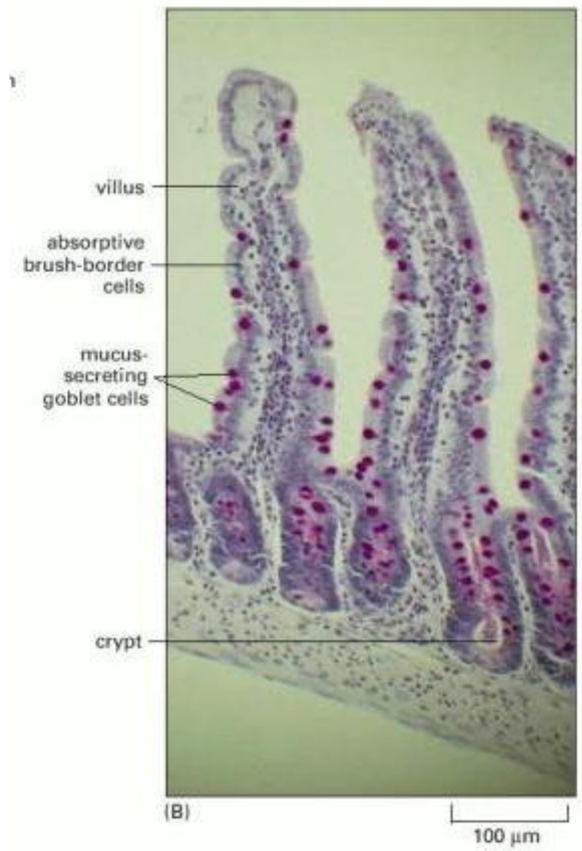
Figure 13. Diabetes in aged rats. After a 6-hr fast, 15-month-old rats were injected with 2g/kg glucose and blood sugar was monitored for 2hrs. Final blood glucose values are given. The ability of the control population to clear blood glucose appears to be impaired. However, it is significantly better than in the AS CFTR group which is diabetic. $p=0.0095$ ($n=12$ control, $n=12$ AS CFTR).



AS CFTR blood glucose at 2hrs: 242
control blood glucose at 2hrs: 178

Figure 14. Diversity of intestinal cell types. A. Photograph of a section of a small intestine showing the basic organization of the epithelium into villi and crypts. Enterocytes and goblet cells are clearly visible; neuroendocrine cells cannot be distinguished morphologically and require immunostaining. **B.** Electron micrographs of the terminally differentiated epithelial cell types of the small intestine. Yellow arrows indicate the secretory of absorptive functions (from Alberts et al. *Molecular Biology of the Cell*, open access at www.ncbi.nih.gov/books).

A.



B.

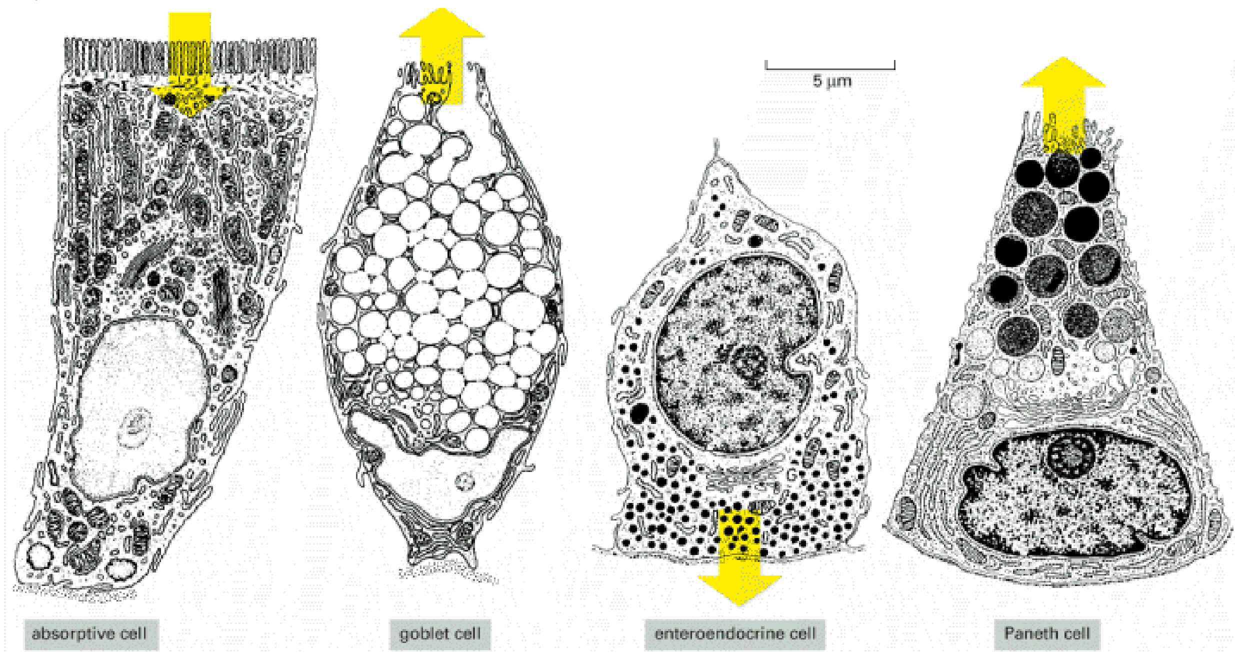


Figure 15. Production of CCK in the duodenum. CCK levels in the duodenum were assayed before (left) and 1hr after a meal (right). In control animals (top), CCK is induced during a meal. In AS rats (bottom), CCK levels remain constant independent of food intake. Three animals were analyzed in each group and images were chosen that represent typical findings.

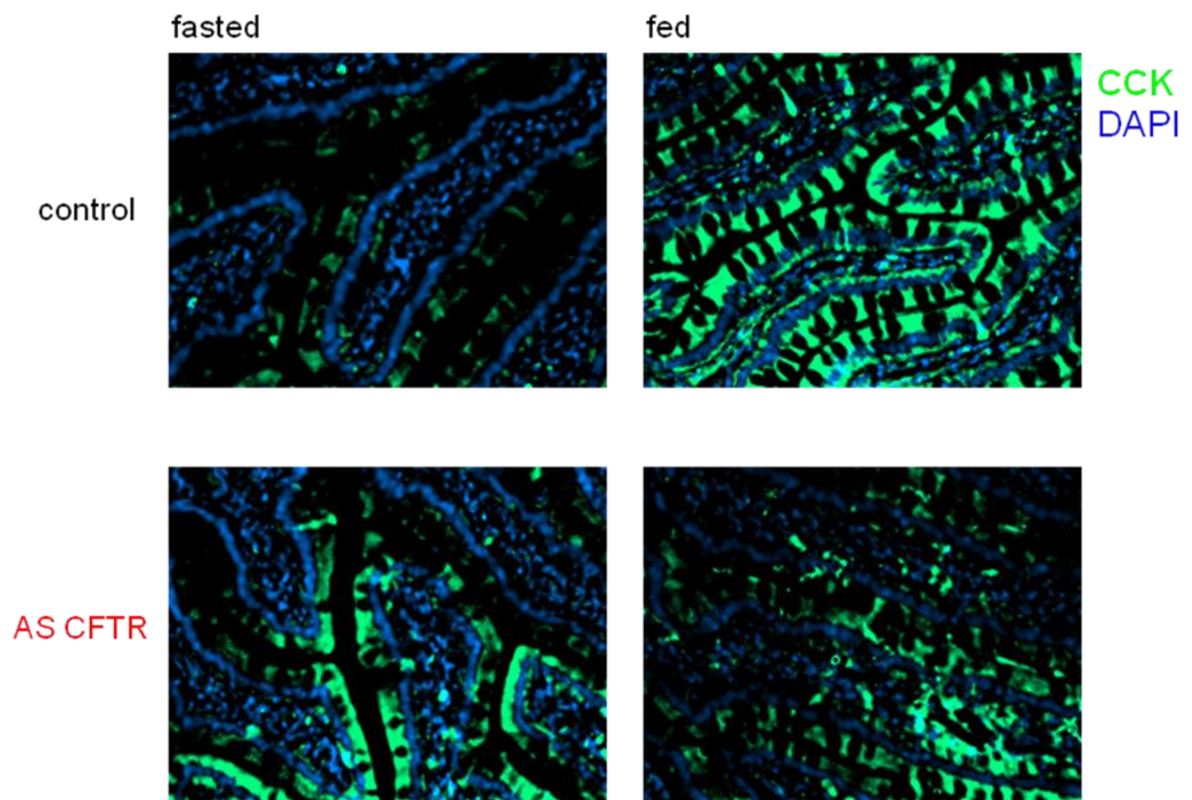

fasted
fed
CCK
DAPI
control
AS CFTR

Figure 16. Substance P production before and after a meal. SP levels in the duodenum were assayed before (left) and 1hr after a meal (right). In control animals (top), SP is induced during a meal. In AS rats (bottom), SP levels remain constant independent of food intake. Three animals were analyzed in each group and images were chosen that represent typical findings.

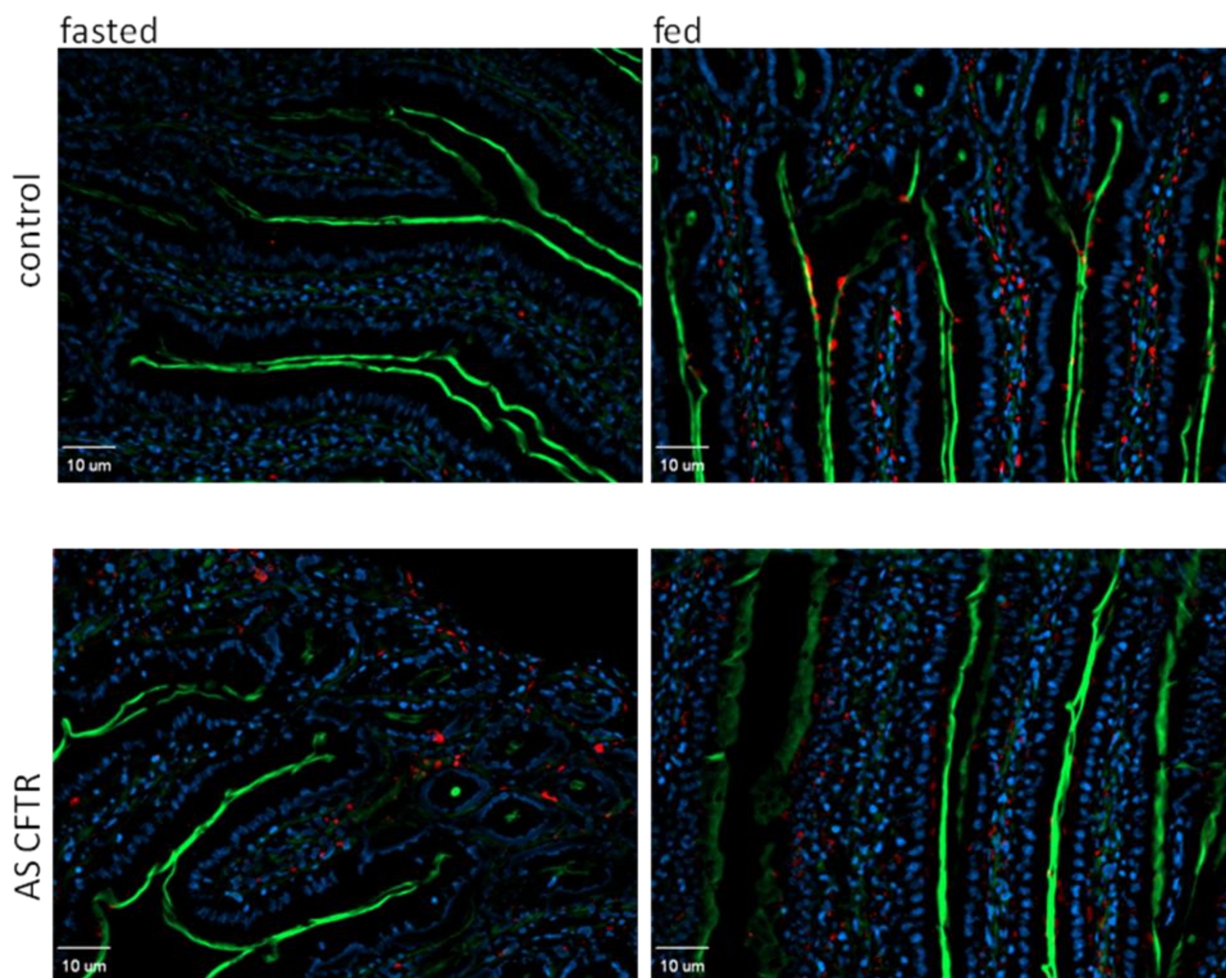
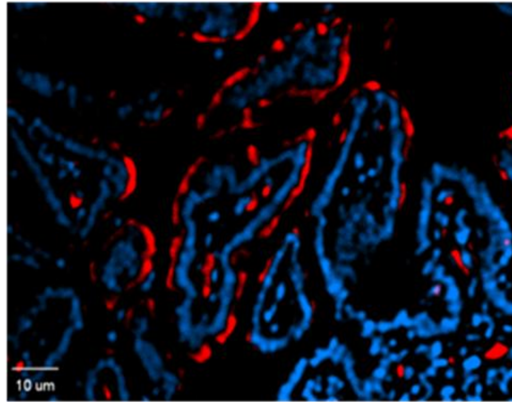
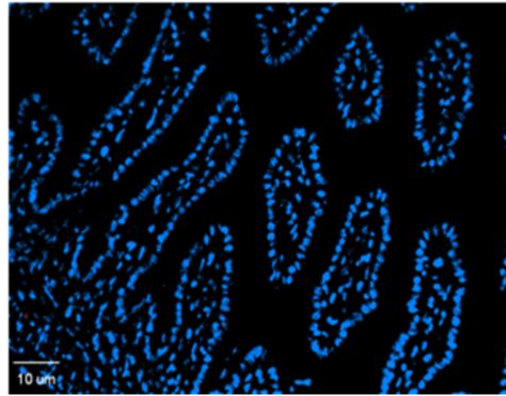

fasted
fed
control
AS CFTR
10 um
10 um
10 um
10 um
DAPI
Substance P
Phalloidin

Figure 17. Claudin-6 expression following CFTR suppression. A. Claudin-6 expression (red) in the fetal (E21) duodenum was assayed by immunofluorescence. Three animals were analyzed in each group and the images shown represent typical findings. **B.** Graphical representation of Claudin-6 mRNA levels found in microarray analysis (Table 4).

A.





B.

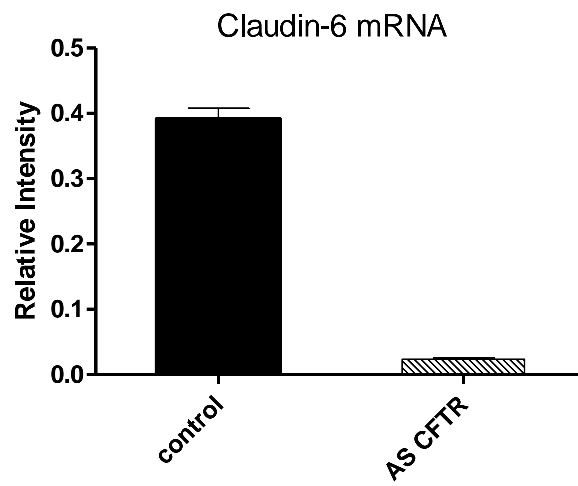
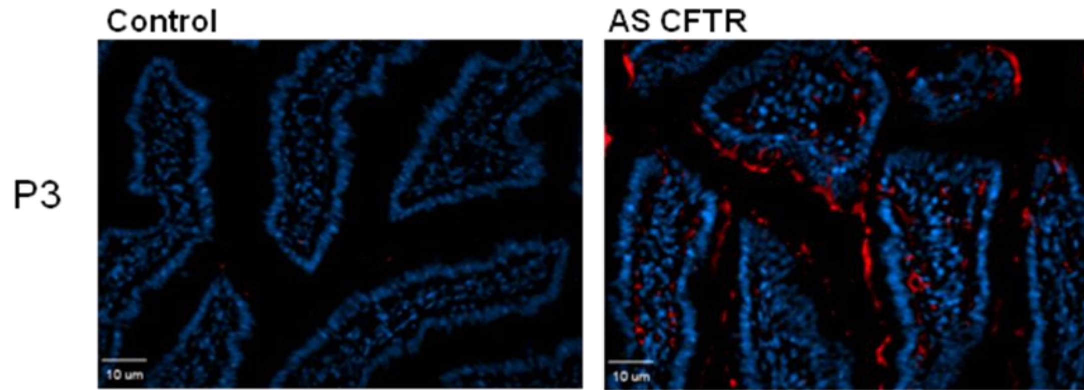
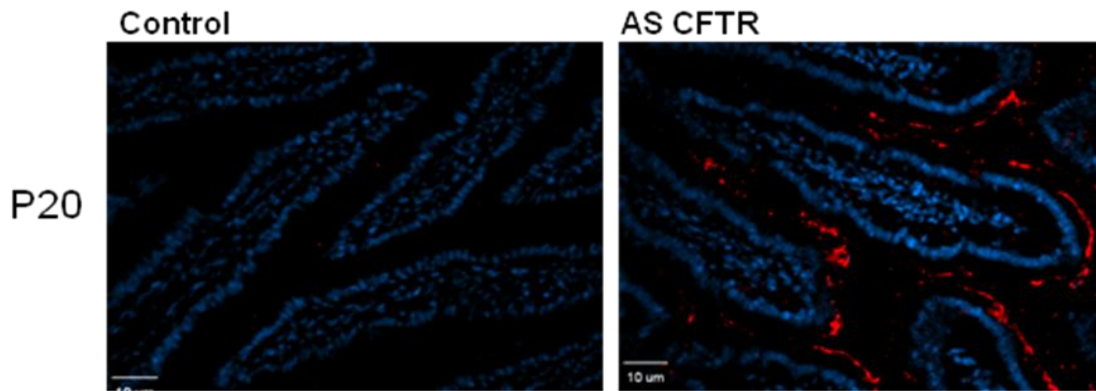


Figure 18. Galanin receptor 2 expression following CFTR suppression. A. Galanin receptor 2 expression (red) in the duodenum is detected by immunofluorescence. The protein is upregulated in newborn (P3) AS animals, and the pattern persists at weaning (P20). Three animals were analyzed in each group and the images shown represent typical findings. **B.** Graphical representation of Galanin receptor 2 mRNA levels found in microarray analysis (Table 4).

A.





DAPI
galanin receptor 2

B.

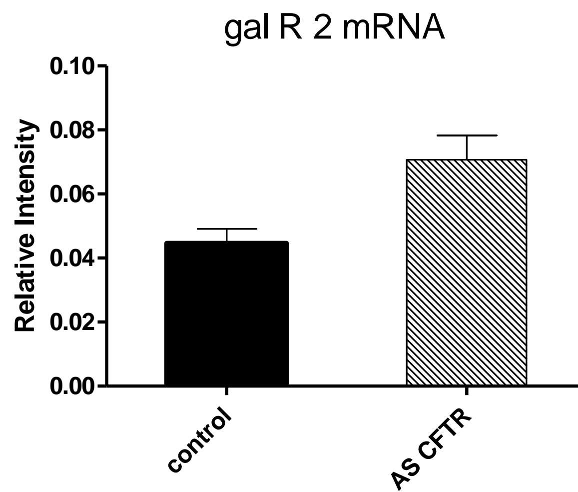
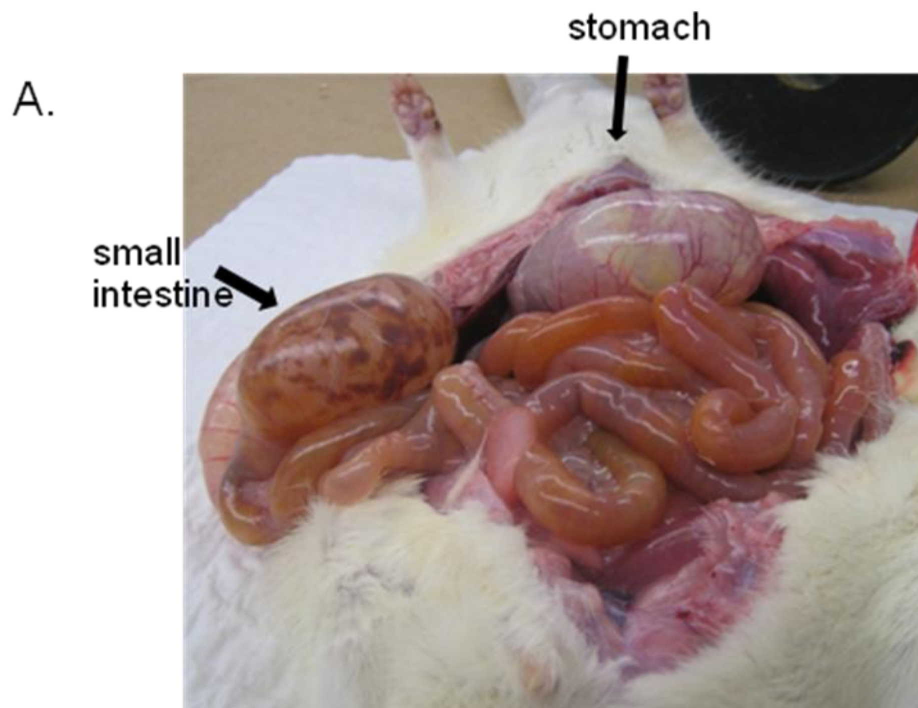
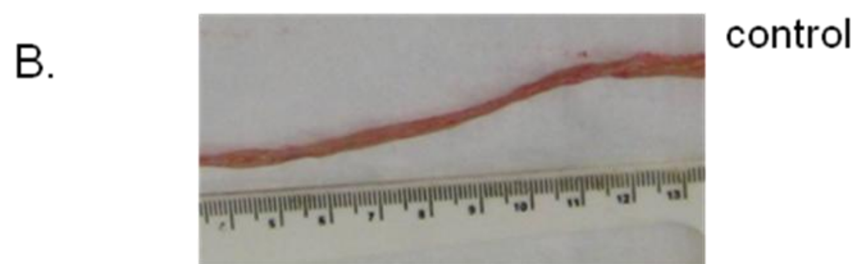


Figure 19. Distended intestines of aged AS CFTR animals. At age 12-18 months, 7 out of 16 AS rats, and none of the age-matched controls, showed these symptoms **A.** AS CFTR rat with severely dialated intestines, indicative of severe functional deficiency of the organ (age 17 months). **B**. control and AS duodenum shown at the same scale for comparison (age 18 months).


A.
stomach
small
intestine


B.
control

AS CFTR

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