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Development of Human Pancreatic Ductal Epithelial Cells as a model of Cystic Fibrosis Pancreatic Disease

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Abstract

Development of Human Pancreatic Ductal Epithelial Cells as a model of Cystic Fibrosis Pancreatic Disease

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Impaired insulin release underlies the development of cystic-fibrosis-related diabetes (CFRD) which affects 40-50% of adults with CF and is associated with significantly increased morbidity and mortality. The initiating site of CF pancreas pathology is the pancreatic duct as CFTR expression is highest in pancreatic ductal epithelial cells (PDECs). While loss-of-function mutations in *CFTR* result in CF, these mutations lead to the destruction of pancreatic exocrine (acinar) tissue which is characterized by the clinical manifestation of pancreatic exocrine insufficiency in 85% of people with CF. Despite this destruction of the pancreas, there is only modest loss of islets. While the pathophysiology of CF pancreas disease and CFRD are not well understood, it is appreciated that paracrine signals in the PDEC-islet axis could be one mechanism contributing to the deficit in insulin release in CFRD. The data supporting the existence of crosstalk between PDECs and islets are limited in translational human models, despite the observation of their close proximity within the pancreas.

The studies presented in this thesis represent an advancement in our tools to study this PDEC-islet axis. Collectively, the experiments enclosed in this thesis provides a better foundational understanding of *ex vivo* and cultured PDEC *CFTR*, *SOX9*, *AQP1*, *KRT7* and *KRT19* mRNA expression. Additionally, these findings represent advancement in developing a model to modulate *CFTR* expression in primary human PDECs. Ultimately, these discoveries set a path for downstream analysis that will prove valuable in the discovery and development of future therapeutics to treat CFRD.

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DEDICATION

"You are not expected to know everything about the universe right now - The whiteboard is not a test – it is a canvas. We want to take what is in your brain and put it on the canvas. From that ***starting point*** *with back-and-forth discussion, going back and reading some literature and* ***you*** *will adjust and refine that picture to something beautiful.*

Think of an artist. An artist does not paint a masterpiece from the very beginning. There is an initial sketch, then thinking about it. Trying something out, thinking about it again. Painting something, then thinking about it. Painting over, then thinking about.

I do not want you to give me the answer, I want to help you discover it because ***the journey*** *is what is valuable. You are never "wrong," you are just at a starting point. You* ***are*** *moving forward. Just keep showing up every day and do your best. You will get there, I am confident about that*" – Christine Krieger, PhD

This is an excerpt taken from a conversation with one of my many life mentors that was very meaningful to me. This dissertation is dedicated to my tribe of mentors who have inspired me to always do better and show up for myself.

PREFACE

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Chapter 1. PANCREAS AND ISLET MORPHOLOGY IN CYSTIC FIBROSIS: CLUES TO THE ETIOLOGY OF CYSTIC FIBROSIS-RELATED DIABETES

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1.1 ABSTRACT

Cystic fibrosis (CF) is a multi-organ disease caused by loss-of-function mutations in *CFTR* (which encodes the CF transmembrane conductance regulator ion channel). Cystic fibrosis related diabetes (CFRD) occurs in 40-50% of adults with CF and is associated with significantly increased morbidity and mortality. CFRD arises from insufficient insulin release from β cells in the pancreatic islet, but the mechanisms underlying the loss of β cell function remain understudied. Widespread pathological changes in the CF pancreas provide clues to these mechanisms. The exocrine pancreas is the epicenter of pancreas pathology in CF, with ductal pathology being the initiating event. Loss of CFTR function results in ductal plugging and subsequent obliteration. This in turn leads to destruction of acinar cells, fibrosis and fatty replacement. Despite this adverse environment, islets remain relatively well preserved. However, islet composition and arrangement are abnormal, including a modest decrease in β cells and an increase in α, δ and γ cell abundance. The small amount of available data suggest that substantial loss of pancreatic/islet microvasculature, autonomic nerve fibers and intra-islet macrophages occur. Conversely, T-cell infiltration is increased and, in CFRD, islet amyloid deposition is a frequent occurrence. Together, these pathological changes clearly demonstrate that CF is a disease of the pancreas/islet microenvironment. Any or all of these changes are likely to have a dramatic effect on the β cell, which relies on positive signals from all of these neighboring cell types for its normal function and survival. A thorough characterization of the CF pancreas microenvironment is needed to develop better therapies to treat, and ultimately prevent CFRD.

1.1 INTRODUCTION

Cystic fibrosis (CF) is the most prevalent life-limiting autosomal recessive disease affecting populations of Northern European descent (Elborn, 2016), although it is important to recognize that CF affects all racial/ethnic groups (Bobadilla et al., 2002; Cystic Fibrosis Foundation, 2021). Mutations

in the cystic fibrosis transmembrane conductance regulator (CFTR) leads to multi-organ disease, with progressive lung disease representing the leading cause of premature death (Elborn, 2016; Welsh & Smith, 1993). With substantial advances in therapies, life expectancy of people with CF in the United States has progressively increased, and is was reported to be 45 years in 2021 (Cystic Fibrosis Foundation, 2021). With this increase in survival, however, other features of CF have emerged, including CF-related diabetes (CFRD). CFRD affects 20% of adolescents and ~40-50% of adults with CF (Cystic Fibrosis Foundation, 2021; Moran et al., 2009) and, importantly, is an independent risk factor for worsened CF lung disease and results in a 4-6 fold greater mortality rate compared to people with CF who do not have diabetes (Kwong et al., 2019; Lewis et al., 2015; Marshall et al., 2005; Olesen et al., 2020). The inadequate release of insulin from the islet β cell underlies the development of CFRD (Merjaneh et al., 2015; Moran et al., 1991; Nyirjesy et al., 2018; Sheikh et al., 2017; Yi et al., 2016), manifest as a characteristic blunted and delayed insulin response to nutrient stimulation. One potential contributor is an impairment in the incretin axis (i.e. action of the hormones glucagon like peptide 1 and gastric inhibitory polypeptide to enhance insulin release in response to glucose) (Frost et al., 2019; Nyirjesy et al., 2022). However, much remains unknown about the etiology of insulin deficiency in CF, limiting the development of improved treatments for CFRD. The focus of this chapter is the islet/pancreas microenvironment whose constituent cell types, under normal conditions, provide critical signals to maintain β cell function, identity and survival (FIG1A). In CF, profound and widespread changes occur in this microenvironment (FIG1B), providing clues to novel mechanisms that may underlie loss of insulin release in this disease (FIG1C).

1.2 PATHOLOGY AND DYSFUNCTION OF THE EXOCRINE AND ENDOCRINE PANCREAS IN CF

Exocrine Pancreas Pathology and Dysfunction in CF

Within the pancreas, CFTR is predominantly expressed, at very high levels, in pancreatic ductal epithelial cells (PDECs) (Marino et al., 1991). There, its regulation of Cl^- and HCO_3^- transport is critical for maintaining luminal pH and water balance and maintaining acinar-derived digestive enzymes in a dilute, inactive form (McKay & Ooi, 2022; Wilschanski & Novak, 2013). Loss of CFTR function results in increased protein concentration and subsequent plugging of the ductal lumen (Wilschanski & Novak, 2013). This results in duct obliteration, which in turn leads to destruction of acinar cells and fatty replacement of the exocrine pancreas. This pathology is strikingly similar to that seen in other diseases of the exocrine pancreas, including chronic pancreatitis (Kloppel & Zamboni, 2023). Fibrosis is also a common feature of CF pancreas disease. Its etiology remains understudied, but likely involves activation of pancreatic stellate cells (PSCs), as has been demonstrated in other exocrine pancreas diseases (Apte et al., 2012). Under normal conditions, PSCs contribute to normal tissue structure and homeostasis (Apte et al., 2012). In disease, however, activation of PSCs results in their proliferation and trans-differentiation to myofibroblasts, leading to synthesis and deposition of a fibrotic, proinflammatory extracellular matrix (Apte et al., 2012; Masamune et al., 2009). The role for PSCs in CF pancreas fibrosis is therefore likely but remains to be clarified.

In CF, exocrine pancreas pathology is initiated very early in life [even *in utero* (Imrie et al., 1979)], and while pancreatic morphology appears relatively preserved in infants (<1 year of age), substantial abnormalities are seen by 5 years of age (Bogdani et al., 2017). Consequently, exocrine pancreas insufficiency occurs in the vast majority (~85%) of people living with CF (Wilschanski & Novak, 2013). Moreover, in the minority that retain exocrine pancreatic function, there is still evidence of damage to their pancreas (Wilschanski & Novak, 2013) with reports of pancreatitis occurring in individuals upon treatment with highly effective CFTR modulator therapies (HEMT) (Redman et al., 2021). Therefore, exocrine pancreas pathology is itself a major, debilitating feature of the CF disease

process. Moreover, as described below in section 3, emerging evidence suggests it may also be a key contributor to the pathogenesis of CFRD.

Endocrine Pancreas Pathology and Dysfunction

Given the extensive disruption of the exocrine pancreas in CF, it is surprising that islets remain relatively intact. However, the number and/or arrangement of endocrine cell types that make up the islet are significantly altered in CF.

Islet β cells

The islet β cell is the most abundant islet endocrine cells and is the source of the hormone insulin, which is required for maintenance glucose homeostasis. Impaired insulin release is common among people living with CF (Merjaneh et al., 2015; Mohan et al., 2009; Moran et al., 1991; Sheikh et al., 2017), and is the key contributing factor to the onset of CFRD (as it is for all forms of diabetes). Defects in processing of insulin from its precursor, proinsulin have been described in CF models (Edlund et al., 2019), a further indication of β cell dysfunction.

In human autopsy pancreas specimens, decreases in β cell area in adult CF subjects without diabetes when compared to non-CF controls have been reported in some (Abdul-Karim et al., 1986; Couce et al., 1996; Hart et al., 2018; Lohr et al., 1989) but not all studies (Bogdani et al., 2017; Hull et al., 2018); data from these studies are summarized in Table 1. β cell loss does not appear to differ based on the major form of exocrine pathology (i.e. fatty vs. fibrotic) (Lohr et al., 1989) although it appears that a greater degree of β cell loss is observed in younger CF subjects (Bogdani et al., 2017). Similarly, most studies report a modest but not severe loss of β cell mass in CFRD (Bogdani et al., 2017; Hull et al., 2018; Iannucci et al., 1984; Soejima & Landing, 1986).

The mechanisms underlying loss of β cells in CF are not well understood. β cell apoptosis may be a contributor, as has been described in one study (Bogdani et al., 2017). However, in T2D, it has been noted that the observed decrease in β cell mass is much greater than can be accounted for by the

rate of β cell apoptosis (Remedi & Emfinger, 2016). This, and other work, led to the concept that loss of β cell identity and not solely β cell death may contribute to the decreased number of functional β cells that are seen in diabetic conditions (Jonas et al., 1999; Remedi & Emfinger, 2016). In β cells, markers of differentiated identity include the transcription factors, MAFA, NKX6.1, PDX1, PAX6, NKX2-2, ISL1, NEUROD1, FOXO1 and FOXA2 (Walker et al., 2021) as well as key components of the β cell secretory pathway (including GCK, SLC2A2, SLC2A1 and INS itself). In CF, islet cell de-differentiation has been suggested based on work identifying islet cells that are positive for chromogranin A but negative for islet hormones, and the presence of polyhormonal cells (Cory et al., 2018). However, which cell type(s) may lose identity markers remains unclear. Only one study to date has investigated β cell identity markers in CF. Bulk RNAseq in islets from CF vs. non-CF donors revealed no significant changes in expression of key β cell transcription factors (*MAFA, NKX6.1, PDX1, PAX6, ISL1*) or components of the β cell secretory pathway (*INS, SLC2A2, SLC2A1, GCK, ABCC8, GLP1R*) (Hart et al., 2018).

This suggests that both β cell mass and identity remain largely unperturbed in CF, despite the extensive destruction of the surrounding exocrine pancreas, and that strategies to improve their function could be a viable means to treat and even prevent CFRD.

Islet α cells

The islet α cell is the second most abundant islet endocrine cell type, whose main function is the secretion of glucagon, a hormone which has a range of metabolic effects, including stimulation of hepatic glucose production (Taborsky, 2010; Wewer Albrechtsen et al., 2023). In contrast to the decrease seen in β cell abundance, α cells have been shown to be increased in autopsy pancreas specimens from donors with CF both with and without diabetes, using immunohistochemical and electron microscopy approaches (Bogdani et al., 2017; Hart et al., 2018; Hull et al., 2018; Lohr et al., 1989). However, despite the increased abundance of α cells, glucagon secretion (like insulin secretion)

is impaired in CF, in response to a range of stimuli including arginine and hypoglycemia (Aitken et al., 2020; Moran et al., 1991).

A possible contributor to α cell dysfunction in CF is loss of cell identity. In islets from CF donors, a significant decrease in *ARX* expression (Hart et al., 2018), one of the transcription factors critical for α cell identity (Walker et al., 2021), was observed. While more research is needed, an imbalance between the abundance vs. function/identity of α cells seems to be a key feature of islet pathology in CF.

Islet δ, γ and ϵ cells

The population of islet δ cells is variable and much less abundant than α or β cells. δ cells secrete somatostatin and act as intra-islet paracrine negative regulators of α and β cells (Gao et al., 2021). Plasma levels of somatostatin have been reported to be increased in CF (Meacham et al., 1993). However, the half-life of islet-derived somatostatin is very short and as such peripheral levels are likely not reflective of those within the islet. Therefore, the status of δ cell function in CF remains unknown. Morphologically, similar to α cells, the abundance of δ cells is significantly increased or shows an upward trend in CF islets based on multiple studies (Abdul-Karim et al., 1986; Bogdani et al., 2017; Hart et al., 2018; Hull et al., 2018; Lohr et al., 1989; Soejima & Landing, 1986), suggesting that local concentrations of somatostatin in the CF islet may be elevated.

γ cells make up only about 5% of islet area and are found more commonly in the head of the pancreas. These cells release pancreatic polypeptide (PP), and PP levels have been shown to be indicative of vagal input to the pancreas. In general, the abundance of γ cells, like α and δ cells, is increased in CF (Bogdani et al., 2017; Hull et al., 2018), although this has not been a universal finding (Iannucci et al., 1984), and γ cells do not seem to be consistently increased in islets from CF donors with and without diabetes (Hull et al., 2018). Despite some inconsistencies in reports of the abundance

of γ cells, there is a profound defect in PP release in CF, revealing another example of a disconnect between the abundance and function of islet endocrine cells in this disease.

ϵ cells are the fifth and final type of endocrine islet cell type in the pancreas. ϵ cells produce ghrelin, which is principally known as a gut hormone that activates the growth hormone secretagogue receptor (Pradhan et al., 2013). Circulating levels of ghrelin (specifically the active, acylated form) are increased in CF (Monajemzadeh et al., 2013). However, a more accurate picture of islet ϵ cell function in CF requires direct examination of ghrelin levels within the pancreatic islet; this has not been determined to date.

Regarding cell identity markers, less is known about the nature of these in δ, ε, and γ cells, although there is overlap between transcription factors expressed in ε and γ cells, with similarity to those expressed in α cells (Walker et al., 2021). Furthermore, HHEX has been shown to contribute to δ cell differentiation (Zhang et al., 2014). Whether these identity markers are compromised in the CF islet and could thereby contribute to impaired function of δ, γ and ϵ cells remains to be determined.

1.3 PATHOLOGICAL CHANGES IN NON-ENDOCRINE ISLET CELLS

Microvasculature

The pancreatic islet has an extensive microvascular capillary network which acts as a conduit for transportation of nutrients to islet endocrine cells and delivery of islet hormones to peripheral tissues (Hogan & Hull, 2017; Jansson & Carlsson, 2019; Peiris et al., 2014; Richards et al., 2010). In addition, the islet microvasculature has emerged as a key source of supportive signals (e.g. growth factors, extracellular matrix components) that are necessary to maintain islet β-cell function and survival. Both of the main constituent cell types, islet endothelial cells which form the capillary wall and the constrictive pericytes which surround them, have been shown to play this positive role in modulating β cell function and survival (Hogan & Hull, 2017; Hogan et al., 2017b; Houtz et al., 2016;

Johansson et al., 2009; Johansson et al., 2006; Nikolova et al., 2006; Peiris et al., 2014; Sakhneny et al., 2021; Sakhneny et al., 2018).

In both T1D and T2D structural defects in the islet vasculature have been observed, including thickening and fragmentation of capillaries and an apparent increase in vascular density (Brissova et al., 2015; Canzano et al., 2018). Moreover, in models of T2D, endothelial cells attain an inflamed, pro-adhesive phenotype (Castillo et al., 2022a; Hogan & Hull, 2017; Hogan et al., 2017a; Lacraz et al., 2009) that renders them incapable of supporting insulin release (Hogan et al., 2017b). In CF, changes in islet microvascular density appear to differ profoundly from what is seen in T1D and T2D. Specifically, preliminary data from our lab demonstrate a substantial decrease in capillary density in islets and exocrine pancreas (Castillo et al., 2022b). Moreover, islet bulk RNAseq data from human donors with CF exhibit increases in inflammatory markers, including key indicators of endothelial inflammation/activation including *SELE* and *IL6* (Hart et al., 2018). Together, these data suggest that while there is a decrease in islet endothelial cell abundance within CF islets, those remaining endothelial cells are highly inflamed.

While some data exist regarding islet endothelial cells in CF, the other main constituent of the islet microvasculature, the pericyte, remains entirely unstudied. Pericytes surround microvascular endothelial cells and are responsible for control of blood flow (Almaca et al., 2018; Landsman, 2019) but are also critical for micro vessel stability and the prevention of vessel leakage and inflammation. Loss of pericyte attachment and/or cell death has been defined as a critical pathological event in multiple vascular diseases including tumor growth, diabetic retinopathy and kidney fibrosis, and the close interaction between endothelial cells and pericytes has been shown to be disrupted in islets in T2D and models thereof (Almaca et al., 2018; Castillo et al., 2022a; Mateus Goncalves et al., 2020). These data suggests that loss of microvascular stability could be a common pathological feature of the diabetic islet. Whether the same is true in CF/CFRD remains an important unanswered question.

Innervation

It has long been appreciated that pancreatic islets are innervated by sympathetic, parasympathetic and sensory neurons (Ahren, 2000; Hampton et al., 2022). Parasympathetic input stimulates, while sympathetic input inhibits insulin release (Ahren, 2000; Hampton et al., 2022). Moreover, sympathetic input to the α cell is required for the glucagon response to hypoglycemia (Taborsky, 2010). Islet innervation in CF has not been well-studied, although a decrease in PGP9.5+ nerve fiber density has been reported in the CF pig pancreas (Uc et al., 2015). The profound defect in PP release in people living with CF is suggestive of a loss of vagal input to the pancreas, and the loss of islet (and exocrine) capillary density would also be expected to be reflective of impaired innervation to both pancreas compartments, given the known close association of the vasculature and autonomic nerve fibers (Hampton et al., 2022). However, more data are clearly needed to delineate the state of pancreatic innervation in human CF.

Immune Cells

While (increased) macrophages within the islet have been reported to have detrimental effects under diabetic conditions (Eguchi & Nagai, 2017), it is now well established that resident intra-islet macrophages are critical for β-cell growth, regeneration following injury and function/glucose homeostasis (Brissova et al., 2014; Chittezhath et al., 2019; Mussar et al., 2017; Nackiewicz et al., 2020; Tessem et al., 2008; Xiao et al., 2014). We previously reported that increased IL-1β positivity is a common and early feature of the islet in CF (Hull et al., 2018). Based on data from T2D, whereby increased IL-1β production is indicative of an increase in the number and/or activation state of macrophages, this observation prompted an investigation of the abundance of islet macrophages in CF. Surprisingly, we and others found an almost complete absence of intra-islet macrophages in adolescents and adults with CF (Bogdani et al., 2017; Hull et al., 2018) (regardless of diabetes status), despite the continued presence of macrophages in exocrine pancreas.

Islets also contain other resident leukocyte populations, including relatively rare T-lymphocytes. Increased T cell infiltration is a hallmark of autoimmune T1D and has also been described in islets in CF pancreas sections taken from children and adults (Bogdani et al., 2017; Hart et al., 2018). Flow cytometry analysis of CF donor islets leukocyte populations revealed a relatively abundant CD3+ T cell population, which largely (~60%) consisted of CD8+ T cells (Hart et al., 2018).

Islet Amyloid

Amyloids are aggregates of misfolded proteins and have been linked to the development of numerous diseases. In the islet, amyloid deposits contain as their unique component islet amyloid polypeptide (IAPP), which is a normal secretory product of the β cell (Hull et al., 2004). Islet amyloid deposition has been recognized as a feature of islets in CF for several decades (Couce et al., 1996) and, unlike many of the other features described above is pathognomonic of CFRD, occurring in 60-70% of cases compared to only 0-20% of individuals with CF without diabetes (Bogdani et al., 2017; Couce et al., 1996; Hull et al., 2018). Moreover, islet amyloid does not appear to be a pathological feature in children with CF (Bogdani et al., 2017), although, deposition in CFRD appears to be markedly accelerated compared to the classically-described islet amyloid seen in subjects with T2D, where amyloid is associated with established disease in individuals around the 6th-7th decade of life (Bell, 1959; Hull et al., 2004).

1.4 POTENTIAL MECHANISMS UNDERLYING CFRD

Investigation of Intrinsic Effects of CFTR in the β cell

Perhaps the most straightforward explanation for β cell dysfunction in CF would be that mutated CFTR has an intrinsic effect to impair insulin secretion in β cells. Indeed, some studies provide evidence for β-cell CFTR expression/activity and an effect on insulin release (Edlund et al., 2019; Edlund et al., 2014; Guo et al., 2014; Ntimbane et al., 2016). Conversely, studies using RNAseq, in situ hybridization and immunohistochemistry show that β cell expression of CFTR is very low and/or

occurs in a small proportion of β cells (Boom et al., 2007; Hart et al., 2018; Norris et al., 2019; Sun et al., 2017; Uhlen et al., 2015; White et al., 2019). Moreover, patch-clamp electrophysiology failed to detect a forskolin-activated chloride currents in human β cells (which would be consistent with CFTR activity) (Hart et al., 2018), and CFTR modulators were unable to alter insulin release in isolated human islets in response to glucose and cAMP-mediated activation (Hart et al., 2018). These latter data are consistent with no impact on insulin release or glucose tolerance in mouse models with β cell deletion of *Cftr* (Hart et al., 2018). Therefore, while intrinsic effects of CFTR in β cells may occur, it seems likely that extrinsic effects of the profoundly altered microenvironment in the CF pancreas are also important in dysregulation of insulin release.

Impact of Pancreatic Ductal Pathology on β cell Function

There is clear support from the literature for a role of exocrine pancreas pathology/dysfunction on insulin release, best illustrated by the fact that CF patients with pancreatic insufficiency have a greater deficit in insulin and glucagon release vs. those with residual exocrine pancreatic function (Moran et al., 1991; Nyirjesy et al., 2018; Sheikh et al., 2017). As mentioned, exocrine pathology is initiated in young children, although it remains relatively normal until around 1 year of age, suggesting there may be a window of opportunity for prevention. Therefore, understanding mechanisms whereby early stages of exocrine pancreas pathology may exert detrimental effects on the β cell is important and timely.

Some evidence exists to suggest that ductal pathology may contribute directly to β-cell dysfunction. First, small intercalating ducts are located in close proximity to islets, making them appropriately positioned to exert effects on β cells (Bertelli & Bendayan, 2005; El-Gohary et al., 2016; Fowler et al., 2018). Second, the limited available data examining an effect of PDECs on β cell function under normal circumstances generally show a positive effect. Specifically, co-incubation of human islets with PDECs ameliorated the decline in β-cell function which is observed with long term culture

(Gatto et al., 2003; Murray et al., 2009). It has also been reported that co-transplantation of islet with PDECs improved islet transplantation outcomes (Benomar et al., 2018; Street et al., 2004; Webb et al., 2012) In contrast, one study showed that PDEC-secreted factors resulted in increased basal insulin release (with no effect on GSIS), and worsened islet transplantation outcomes (Marin-Canas et al., 2019). The reason for the discrepant data is not clear, but the authors of the latter study suggest that "inflammation may mediate the deleterious effects of ductal cells on islet cells".

Several studies suggest that loss of CFTR expression/activity from PDECs can impair insulin release (Norris et al., 2019; Shik Mun et al., 2019; Sun et al., 2017). Knockdown of CFTR in isolated islets (from ferrets or human donors) was sufficient to impair insulin release (Norris et al., 2019; Sun et al., 2017). This interpretation is based on the authors' demonstration that CFTR expression in isolated islets was restricted to ductal epithelial cells (the presence of ductal cells within isolated islet preparations is well supported in the literature (Ichii et al., 2008; Keymeulen et al., 2006; Segerstolpe et al., 2016; Sun et al., 2017; Warnock et al., 2005)). A separate study developed a model whereby islets were cultured in close proximity to human PDECs (Shik Mun et al., 2019). Here, acute CFTR inhibition in those PDECs resulted in decreased insulin release from islets. While this study had the advantage of a PDEC-selective intervention, it had some limitations, such as use of the pharmacological inhibitor CFTR (inh)-172, which has been shown to have non-specific effects (Friard et al., 2017; Kelly et al., 2010; Melis et al., 2014; Sun et al., 2017).

Further, PDECs with defective CFTR function likely release proinflammatory mediators that could impair insulin release from β cells. Human pluripotent stem cell (hPSC)-derived PDECs from CF donors express a proinflammatory transcriptome (Hohwieler et al., 2017). Moreover, PDECs from CF ferrets release a pro-inflammatory secretome (Rotti et al., 2022). Several of the differentially regulated proteins identified in the latter study have been reported to impact pathways known to affect β cell/glucose metabolism (Amyot et al., 2012; Arima et al., 2020; Assayag-Asherie et al., 2015; Garay-

Malpartida et al., 2011; Gurlo et al., 2016). For example, IGFBP7 was found to be downregulated in PDECs from CFTR$^{-/-}$ ferrets, and exogenous treatment of wild-type whole islets from ferrets, IGFBP7 resulted in altered insulin secretion (Rotti et al., 2022). Together, these data strongly suggest that PDEC dysfunction, especially in the early stages of CF pancreas disease may contribute to impaired β cell function/survival.

Impact of Acinar Pathology on β cell Function

It is well-established that pancreatic ductal pathology is also the key initiator of pancreatic acinar destruction in CF (Andersen, 1958; Bogdani et al., 2017; Imrie et al., 1979; Wilschanski & Novak, 2013), and in turn the profound destruction and remodeling of acinar cells is a likely source of signals that may negatively impact the β cell.

There is evidence in the literature to support the existence of an acinar-islet axis: insulin is known to modulate pancreatic acinar cell function (Adler & Kern, 1975; Söling & Unger, 1972), while pancreatic acinar-derived products affect islet cell function and proliferation (Aida et al., 2018; Aida et al., 2014; Egozi et al., 2020; Henderson, 1969). This suggests that normal acinar function is important in maintaining β cell function and, conversely, that acinar pathology or loss in CF could contribute to impaired insulin release.

Potential mediators of negative effects of inflamed acinar cells on the islet come from *in vitro* studies of chronic pancreatitis (which also manifests acinar dysfunction/destruction) (Lugea et al., 2017). These studies show increased production of proinflammatory cytokines such as TNF-α and IL-1β from pancreatic acinar cells, which are known to mediate impaired insulin release and/or β cell death. Recent work focused on MODY8 (which occurs due to mutations in the acinar lipase *CEL*) showed that defective acinar cells were able to induce ER stress and β cell secretory dysfunction (Kahraman et al., 2022). Additionally, the extensive fibrosis and infiltrating fat that characterize the

CF pancreas may themselves release factors that could impact β cell survival and function, although the nature of such factors remain unknown.

Endocrine Cell Cross-Talk

Disruptions to the islet microenvironment likely also contribute to the insulin deficiency that characterizes CF. In α cells, several studies suggest that CFTR may have intrinsic effects (Edlund et al., 2017; Huang et al., 2017), although whether CFTR is expressed/active in α cells remains controversial and understudied. It is now well recognized that paracrine effects of α cell products (predominantly glucagon and GLP-1) are required for optimal β cell function. However, the increased α cell abundance in the face of impaired insulin release in CF suggests an impairment in that paracrine axis. Indeed, new data suggests that gene expression related to cell identity and hormone secretion is altered in α cells from CF donors (Hart et al., 2018; Vemireddy et al., 2023), and that β cell GLP-1R expression is also decreased (Vemireddy et al., 2023), both of which would be consistent with impaired α-to-β cell communication within the CF islet.

For other islet endocrine cells, fewer data exist. However, the increased abundance of δ cells suggests that local islet somatostatin levels may be elevated, which would be expected to suppress both insulin and glucagon release. For PP cells, these may also be increased in CF, despite a suppression of circulating PP levels, suggesting again that local islet levels may be increased. Given that PP has been shown to inhibit glucagon secretion through the PPYR-1 receptors in α cells (Aragon et al., 2015), an increase in islet PP could be another means by which glucagon (and thereby insulin) release could be impaired in CF. For ϵ cells, as mentioned, the status of islet ghrelin production in CF is unknown. However, circulating levels are increased (Monajemzadeh et al., 2013) which, given the known effect of ghrelin to suppress insulin release (but enhance glucagon release) could also impact islet function. Taken together, dysregulation of islet endocrine cell composition and/or function likely play a critical role in perturbing insulin release in CF.

Changes in the Pancreas/Islet Microenvironment

It is highly likely that the decrease in islet vascular density reported in our preliminary study has a profound effect on insulin secretion into the peripheral circulation. In fact, the profile of insulin release classically seen in CF, namely a blunted and delayed insulin response (Moran et al., 1991; Nyirjesy et al., 2018; Sheikh et al., 2017), could be explained almost entirely by the impaired delivery of nutrients to the islet along with inefficient delivery of insulin to the peripheral circulation that would be expected to occur in the face of decreased islet vascularization. The concept that impaired insulin release may be at least partly dependent on this loss of vascularity, also fits with data showing that β cell mass (Abdul-Karim et al., 1986; Bogdani et al., 2017; Couce et al., 1996; Hart et al., 2018; Hull et al., 2018; Lohr et al., 1989) and identity (Hart et al., 2018) are relatively preserved in CF, suggesting again that "indirect" mechanisms likely explain the deficient insulin release. And, moreover, this is also consistent with data showing that when isolated islets from CF donors are studied *ex vivo* (i.e. under conditions where they are no longer dependent on vascularization for nutrient delivery and hormone release), insulin release is relatively intact (Hart et al., 2018). Of course, additional data will be required to fully test this hypothesis.

Similarly, the anticipated loss in autonomic innervation to the CF pancreas is also likely to have a major impact on islet function. This decreased innervation is likely to be a generalized effect, affecting sympathetic, parasympathetic and sensory input, as it is anticipated to occur secondary to exocrine pancreas destruction (and loss of vasculature). Therefore, CF islets may be essentially denervated, which would certainly be consistent with known defects such as impaired insulin release (e.g. due to reduced cholinergic tone), loss of the glucagon response to hypoglycemia (due to loss of sympathetic input) and essentially absent PP secretion (due to loss of vagal input).

While the activation of PSCs during fibrosis has been most widely studied in the context of pancreatic cancer and pancreatitis (Apte et al., 2012; Masamune et al., 2009) there is also some

evidence to suggest the activated PSCs can be detrimental to β cells. Several studies demonstrate that activated PSCs can impair β cell development (Li et al., 2021) induce β cell death (Kikuta et al., 2013; Kim et al., 2020; Zang et al., 2015), with some (Zha et al., 2014) but not all studies (Lee et al., 2017) also suggesting a negative impact on β cell function. Analysis of secreted factors from PSCs has been published; investigation of these data may inform our understanding of potential mechanisms underlying β cell dysfunction in CF.

Several lines of evidence suggest that the islet microenvironment in CF is highly inflamed. As described above, a diverse array of proinflammatory molecules are produced by both CFTR-defective PDECs and inflamed acinar cells in the surrounding exocrine pancreas, as well as CD8+ T-cells, which are known to produce proinflammatory cytokines, and inflamed/activated endothelial cells within the islet itself. Deposition of islet amyloid, in addition to its well-known toxic effects on β cells, has proinflammatory effects on both leukocytes and endothelial cells (Castillo et al., 2022a; Masters et al., 2010; Westwell-Roper et al., 2011). Moreover, the proinflammatory cytokines IL-1β and IL-6 have both been shown to be produced by human or ferret islets, respectively, in CF (Hull et al., 2018; Sun et al., 2017); these may derive from endocrine cells themselves, although the cellular source has not been definitively determined. Finally, the loss of intra-islet macrophages would further compromise the islet's ability to clear cell debris or pathological material such as amyloid deposits, which are known to stimulate pathways such as inflammasome and toll like receptor signaling, further exacerbating the inflammatory milieu of the CF islet. Overall, this inflammatory assault on the islet is a prime target for therapeutic intervention, with approaches ideally targeting multiple cell types and/or inflammatory mediators.

1.5 CONSIDERATIONS FOR TREATMENT OF CFRD

The ideal treatment for CFRD would be correction of *CFTR* mutations to reverse or even prevent exocrine and endocrine pancreas disease. Highly effective CFTR modulator therapies (HEMT)

have had major positive benefit in treating CF lung disease and improving nutritional status/body mass index (Accurso et al., 2010; Burgel et al., 2020; Middleton et al., 2019; Volkova et al., 2020). However, only limited data are available demonstrating their impact on insulin release and glucose tolerance [reviewed in (Merjaneh et al., 2022)].

Since exocrine and endocrine pancreatic defects begin very early in life (Andersen, 1958; Ode et al., 2010; Yi et al., 2016), CFTR modulators may need to be initiated in very young children to see substantial benefit. Encouragingly, in young children (between 4 months and 5 years old), the CFTR modulator ivacaftor shows improvements in exocrine pancreas function (Davies et al., 2016; Davies et al., 2021; Rosenfeld et al., 2019), with small studies (including children as young as 5 years old) also showing improved insulin release (Bellin et al., 2013; Kelly et al., 2019). However, it is important to recognize that many people with CF may not be able to fully benefit from these interventions (e.g. those with already-established pancreas disease or whose CFTR mutations do not have available modulator therapies). Moreover, concerningly, the improved nutritional status with the administration of HEMTs have resulted in increased prevalence of overweight and obesity in people with CF as well as the emergence of features of metabolic syndrome (Kutney et al., 2021), both of which may further increase the prevalence and/or severity of CFRD.

It seems that early adoption of HEMT may be possible in some people with CF. However, even in those people, combination of HEMT with therapies aimed at reversing or compensating for aspects of the disrupted pancreas/islet microenvironment (illustrated in Figs. 1B and 1C) may well be necessary to improve β cell function. These include three major areas of intervention. First, the replacement of factors that are lost or whose release is impaired in CF may be beneficial. Incretin-based therapies fit into this category and have been shown to be efficacious in small studies of people with CF (Gnanapragasam et al., 2020; Nyirjesy et al., 2022) but their broad applicability remains to be demonstrated. Other islet-specific mediators may include glucagon or factors elicited from the islet

microvasculature which are required for optimal insulin release. Second, correcting aberrant restraint on the β cell due to the upregulation of paracrine inhibitors such as somatostatin or ghrelin may be effective in restoring insulin release. Finally, a key component will likely be blockade of inflammatory mediators, which are released by multiple islet/pancreatic cell types (e.g. exocrine, vascular and adaptive immune cells; see Fig 1C) and which are well known to impair β cell function and identity. To develop such approaches will require efforts to generate a more comprehensive picture of the underlying pathways and mediators which result in a compromised pancreatic/islet microenvironment in CF.

1.6 ACKNOWLEDGEMENTS

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1.7 FIGURES

TABLE

Table 1. Summary of findings from morphometric studies examining islet endocrine cell types in donors with CF (no diabetes) and/or CFRD.

	Donor information		Comparison: CF vs non-CF					Comparison: CFRD vs CF no diabetes				
Study	Donor samples/ group	Donor age range	Beta cell		Alpha cell	Delta cell	PP cell	Beta cell		Alpha cell	Delta cell	PP cell
			per islet	per pancreas				per islet	per pancreas			
Iannucci et al 1984	4-6	Adolescent -adult	-	-	-	-	-	Decreased	-	Increased	Increased	No change
Abdul-Karim et al 1986	4-11	Adolescent -adult	Decreased	-	No change	Increased	-	Decreased	-	No change	Increased	-
Soejima et al 1986	6-34	Pediatric-adult	-	-	-	-	-	Decreased	-	No change	Increased	-
Lohr et al 1989	6-23	Adolescent -adult	Decreased	-	Increased	Increased	Increased	-	-	-	-	-
Couce et al 1996	12-16	Adolescent -adult	Decreased	-	-	-	-	No change	-	-	-	-
Bogdani et al, 2017	3-8	Adult	Decreased	No change	Increased	Increased	No change	Decreased	No change	No change	No change	No change
Hart et al, 2018	5-7	Adult	No change	Decreased	Trend	Increased	-	-	-	-	-	-
Hull et al 2018	17-20	Pediatric-adult	No change	No change	Increased	Trend	Increased	Trend	No change	Increased	No change	No change
Bogdani et al, 2017	11-16	Young (<4 years)	Decreased	Decreased	Increased	Increased	Increased	-	-	-	-	-

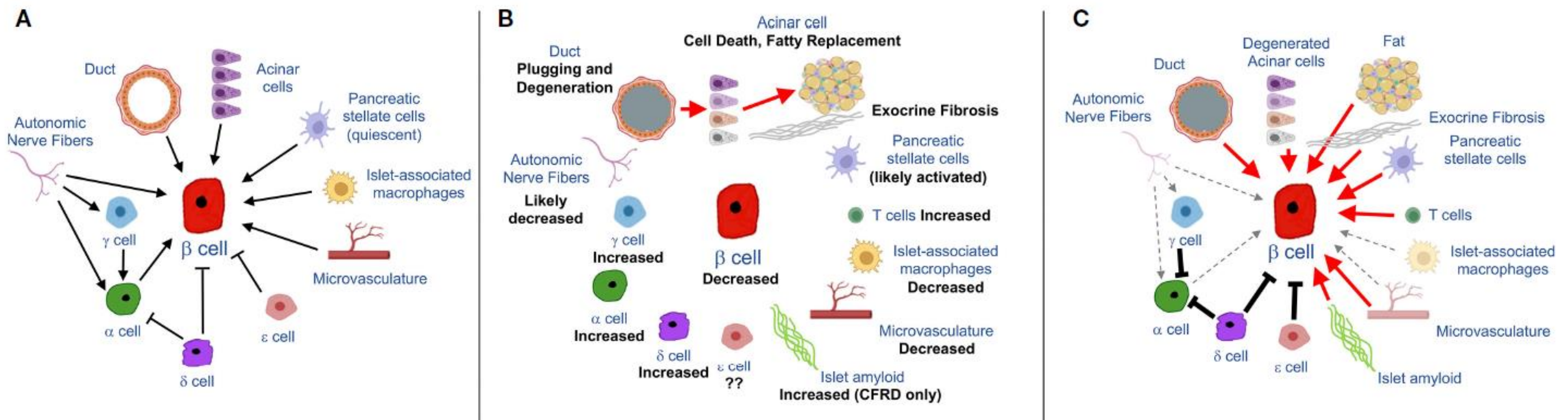

Figure 1. Schematics showing input from cells of the pancreas/islet microvasculature cells under normal (A) and CF (B, C) conditions. A: Summary of physiological/homeostatic input to β cell from exocrine-, endocrine or islet-associated cell types. B: Pathological features of the exocrine pancreas in CF, emanating from ductal pathology, as indicated by red arrows, along with changes in abundance of islet endocrine cells and support cells (nerve, vascular and immune cells). C: hypothesized implications for β cell function and/or survival based on CF pancreatic pathology. Red arrows denote detrimental (chiefly inflammatory) signals derived from diseased cell types. Dashed grey arrows denote decreased input to the β cell due to loss of nerve fibers, macrophages or vasculature. Solid black lines denote increased inhibitory input to β and α cells (due to increased abundance of δ, γ and potentially also ϵ cells).

Chapter 2. GENERATION AND MAINTENANCE OF PRIMARY HUMAN PANCREATIC DUCTAL EPITHELIAL CELLS WITH *CFTR* EXPRESSION

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2.1 ABSTRACT

CFTR is highly expressed in pancreatic ductal epithelial cells (PDECs) and facilitates anion transport into the ductal lumen to maintain pH and concentration of digestive enzyme secretions from acinar cells. In CF, destruction of pancreatic exocrine (acinar) tissue is common, leading to pancreatic exocrine insufficiency in 85% of people with CF. Endocrine pancreatic dysfunction ,such as impaired insulin release, is also a common feature of CF resulting in CF-related diabetes (CFRD) in 40-50% of adults. The pathophysiology of CF pancreas disease, especially CFRD, is not well understood, but the pancreatic duct is the initiating site of pathology. Based on data from animal models, we hypothesize that PDECs secrete factors that may impair islet function. In order to study these effects, we have developed an approach to isolate, culture and modulate CFTR in primary human PDECs as a model to study CF pancreas disease. PDECs were isolated from cadaveric human pancreatic tissue (from donors without CF or diabetes) via HPd1+ antibody-magnetic beads, which selectively binds to ductal epithelial cells in human pancreas. PDEC genes of interest were enriched in HPd1+ cells. Additionally, HPd1+ cells were amendable to expansion in culture. However, HPd1+ cells cultured for 1-4 weeks resulted in a decrease of CFTR mRNA expression to 6.4% of the levels seen on the day of isolation. Despite the observation that CFTR expression was downregulated, cultured primary PDECs still expressed CFTR at substantially greater levels than that seen in commercially available pancreatic ductal cell lines (Capan 1, PANC1, and H6C7). A LentiCRISPRv2 plasmid encoding synthetic guide RNA (sgRNA) was designed to target the region of CFTR near exon 11 to generate LentiCRISPRv2.hCFTRsgRNA73 (LentiCRISPR2-CFTR). Using our in vitro culture model of human PDECs, we have shown that LentiCRISPR2-CFTR is effective at decreasing expression of full length CFTR mRNA in a consistent manner in primary HPd1+ hPDECs. In summary, we have demonstrated the ability to isolate human PDECs which will retain key PDEC genes of interest in culture and are amenable to inhibition of CFTR expression using a LentiCRISPR approach. These approaches will serve as tools to generate a potentially valuable model system for investigation of CF pancreas disease.

2.2 INTRODUCTION

Within the pancreas, CFTR is most highly expressed in ductal epithelial cells (Venglovecz et al., 2018). Under normal conditions, CFTR is responsible for HCO_3^- transport which is essential for maintaining alkaline pH and water balance in the pancreatic ductal lumen, maintaining digestive enzymes secreted from acinar cells in a dilute, inactive form. In CF pancreas, loss of

CFTR function results in protein precipitations which plug the ducts and lead to their obliteration (Malik et al., 2023, Wilschanski & Novak, 2013). The subsequent destruction of the acinar tissue in the exocrine pancreas leads to pancreatic exocrine insufficiency in 85% of people with CF (Malik et al., 2023, Wilschanski & Novak, 2013). In addition to exocrine pathology, endocrine pancreas pathology is also common, with CF-related diabetes (CFRD) affecting 40-50% of adults living with CF (Moran et al., 2009). While the mechanisms underlying CFRD remain unclear, several groups have hypothesized a role for ductal-islet crosstalk (Shik Mun et al., 2019, Rotti et al., 2022). The literature largely supports the concept that pancreatic ductal epithelial cells (PDECs) exert a positive effect on islet function and/or survival (Ilieva et al., 1999, Street et al., 2004). Conversely, human PDECs pre-treated with a pharmacological CFTR inhibitor elicited reduced glucose-stimulated insulin release from islets using an organ-on-a-chip model (Shik Mun et al., 2019). Clues to mechanisms underlying this reduced insulin release come from PDECs isolated from CF ferrets, which produce a proinflammatory secretome (Rotti et al., 2022). Indeed, some of the factors (e.g. IGFBP7) produced by CF ferret PDECs have detrimental effects on islet function/survival (Rotti et al., 2022). However, whether human PDECs with defective CFTR also exhibit an altered secretome which may have a detrimental effect on insulin release remains unknown. Progress in this area is hampered by the lack of readily available model systems that recapitulate primary human PDECs. Commercially available immortalized PDEC lines are derived from adenocarcinoma (e.g. PANC1 or H6C7 cells) and/or have been reported to have poor (Sood et al., 1992) or unknown *CFTR* expression. Several groups have reported the generation of PDECs from human pancreas, but these studies have not consistently reported CFTR expression or activity. In the present study, we sought to determine whether PDECs can be isolated from islet depleted pancreas samples available from human cadaveric donors through the NIH-funded Integrated Islet Distribution Program (IIDP) and whether these cells are amenable to culture and modulation of *CFTR* expression.

2.3 METHODS

Isolation and culture of primary human pancreatic ductal epithelial cells

Islet-depleted pancreatic samples (designated “input” for data analysis) were obtained from 17 human donors (characteristics in Suppl Table 1) from the Integrated Islet Distribution Program (IIDP). Samples were accepted between June 2021 to November 2023 without restriction on the basis of donor characteristics; all samples that could be practically accommodated were accepted.

Pancreas samples were dissociated to single cells (Cell Dissociation Buffer; Gibco), incubated with biotinylated HPd1 antibody (Novus Bio), which targets ductal epithelial cells (Dorrell et al., 2008) and purified over a streptavidin-magnetic microbead columns (Miltenyi Biotec, Bergisch Gladbach, Germany; Experimental design shown in Suppl Fig 1)

On average HPd1+ cells were seeded at density of 50,000 cells/cm^2 onto Collagen 1A-coated plates and grown in PneumaCult EX+ media (Stem Cell Technologies, MA, USA) as in (Rotti et al., 2022) supplemented with 0.5% Penicillin/Streptomycin (Pen/Strep; Sigma-Aldrich) and 10 µg/ml of Soybean Trypsin Inhibitor (Gibco). Media were changed every 24-48h. RNA was harvested 1-4 weeks after isolation, due to differences in growth rates among cells derived from different donors. Passage 1 was defined as the first trypsinization of cells.

RNA isolation and real time quantitative PCR

RNA extraction was performed using High Pure RNA Isolation Kit (Roche, Basel Switzerland) and reverse transcribed (High-Capacity cDNA Reverse Transcription Kit; Thermo Fisher Scientific). Gene expression was determined using TaqMan real-time quantitative PCR (Life Technologies, Corp., Carlsbad, CA) for *GAPDH* (Hs02786624_g1), *CFTR* (Hs00357011_m1), *INS* (Hs00355773_m1), *GCG* (Hs01031536_m1), *PNLIP* (Hs00609591_m1), *CPA1* (Hs01056157_m1), *AMY1BA* (Hs00420710_g1), *AQP1* (Hs01028916_m1), *KRT7* (Hs00559840_m1), SOX9 (Hs00165814_m1), and KRT19 (Hs00761767_s1). Each sample was run in triplicate. *GAPDH* was compared to *18S* (Hs99999901_s1) and *ACTB* (Hs01060665_g1) and found to be appropriate for use as a housekeeping gene. Results were calculated using the $\Delta\Delta C_t$ method.

Cell lines and cell line culture

The transformed *CFTR*-expressing PDEC line Capan1 (ATCC, accession number HTB-79) was seeded at 66,000 cells/cm^2 and cultured in RPMI 1640 medium with 11.1 mM glucose, 20% fetal bovine serum (FBS), 0.5% penicillin streptomycin and 0.5% sodium pyruvate (all by volume) media (Chambers & Harris, 1993). PANC-1 cells (ATCC, accession number CRL-1469) were seeded at 18,000 cells/cm^2 and cultured in RPMI media as above. H6C7 cells were seeded at 19,000 cells/cm^2 and grown in Keratinocyte serum-free (KSF) medium (Gibco/Invitrogen) supplemented with 50 mg/ml bovine pituitary extract (BPE), 5 ng/ml epidermal growth factor (EGF), and 1× antibiotic–antimycotic cocktail (Gibco/Invitrogen) as described (Ouyang et al., 2000). The colorectal adenocarcinoma cell line Caco2 (ATCC, accession number HTB-37) was used as a positive control for siRNA and lentiviral transduction (described below) as it is known to stably express *CFTR* (Sood et al., 1992). Cells were

seeded at average 13,000 cells/cm^2 in RPMI 1640 medium, 20% FBS, 0.5% Pen/Strep, and 0.5% sodium pyruvate (supplemented RPMI). For all cell lines, samples were generated from cells at passage 6 or less relative to the time of receipt from ATCC.

siRNA Knockdown of CFTR

Caco2 cells were plated at a density of 13,000/cm^2 in supplemented RPMI. After 24 h, media were removed, and cells were washed 2x in 500ul of supplemented RPMI 1640. The cells were then treated with 200ul of either siCFTR or siScramble (10nM) and Lipofectamine RNAiMAX in serum-free/antibiotic free RPMI 1640 for 6 hours. After 6 hours, 50ul of RPMI 1640 containing 25% fetal bovine serum, 0.5% Pen/Strep, and 0.5% sodium pyruvate was added to the cells for a final concentration of 5% of fetal bovine serum. Caco2 cells were treated for 24 hours before lysing for RNA isolation.

HPd1+ hPDECs were plated at 35,000 cells/cm^2 and cultured in PneumaCult EX+ media and supplemented with 0.5% Pen/Strep. Passage 8 was used for experiments. HPd1+ hPDECs transfection with siRNA followed the same procedure as described with Caco2 cells. HPd1+ hPDECs were also treated for 24 h before lysing for RNA isolation.

Lenti-CRISPR-Cas9 mediated CFTR gene disruption

A lentiviral-delivered CRISPR-Cas9 construct targeting *CFTR* was designed as a knockdown strategy using the University of Washington Diabetes Research Center's CRISPR Virus and Transgenic Mouse Core. A synthetic guide RNA (sgRNA) targeting the region of *CFTR* which encodes G542 near exon 11 was cloned into the LentiCRISPRv2 plasmid (addgene.com) to generate LentiCRISPRv2.hCFTRsgRNA73 (LentiCRISPR-*CFTR*). Caco2 cells were seeded 22,000/cm^2 in RPMI 1640 media with 11.1 mM glucose, 20% FBS, 0.5% Pen/Strep, and 0.5% sodium pyruvate. Caco2 cells were grown to sub-confluency prior to treatment with LentiCRISPR-*CFTR*. On average sub-confluency was reached between 4-7 days after seeding Caco2 cells.

LentiCRISRP2-*CFTR* was diluted 10ul/ml in RPMI 1640 medium with 11.1 mM glucose, 20% FBS, 0.5% Pen/Strep, 0.5% sodium pyruvate and Polybrene (2ug/ml; EMD Sigma TR-1003-G). Caco2 cells were incubated with this LentiCRISPR2-*CFTR* solution for 24 h, followed by 3-day culture in fresh media (no virus) and then puromycin (10ug/ml) selection for 12 days.

Primary HPd1+ hPDECs (passage 1) were seeded at 60,000/cm^2 and cultured to sub-confluency prior to treatment with LentiCRISPR-*CFTR*. On average sub-confluency was reached in between 7-10 days after seeding HPd1+ hPDECs. This was followed by a 24h incubation with *LentiCRISPR2-CFTR*

(10ul/ml) in PneumaCult EX+ media with 0.5% Pen/Strep, Soybean Trypsin Inhibitor (10 µg/ml) and Polybrene (2ug/ml). Cells were cultured for 4–5-days after transduction before initiating puromycin selection (10ug/ml) for ~8 days.

Statistical analysis

Data are presented as mean ± SEM. Student's *t* test (Wilcoxon matched pairs signed rank test) was used for two-group comparisons. One-way ANOVA with Dunnett's multiple comparisons test was used to detect significant differences for datasets with more than two groups. Prism (version 10.2.0; GraphPad Software, USA) was used for all statistical analyses. Numbers of experimental replications are represented by individual data points in figures or are provided in figure legends.

2.4 RESULTS

Isolation and analysis of Hpd1+ cells.

HPd1+ cells were generated from 17 donors (14 male, 3 female) with an average age of 43±16 years and average BMI of 32.1±5.8 kg/m^2 (Suppl Table 1). Compared to input pancreas tissue, HPd1+ cells showed increased mRNA levels of *CFTR* (FIG 1A; p=0.030) as well as ductal markers *SOX9*, *AQP1 KRT19* and *KRT7*. HPd1+ samples were also positive for acinar cell markers *PNLIP*, *AMY2A* and *CPA1* (FIG 1B), while levels for islet markers *INS* and *GCG* were significantly decreased in HPd1+ samples compared to input samples (FIG 1C; p=0.017 and p=0.002 respectively).

Short-term propagation of Hpd1 + cells.

A subset of preparations (n=9) was propagated in culture. Cultured cells retained the expected cobblestone morphology (FIG 2A). Following culture (ranging from 1-4 weeks due to donor variability) *CFTR* mRNA expression was decreased to 6.4% of the levels seen on the day of isolation (FIG 2B; p=0.003) but was still readily detectable (average Ct 24 ±2). mRNA levels of *SOX9* (FIG 2B; p=0.004) and *AQP1* (FIG 2B; p=0.004) were also decreased but still detectable (average Ct of 28±1 and 28 ± 3, respectively), while mRNA levels of *KRT7* and *KRT19* mRNA were significantly increased in cultured HPd1+ cells (FIG 2B; p=0.128 and p=0.203). In contrast, acinar markers *AMY2A, CPA1,* and *PNLP* were all substantially decreased vs. the day of isolation (average Ct 34 ± 07, 31±2, and 33 ± 7, respectively; FIG 2C; p=0.004, p=0.004, and p=0.004) and mRNA levels of *INS* and *GCG* were undetectable (data not shown) in HPd1+ cells following culture.

CFTR mRNA expression in commercially available cell lines.

Next, we compared *CFTR* mRNA expression in HPd1+cells, on the day of isolation and following culture, to that in commercially available PDEC lines (Capan1, PANC1, and H6C7 cells) using Capan1 cells, which are known to express *CFTR*, as a positive control. We normalized *CFTR* mRNA expression to HPd1+ cells on the day of isolation (FIG 2D). *CFTR* mRNA in HPd1+ cells on the day of isolation were 50-fold higher than even the positive control cell line Capan 1 (FIG 2D; $p<0.0001$). *CFTR* expression among commercially available PDEC lines Capan1, PANC1, and H6C7 was substantially lower than that seen in freshly isolated and cultured HPd1+ cells ($p<0.0001$ for all). We next sought to determine whether *CFTR* was the only lowly expressed gene in these cell lines or whether there was a generalized decrease in well-established PDEC markers. Capan 1 cells had increased expressed *SOX9* compared to cultured HPd1+ cells (FIG 2D). In contrast, day of isolation HPd1+ samples had a much greater expression of *SOX9* compared to Capan 1, PANC1, and H6C7 cells (FIG2D). Day of isolation HPd1+ samples had significantly higher *AQP1* expression compared to cultured HPd1+ cells, Capan 1, PANC1, and H6C7 cells (FIG2D). Notably, KRT7 expression was increased in cultured HPd1+ cells compared to day of isolation HPd1+ samples (FIG2D). Additionally, H6C7 cells had a similar expression of KRT7 to cultured HPd1+ cells and Capan 1 cells (FIG2D). Cultured HPd1+ cells had a higher expression of KRT19 compared to day of isolation and the PDEC cell lines (FIG2D).
We found that Capan1 cells expressed *SOX9, AQP1,* and *KRT19* at much higher levels compared to PANC1 and H6C7 cells (FIG2D).

CFTR expression modulated by siRNA.

Next, we determined whether it was possible to modulate *CFTR* expression in our HPd1+ cells. We first tested Capan1 cells as a positive control (since, as mentioned they express *CFTR*). However, we were not able to detect a decrease in *CFTR* mRNA with siRNA relative to scramble controls (n=2, data not shown). We therefore utilized the widely used Caco2 cell as our positive control as Caco2 cells both express CFTR and have been shown to be amenable to siRNA transfection (Crites et al., 2015). As expected, we observed a statistically significant 55% decrease in *CFTR* mRNA expression in si-CFTR compared to the si-scramble control in Caco2 cells (FIG 3A, $p<0.05$). However, si-CFTR was not effective at modulating *CFTR* mRNA in cultured HPd1+ cells (FIG 3B). As these cells are precious and require donated tissue, we moved on to alternative strategies of modulation of *CFTR*.

CFTR expression modulated by LentiCRISPR2-CFTR

Again, initial testing and optimization of LentiCRISPR2-*CFTR* was done in Caco2 cells. Caco2 cells treated with LentiCRISPR2-*CFTR* compared to control cells which were not exposed to virus or

puromycin, resulted in a statistically significant decrease (an average of ~81%) of *CFTR* mRNA expression (FIG 3C, $p<0.001$). Similarly, HPd1+ hPDECs treated with LentiCRISPR2-*CFTR* compared to control cells which were not exposed to virus or puromycin, resulted in a statistically significant decrease (an average of ~70%) of *CFTR* mRNA expression (FIG 3D, $p<0.05$).

2.5 DISCUSSION

We successfully utilized the HPd1 antibody to isolate a population of primary human pancreatic cells that express PDEC markers and are amenable to propagation. While this cell population loses *CFTR* expression (along with *SOX9* and *AQP1*) over a few weeks in culture, *CFTR* mRNA levels still remain much higher than in commercially available human pancreatic ductal epithelial cell lines (Capan1, PANC1, and H6C7), suggesting HPd1-mediated isolation may be a viable approach to generate human PDECs for use in the CF research community. While modulation of *CFTR* by siRNA was not effective in HPd1+ cells, we successfully used LentiCRISPR2-*CFTR* to down regulate *CFTR* mRNA in primary HPd1+ PDECs. Our observation of decreased expression of *CFTR* in LentiCRISPR2-*CFTR* treated HPd1+ cells suggest these cells may be a useful a model to study the comparison of the impact of *CFTR* loss in primary human PDECs, from a range of human donors which may be of value to the CF research community.

This study is not the first to attempt to generate PDECs from human pancreas. Several approaches have been reported. Kolar et al. reported the use of an enzymatic approach on islet depleted pancreatic tissue from a single donor to generate CFTR expressing cells that were amenable to culture (Kolar et al., 1997), similar to our findings. Hoesli et al also used islet-depleted pancreatic tissue and magnetic-activated sorting (targeting KRT19+ cells) as a successful isolation strategy in 10 donors (Hoesli et al., 2012). Other studies have taken advantage of the availability of intact pancreatic ducts, a more specialized source of starting material. Trautman et al performed enzymatic digestion of the pancreatic duct to yield cells with epithelial cobblestone morphology that could be propagated in culture for many weeks (Trautmann et al., 1993). Both this and the Hoesli paper used KRT19 as a marker of PDEC phenotype; this is a well-established marker, but our data suggest KRT19 expression can remain high even under conditions where CFTR is decreased. Therefore, it is not clear whether the procedures used in these studies would successfully yield cells that retain CFTR expression over time.

Mun et al also obtained primary PDECs from dissected whole pancreatic ductal tissue derived from patients undergoing total pancreatectomy and islet auto transplantation (Shik Mun et al., 2019). A major strength of this study was the determination of CFTR activity in the resulting cells, which we

have not yet performed in HPd1-derived cells. However, whether CFTR activity is maintained over time in culture was not determined in their study and remains an open question.

The approach described in the present study can certainly be refined. The enrichment *CFTR* in HPd1+ samples were only ~2 fold and we noted significant remaining contamination of acinar markers. This is not unexpected given the vast excess of acinar cells relative to all other pancreatic cell types but could be improved by, for example, added wash steps. However, additional steps that lengthen the purification procedure may actually be detrimental to PDECs; therefore, our choice in the present study was to prioritize getting the cells into culture media as quickly as possible. Another obvious limitation of the present study is the lack of protein or activity data. This is the focus of ongoing work, but the differences we found in CFTR mRNA levels among primary vs. immortalized cell lines are so striking that we feel they are informative for the field by themselves. Our ability to knockdown CFTR expression in primary human PDECs allows for an experimental control within donors. Moreover, these cells are capable of persisting in culture for weeks in puromycin containing cell culture media.

The strategy of using LentiCRISPR2-*CFTR* to introduce *CFTR* mutations may contribute to some variability. However, in order to introduce a specific edit (as was the initial goal of this work), cells would have needed to be co-transduced with two viral vectors and then undergone clonal expansion, necessitating extensive culture and passage number to generate enough cells to work with. The decision not to introduce specific edits was made following initial work showing human PDECs continue to progressively lose *CFTR* expression following multiple passages in culture (*data not shown*), making the extensive culture required to generate a population of cells that express CFTR with a specific edit unfeasible.

One additional component we have not yet explored but may be useful in retaining CFTR expression over time, is the use of 3D culture systems. This was used by Mun et al in their pancreatic duct-derived cells. Other groups have successfully generated pancreatic organoids from human pancreas samples (Broutier et al., 2016) or using human induced pluripotent stem cell-derived ductal-like cells (Wiedenmann et al., 2021).

In summary, we describe a simple approach to obtain PDECs from a relatively abundant and easy to access source of human pancreas tissue. This approach can be used to generate CFTR-expressing cells from multiple donor pancreata and has the potential to be scalable, providing a source of these cells for downstream analysis. Despite the potential limitations in the LentiCRISPR2-*CFTR* approach, the data clearly show a reproducible loss of ~85% of CFTR expression across n=3 in HPd1+ PDEC.

This work therefore provides the field with a tool that may help clarify the poorly understood mechanisms that underlie exocrine and endocrine pancreatic disease in many people with CF.

2.6 ACKNOWLEDGEMENTS

We thank Drs. Sakeneh Zraika and Steven Kahn as well as, Daryl Hackney and Breanne Barrow for Research Service at VA Puget Sound Health Care System, Seattle, WA, USA, as well as Dr. G. Stanley McKnight for valuable discussions related to the writing of this article. This work was supported by the Department of Veterans Affairs, VA Puget Sound Health Care System (Seattle, WA), Seattle Institute for Biomedical and Clinical Research and the Cystic Fibrosis Foundation award HULL21P0. SSM was supported by T32 GM007750 and by a fellowship funded by the University of Washington CFF Research and Development Program (SINGH19R0).

2.7 FIGURES

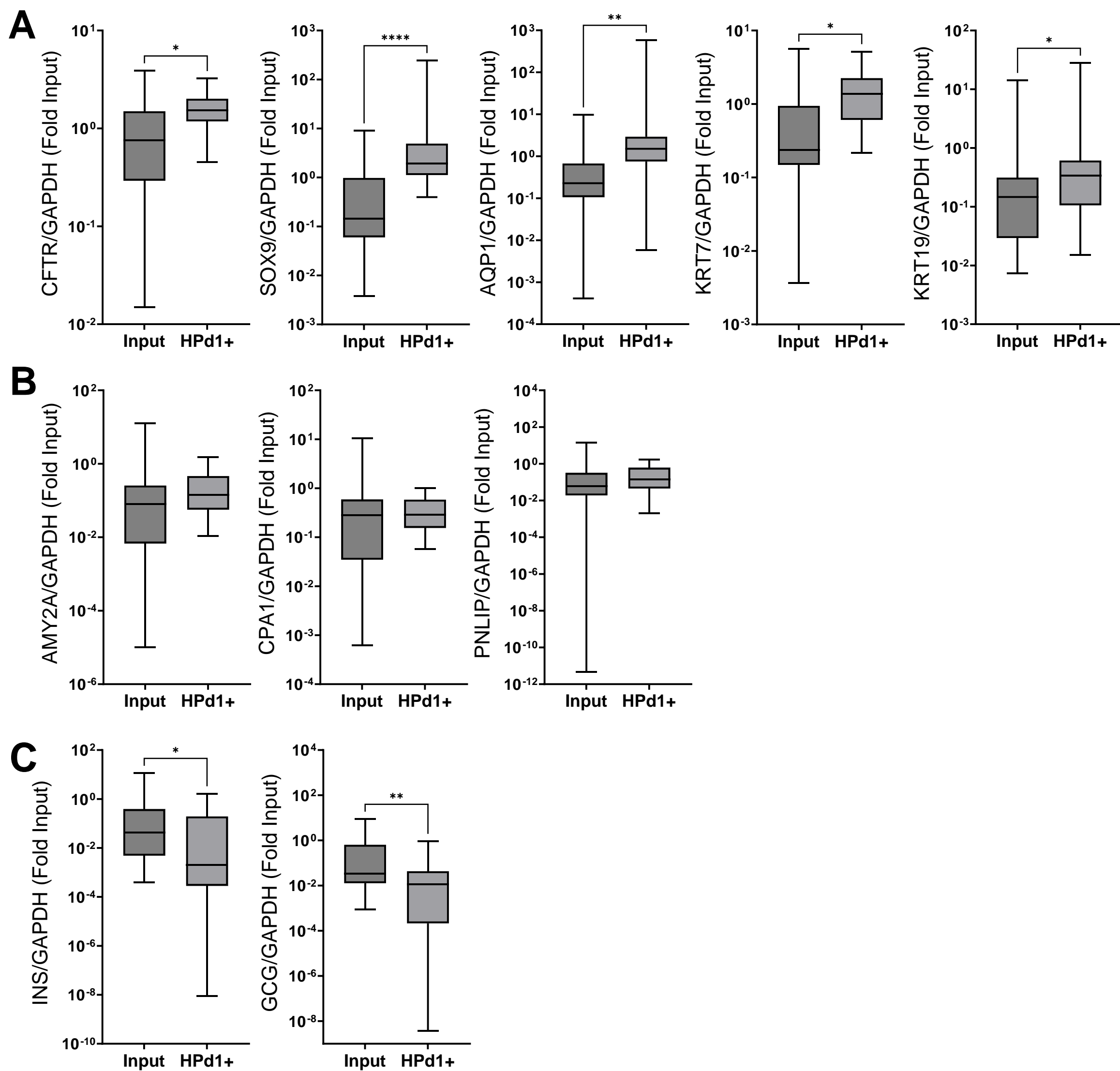


Figure 1: **Assessment of mRNA expression of ductal, acinar, and islet markers in HPd1+ cells isolated from cadaveric human pancreas tissue**. (A) Box and whisker plots showing the comparison of the PDEC markers (*CFTR, SOX9, AQP1, KRT7,* and *KRT19*) mRNA expression between input pancreas tissue and HPd1+ samples on day of isolation. (B) Box and whisker plots showing the comparison of the acinar markers (*AMY2A, CPA1,* and *PNLIP*) mRNA expression between input pancreas tissue and HPd1+ samples on day of isolation. (C) Box and whisker plots showing the comparison of the islet markers (*INS* and *GCG*) mRNA expression between input pancreas tissue and HPd1+ samples on day of isolation. mRNA levels were normalized to expression in the input pancreas sample. $n=17$; *p<0.05, **p<0.01, and ****p<0.005.

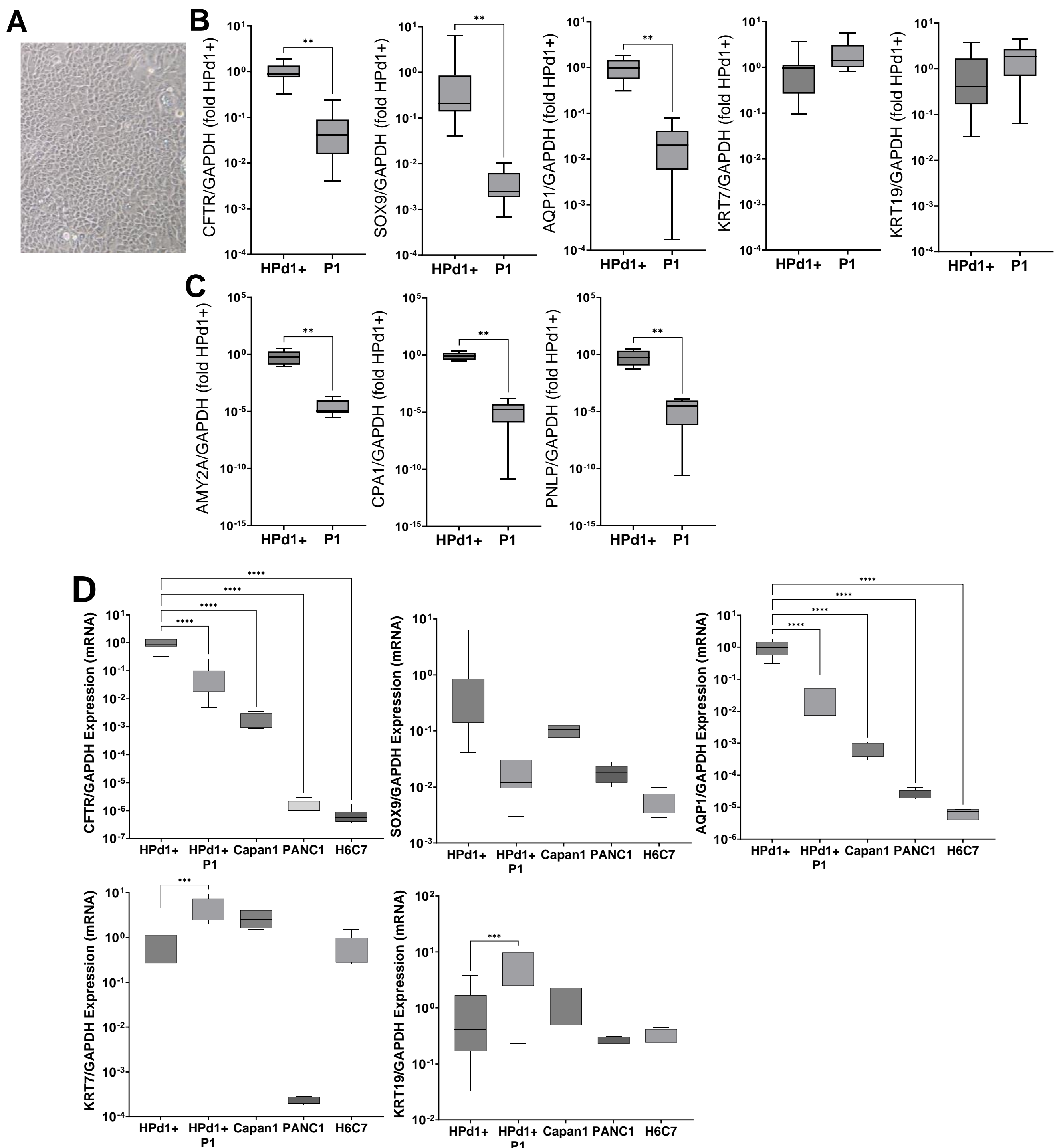


Figure 2: Morphology of HPd1+ cells in culture and assessment of PDEC and acinar cell markers in short-term culture. (A) Image of HPd1+ cells in short-term culture at 4x magnification using a bright field microscope. (B) Box and whisker plot of showing the comparison of PDEC markers (*CFTR, SOX9, AQP1, KRT7,* and *KRT19*) mRNA expression between day of isolation HPd1+ samples and HPd1+ passage 1 "P1" cells. (C) Box and whisker plot showing the comparison of acinar cell markers (*AMY2A, CPA1,* and *PNLIP*) mRNA expression between day of isolation HPd1+ samples and HPd1+ passage 1 "P1" cells. (D) Box and whisker plot demonstrating the comparison of PDEC markers (*CFTR, SOX9, AQP1, KRT7*, and *KRT19*) mRNA expression between day of isolation HPd1+ samples, HPd1+ passage 1 "P1" cells, Capan1, PANC1, and H6C7. Samples were normalized to the mRNA expression in the day of isolation HPd1+ sample. HPd1+ and HPd1+ n=9, Capan1 n=4, PANC1 n=6, H6C7 n=6, ***$p<0.001$, and ****$p<0.005$.

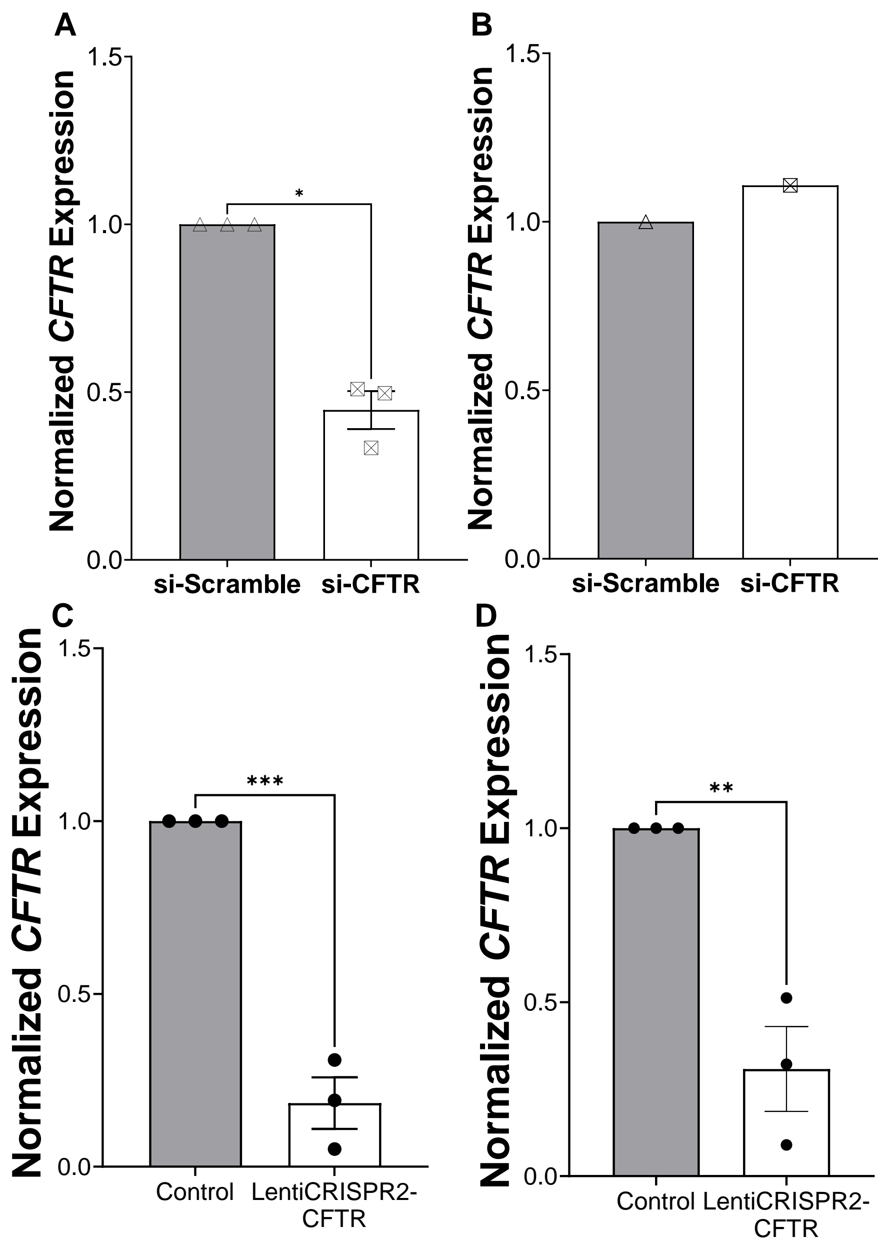
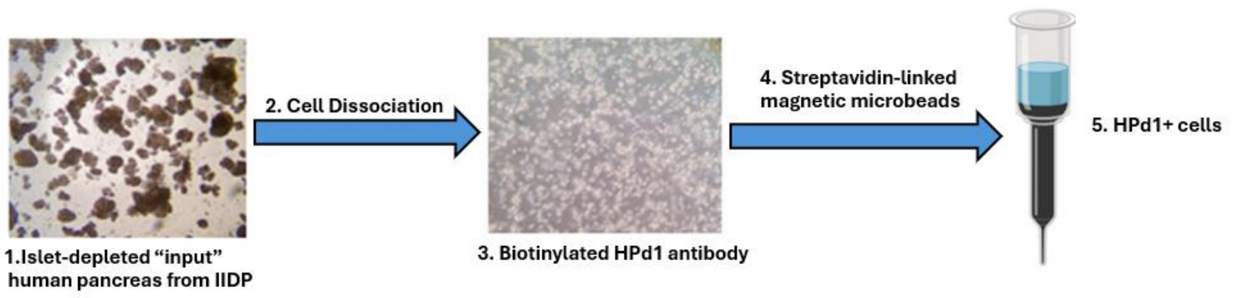


Figure 3: Modulating CFTR expression in HPd1+ cells. *CFTR* mRNA levels in Caco2 cells (A) or HPd1+ hPDECs (B) treated with siRNA control (si-Scramble) or siRNA against *CFTR* (si-CFTR). For A: n=3, *p<0.05; for B: *n=1* (Donor 2). Knockdown of *CFTR* mRNA levels in Caco2 cells (C) or HPd1+ hPDEC (D) treated with lentiCRISPR2-*CFTR*. The achieved decrease in *CFTR* expression was measured by qPCR with ACTB (C) or *GAPDH* (D) as an endogenous control and normalized to expression of *CFTR* mRNA in untransduced control. For C: n=3, ***p<0.0011 ; for B: n=3, **p<0.01.

Donor #	Sex	Age (years)	BMI (kg/m^2)	HbA1c (%)	Institution	Used for culture	Resource ID# (RRID)
Donor 1	M	61	29.3	5.9	SCICRC	No	SAMN19588420
Donor 2	M	66	29.9	5.8	SCICRC	Yes	SAMN20064637
Donor 3	F	62	26.8	6.2	SCICRC	No	SAMN20926065
Donor 4	M	47	25.9	5.5	U. Miami	No	SAMN27057448
Donor 5	M	36	29.6	5.4	SCICRC	No	SAMN28867623
Donor 6	M	38	28.4	6.1	SCICRC	Yes	SAMN30181460
Donor 7	M	50	36.3	4.9	SCICRC	No	SAMN30927410
Donor 8	F	30	39.9	5.3	SCICRC	Yes	SAMN31525424
Donor 9	M	16	29.5	5.4	SCICRC	Yes	SAMN32641506
Donor 10	M	39	33.4	5.0	SCICRC	Yes	SAMN34033793
Donor 11	M	39	41.8	5.5	Imagine Pharma	Yes	SAMN34075902
Donor 12	M	20	38.5	5.5	SCICRC	Yes	SAMN34356134
Donor 13	M	52	25.9	4.9	Imagine Pharma	No	SAMN36704820
Donor 14	M	40	32.3	5.1	SCICRC	Yes	SAMN37158107
Donor 15	M	63	33.9	6.7	SCICRC	No	SAMN37680486
Donor 16	M	59	41.7	5.4	VCU	Yes	SAMN37871874
Donor 17	F	20	22.4	4.9	U. Miami	Yes	SAMN38040976

Supplemental Table 1: **Donor demographics of cadaveric human pancreas tissue.** Donor demographics for all 17 donors with information on age, BMI, HbA1c% and acinar resource ID# RRID from IIDP.



Supplemental Figure 1: **Scheme of HPd1+ cell isolation procedure.** Schematic showing isolation process of pancreatic ductal epithelial cells from cadaveric human islet-depleted "Input" pancreas. (Crude/input Exocrine). Cells are dissociated into single cells and incubated with biotinylated HPd1 antibody and then placed on streptavidin linked magnetic microbeads. HPd1- cells are washed off the column and HPd1+ are recovered.

Chapter 3. AN ALTERNATIVE APPROACH FOR PURIFICATION OF PRIMARY HUMAN PANCREATIC DUCTAL EPITHELIAL CELLS

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3.1 ABSTRACT

In this chapter, we compare isolations using different monoclonal antibodies that bind to PDECs. We performed a side-by-side comparison of HPd1 versus HPd3 and measured percentage cell recovery and expression of genes of interest for each antibody-bound cell sample. Similar to chapter 2, PDECs were isolated from cadaveric islet depleted input human pancreatic tissue (in donors without CF or diabetes) via either HPd1+ or HPd3+ antibody-magnetic beads, which either selectively binds to large pancreatic ductal epithelial cells or all pancreatic ductal epithelial cells in human pancreas, respectively (n=5 for each). The HPd3 antibody demonstrated the ability to isolate 2x the number of cells from cadaveric islet depleted input human pancreas tissue. PDEC genes of interest were enriched similarly between HPd1+ and Hpd3+ samples relative to the input pancreas sample. While HPd3+ cells exhibited decreased *CFTR* expression cultured HPd3+ cells demonstrated expression of *CFTR* at substantially greater levels than that seen in commercially available pancreatic ductal cell lines (Capan 1, PANC1, and H6C7).

In summary, we have demonstrated an alternative approach to isolation of PDECs using the HPd3 antibody. The HPd3 antibody demonstrates the ability to isolate a greater number of human PDECs which will persist in culture retaining key PDEC genes of interest while losing key acinar cell markers. This alternative approach may improve the feasibility of downstream analysis of primary human PDECs with modulated *CFTR* expression.

3.2 INTRODUCTION

The PDEC-islet axis is an understudied area. There are not many established models of human PDECs that retain *CFTR* at levels similar to *in vivo* expression (as described in chapter 2). In chapter 2, we discussed the use of monoclonal antibody HPd1, which targets large intralobular ductal PDECs (Dorrell et al., 2008). We showed that HPd1 can select for a subset of *CFTR*-expressing PDECs which retain *CFTR* through short-term culture at increased levels (0.07 v 0.002) compared to established *CFTR*-expressing PDEC models such as Capan1 cells. However, the relatively low yield of HPd1 cells makes it difficult to obtain a sufficient number of cells for downstream applications. This limitation prompted the search for an alternate antibody for PDEC purification. For this, we chose HPd3, another mouse monoclonal antibody developed by Dorrell and colleagues which has been shown to bind to a much greater proportion of PDECs than HPd1 (Dorrell et al., 2011).

Previous work evaluated immunoreactivity of HPd1 and HPd3 on adult human pancreatic cryosections. In that study, pancreatic sections were co-stained with HPd1 and HPd3 to see what fraction of HPd3+ PDECs could be detected by HPd1. A similar approach was taken with HPd3 and

KRT19. In sections stained with HPd1, only cells comprising larger ducts were detected (FIG1A, from (Dorrell et al., 2011)), however many more cells were labeled in sections stained with HPd3 (FIG1A). When HPd3+ staining is overlayed with KRT19+, it is apparent that HPd3+ staining occurs in KRT19+ cells, which would suggest these are PDECs (FIG1A, (Dorrell et al., 2011)). While we were successful in acquiring *CFTR*-expressing HPd1+ cells from cadaveric islet-depleted pancreas samples as described in chapter 2, the work in this chapter was designed to test the hypothesis that utilizing HPd3 antibody would yield a (i) greater number of *CFTR*-expressing cells, (ii) cells with higher *CFTR* expression and (iii) cells that would retain more *CFTR* expression through short-term culture.

3.3 METHODS

Biotinylation of HPd3 Antibody

The HPd3 antibody is only available in its unconjugated form (EMD Millipore, MABS2040). Therefore, biotinylation of HPd3 was necessary prior to use in our streptavidin-linked magnetic bead approach for PDEC purification (as described in chapter 2). Biotinylation was performed on unconjugated HPd3 antibody with a 1:10 ratio of modifier and quencher reagent for every 10ul of HPd3 antibody using the Abcam Biotin Conjugation kit per manufacturer instructions (Abcam, Fast, Type A- Lightning Link® ab201795). To determine if this HPd3 biotinylation conjugation procedure interfered with the availability of the epitope to bind the cell surface of PDECs, sections of deidentified human autopsy pancreas specimens were stained with either unconjugated HPd3 antibody or biotinylated HPd3 antibody. Formalin fixed paraffin embedded sections were deparaffinized and rehydrated prior to staining. Two sections were used for this procedure. The first section was stained with unconjugated mouse monoclonal HPd3 (1:200, EMD Millipore MABS2040) followed by AlexaFluor 548-goat anti-mouse IgG (Invitrogen A11004) (FIG1B) and the second section was stained with biotinylated mouse monoclonal HPd3 (1:200, EMD Millipore MABS2040) followed by AlexaFluor 488-streptavidin (Invitrogen S32354) (FIG1C). The sections were counterstained with Hoechst 33258 (2ug/ml) to visualize cell nuclei. Multichannel fluorescence images (FIG1B-C) were acquired using 20X objective magnification on a Nikon Ni-E microscope system (Nikon USA, Melville, NY, USA).

Isolation and analysis of primary human HPd3+ cells

We used cadaveric human pancreas tissue from a subset of the donors previously described in chapter 2 (donors 10-14; Table 1). All samples available for this study were from male donors (Table 1). The techniques to isolate cells from islet-depleted pancreas tissue (defined as "input" as in chapter 2) were performed as described in chapter 2. However, after pancreatic samples were dissociated to single cells using a non-enzymatic approach, the resulting cell suspension was then equally split into two samples. The first group were then incubated with biotinylated Hpd1 antibody (Novus Bio). The second group was incubated with biotinylated HPd3. On average HPd3+ cells were seeded at density of 75,000 cells/cm^2 onto Collagen 1A-coated plates and grown in PneumaCult EX+ media supplemented as described in chapter 2. Additionally, HPd3+ cells were maintained in culture and harvested 1-4 weeks after isolation for RNA isolation, similar to what was described in chapter 2.

RNA isolation and real time quantitative PCR

RNA extraction, isolation, and reverse transcription to cDNA were all performed similarly as described in chapter 2. Gene expression was also measured using TaqMan probes as similarly described in chapter 2 as well as, results calculated using the $\Delta\Delta C_t$ method.

Statistical analysis

Data are presented as mean ± SEM. Student's t test (Wilcoxon matched pairs signed rank test) was used for two-group comparisons. One-way ANOVA with Dunnett's multiple comparisons test was used to detect significant differences for datasets with more than two groups. Prism (version 10.2.0; GraphPad Software, USA) was used for all statistical analyses. Numbers of experimental replications are represented by individual data points in figures.

3.4 RESULTS

Comparison of isolation and analysis of HPd1+ vs. HPd3+ cells.

HPd1+ and HPd3+ cells were generated from 5 donors (all males) with an average age of 38±11.5 and average BMI of 34.4±6.1 (Table 1). The average starting cell number was ~115 million cells with a recovery of HPd-bound cells between 2-4 million cells (Table 2). The average cell yield percentage of HPd1+ within these donors was 3.07% compared to 6.82% of HPd3+ (Table 2). Therefore, there were 2x more cells recovered in preparations using the HPd3 antibody.

The mRNA expression for key ductal, acinar and islet genes were measured in both HPd1+ and HPd3+ samples for all 5 donors (FIG2B-D). The samples were normalized to housekeeping control (GAPDH) and expressed relative to mRNA expression of input samples for these 5 donors as

indicate by the dashed line (FIG2B-D). *CFTR* expression was similar between HPd1+ samples versus HPd3+ samples (1.18 v 1.01) compared to input samples (input = 1) (FIG2B; p=0.187). *KRT7* expression was much greater in HPd1+ samples compared to HPd3+ samples (1.01 v 0.42) (FIG2B; p=0.063). Similarly, *KRT19* expression was much more robustly expressed in HPd1+ samples than HPd3+ samples (2.03 v 0.47) (FIG2B; p=0.062). Both *SOX9* and *AQP1* expressions were expressed similarly between HPd1+ samples and HPd3+ samples (FIG2B; p=0.812 and p=0.438). However, both HPd1+ and HPd3+ samples had an increase in *SOX9* expression (4.33 and 3.83) compared to input samples (input = 1) (FIG2B).

HPd3+ cells did not show any significant increase in mRNA levels for *CFTR* (FIG2B; p=0.187) or ductal markers *SOX9*, *AQP1 KRT7* and *KRT19*, compared to HPd1+ samples (FIG2B; p=0.812, p=0.438, p=0.063, and p=0.062). Furthermore, HPd3+ samples had less *KRT7* and *KRT19* mRNA expression compared to HPd1+ samples (FIG2B; p=0.063, and p=0.062). Both HPd1+ and HPd3+ samples had a decrease of acinar cell markers mRNA expression; *AMY2A (*0.21 and 0.22) CPA*1* (0.14 v 0.11), and PNLIP (0.23 and 0.19), compared to input (input = 1) samples (FIG2C; p=0.625, p=0.437, and p=0.063). The levels for islet markers *INS* (0.06 and 0.01) and *GCG* (0.10 and 0.04) were decreased in both HPd1+ and HPd3+ samples compared to input (input = 1) samples (FIG2D; p=0.125 and p=0.125).

Short-term propagation of HPd3 + cells.

A subset of preparations (n=4) was propagated in culture. Cultured cells retained the expected cobblestone morphology (FIG3A). *CFTR* mRNA expression was decreased to 9% of the levels seen on the day of isolation (FIG3B; p=0.003) but was still readily detectable (average Ct 24 ±2). mRNA levels of *SOX9* and *AQP1* were also decreased but still detectable (average Ct of 28±2 and 27 ± 1, respectively; FIG3B; p=0.010 and p=0.031), while mRNA levels of *KRT7* and *KRT19* mRNA were significantly increased in cultured HPd3+ cells (FIG3B; p=0.033 and p=0.005, respectively). In contrast, acinar markers *PNLIP, AMY2A,* and *CPA1* were all substantially decreased (average Ct 32 ± 6, 28±3, and 31 ± 6, respectively; FIG3C) and mRNA levels of *INS* and *GCG* were undetectable (data not shown) in HPd3+ cells following culture.

Comparison of CFTR mRNA expression in HPd3+ cells vs. commercially available cell lines.

Next, we compared *CFTR* expression in HPd3+ samples (on the day of isolation and following culture) to HPd1+ cells and available cell lines, using the data set from chapter 2 (FIG4). *CFTR* mRNA levels were almost identical in HPd1+ and HPd3+ cells on the day of isolation (1.00 v

1.12). *CFTR* mRNA decreased to a similar extent in cultured HPd1+ and HPd3+ cells (0.07 and 0.08) (FIG4). As expected, therefore, *CFTR* mRNA expression in HPd3+ day 0 samples was significantly higher than Capan 1, PANC1 and H6C7 cells ($p<0.0001$ for all). Cultured HPd3+ cells were found to express *CFTR* significantly higher than Capan 1, PANC1, and H6C7 cells (0.08 compared to 0.002, 1.69 $x10^{-6}$ and $7.06x10^{-7}$) (FIG4; $p<0.0001$ for all).

3.5 DISCUSSION

We were successful in isolating HPd3+ cells from islet-depleted input human pancreas fractions with the HPd3 antibody similar to methods utilizing HPd1. Additionally, we were successful in demonstrating HPd3+ cells can persist in short-term culture. The comparison between mRNA levels of *CFTR, SOX9*, and *AQP1* between day of isolation HPd1+ and HPd3+ samples show no significant difference among these genes. The acinar cell markers (*AMY2A, CPA1*, and *PNLIP*) and islet cell markers (*INS* and *GCG*), were comparably decreased compared to input pancreas samples for both HPd1+ and HPd3+ samples. We also demonstrated that similarly to HPd1+ cells, HPd3+ cells can grow in culture but lose mRNA expression of *CFTR, SOX9*, and *AQP1* but not *KRT7* and *KRT19,* as well as showing substantial loss of mRNA expression of acinar markers (*AMY2A, CPA1*, and *PNLIP)*.

There are many different types of PDECs and these different types of PDECs do not express CFTR equivalently. It has been observed that PDECs, such as centroacinar and intercalated ducts, which are closest to acinar cells have the highest CFTR expression (Marino et al., 1991). Moreover, this subset of PDECs often do not survive the harsh conditions of acinar destruction which is observed in CF. While large/main PDECs do not express CFTR similarly to PDECs which are closest to acinar cells, it has been suggested they may be resistant to progressive acinar destruction of the exocrine pancreas that is observed in CF (Norris et al., 2019). We decided to utilize HPd3, a pan-ductal marker, due to its capability of binding to all types of PDECs which could increase the yield of PDECs that have higher *CFTR* expression compared to HPd1+ cells.

Therefore, our expectation was that HPd3+ samples would express a greater amount of *CFTR* as compared to HPd1+ samples. This work was also motivated by the necessity to acquire more primary human PDECs for the purpose of scaling up experiments designed to inhibit *CFTR* expression in primary human PDECs. The cell yields observed with methods solely utilizing HPd1 antibody were often not sufficient for downstream experiments.

While our rationale was to increase the amount of viable PDECs with robust *CFTR* expression by utilizing HPd3, upon reflection of a comparison of mRNA levels between HPd1+ and HPd3+

samples, HPd3+ samples did not illustrate a significant improvement in *CFTR* expression. This could be due to a variety of limitations such as a partial loss of the epitope of HPd3 antibody as well as similar technical limitations stated in chapter 2. Additionally, the subset of donors (donors 10-14) used in this study account for less enrichment of *CFTR* mRNA expression among the entire data set from donors 1-17 examined in chapter 2. While 2x number of cells were recovered using HPd3, it is possible that characteristics of the donors chosen for these studies account for the lackluster *CFTR* mRNA expression due to possible loss of highly expressing *CFTR* PDECs in the islet isolation procedure performed by IIDP. Additionally, the donor-to-donor variability of the input pancreatic tissue that is available from human donors could account for the lackluster *CFTR* mRNA expression. As HPd1 binds to PDECs in the large ducts, which are only one type of ductal cells, it is possible that the preparations used in these studies (HPd1 versus HPd3) did not contain as many of these PDECs that have higher *CFTR* expression. However, while HPd3+ cells did not have greater enrichment of *CFTR*, our PDEC isolation approach with HPd3 will likely yield a greater number of cells. In fact, sorting with HPd3 compared to HPd1 allows for almost double the number of cells available for experiments, which is advantageous because these cells lose a significant amount of *CFTR* mRNA expression in culture. Therefore, starting with a greater number of cells allows for more experiments during the window of peak *in vitro* *CFTR* mRNA expression. Notably, while we discovered that *CFTR* mRNA expression is equivalent between HPd3+ and HPd1+ isolated cells, both express *CFTR* at a much higher level than commercially available PDEC lines. In addition to continuing work with HPd3 among more donors, it is imperative to determine if these findings are recapitulated by protein expression of CFTR or channel activity. Moreover, RNAseq could be a helpful tool to determine the impact of inhibition of *CFTR* on the transcriptome in PDECs.

To conclude, we describe an alternative strategy whereby HPd3 yields approximately double the number of cells compared to HPd1 isolation. Moreover, these cells can be propagated in culture, after which they do not show appreciable residual acinar and islet cell markers. This approach may be useful when scaling experiments which require *CFTR* expressing cells isolated from primary human pancreas tissue for downstream analyses.

3.6 ACKNOWLEDGMENTS

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3.7 FIGURES

Table 1

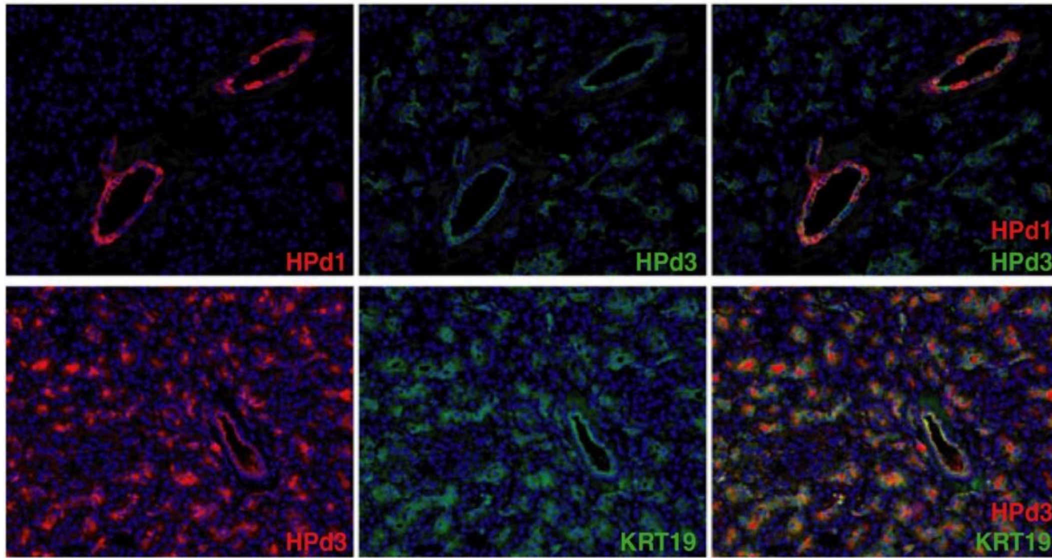
Donor #	Sex	Age (Years)	BMI	HbA1c%	Instituion	Culture	Acinar Rescource ID# RRID
Donor 10	M	39	33.4	5	SCICRC	Yes	SAMN34033793
Donor 11	M	39	41.8	5.5	Imagine Pharma	Yes	SAMN34075902
Donor 12	M	20	38.5	5.5	SCICRC	Yes	SAMN34356134
Donor 13	M	52	25.9	4.9	Imagine Pharma	Yes	SAMN36704820
Donor 14	M	40	32.3	5.1	SCICRC	No	SAMN37158107

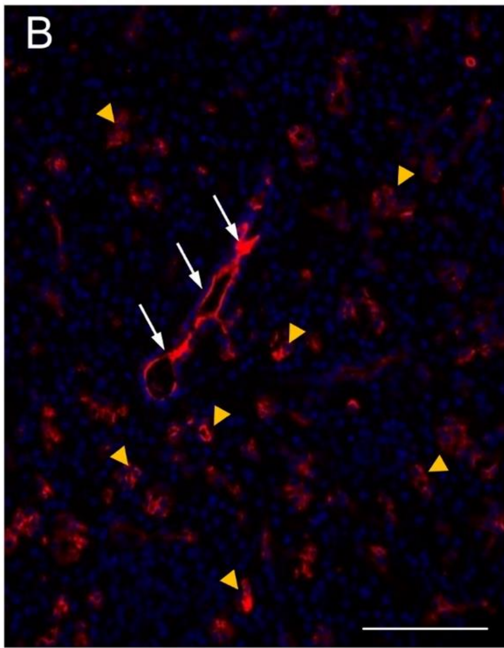
Table 1: Donor demographics of cadaveric human pancreas tissue. Donor demographics for all 5 donors with information on age, BMI, HbA1c% and acinar resource ID# RRID from IIDP.

Table 2

Donor #	Starting Cell #	HPd1+ cells	% HPd1+ Recovery	HPd3+ cells	% HPd3+ Recovery
Donor 10	240,000,000	100,000	0.04%	1,600,000	0.67%
Donor 11	50,000,000	3,000,000	6.00%	4,500,000	9.00%
Donor 12	230,400,000	4,500,000	1.95%	9,000,000	3.91%
Donor 13	11,000,000	105,000	0.95%	1,280,000	11.64%
Donor 14	45,000,000	2,880,000	6.40%	4,000,000	8.89%
Average	115,280,000	2,117,000	3.07%	4,076,000	6.82%

Table 2: Comparison of HPd1+ and HPd3+ isolation yields
Comparison of isolation yields using HPd1+ vs. HPd3+ from 5 individual donors. The percentage of HPd1+ or HPd3+ recovery is defined by dividing the amount of collected HPd1+ or HPd3+ cells by the starting cell number.





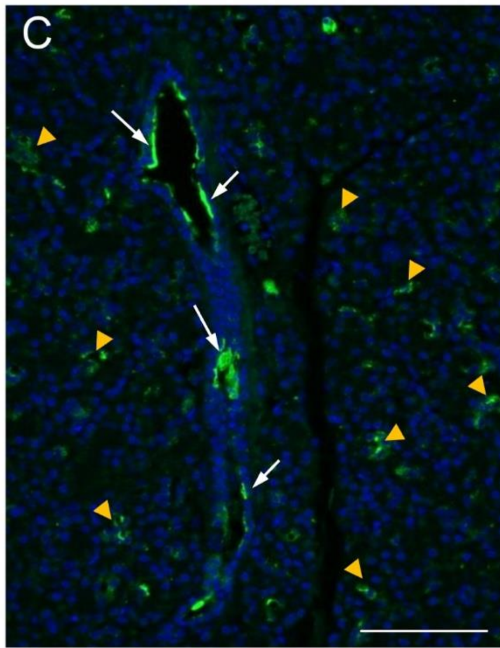


Figure 1: HPd1 and HPd3 antibody staining of human pancreas sections.

(A) Top Panel: Human adult pancreas cryosections stained for HPd1 (top left; red), HPd3 (top middle; green) and the overlay (top right). (A) Bottom Panel: HPd3 (bottom left; red) is co-stained with KRT19, a positive control PDEC marker (bottom middle; green), with extensive colocalization being shown in the overlay image (bottom right). This figure was adapted from Dorrell et al (Dorrell et al., 2011). Deidentified human autopsy pancreas section stained with unmodified (non-biotinylated) HPd3 antibody (red immunoreactivity; B) and biotinylated HPd3 antibody (green immunoreactivity; C). For both panels, white arrows denote immunoreactivity in large ducts; orange arrowheads show examples of immunoreactivity in small ducts. Scale bar is 100 µm.

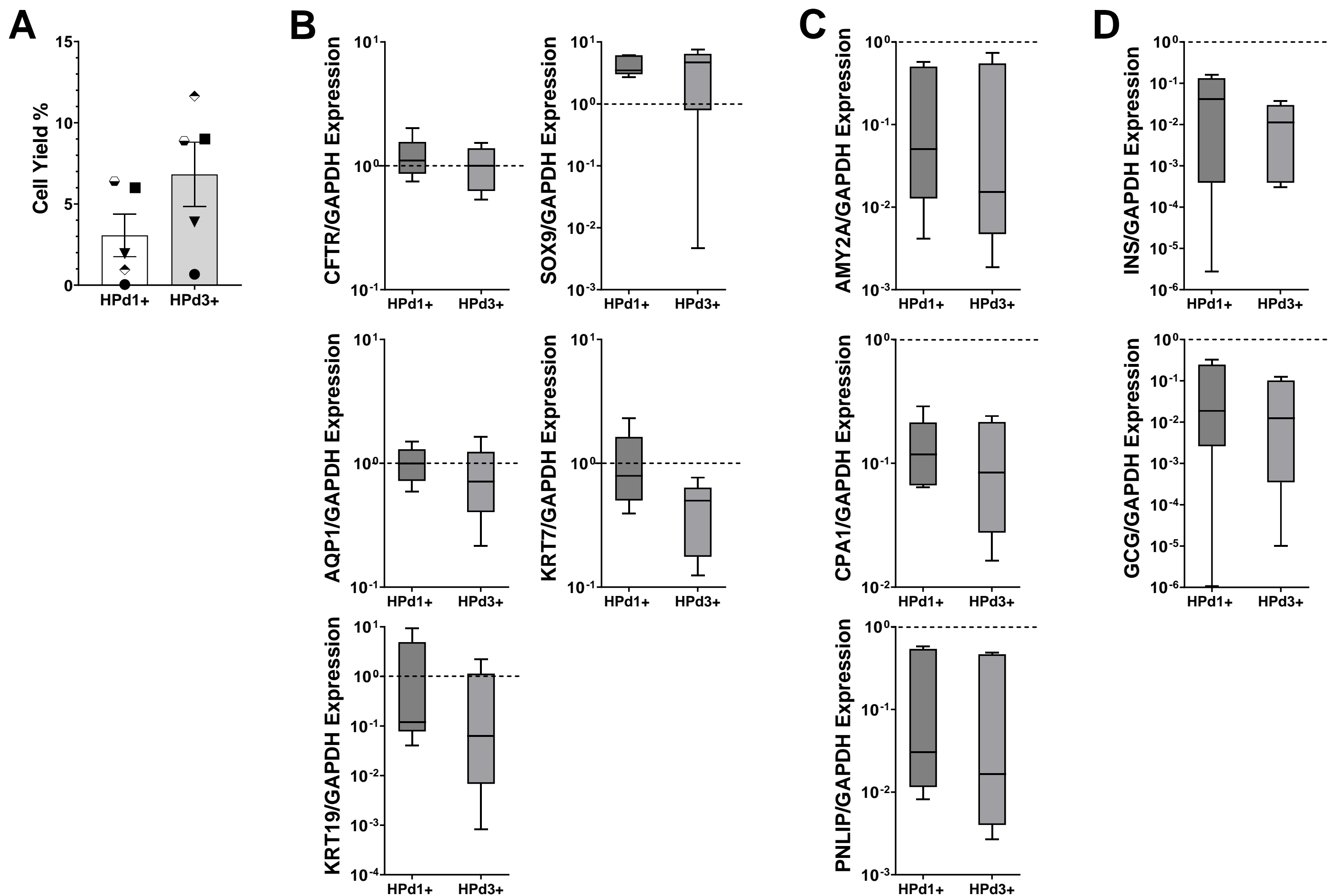
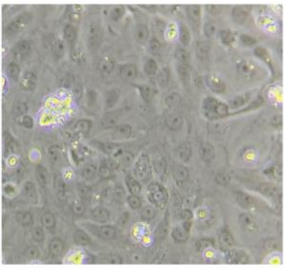
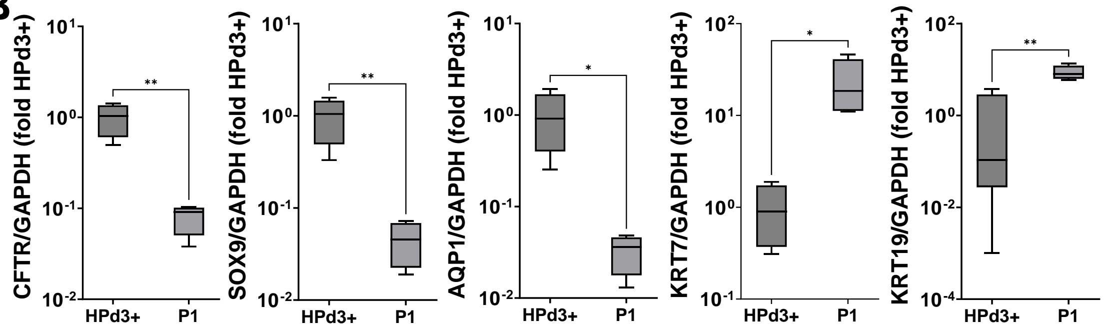


Figure 2: Assessment of mRNA expression of ductal, acinar, and islet markers in HPd3+ cells isolated from cadaveric human pancreas tissue. (A) Bar graph (mean ± SEM) representing percentage of cell yield recovery between HPd1+ (open bar) and HPd3+ (shaded bar) isolation methods. Each donor is represented as a different symbol. (C) Box and whisker plots showing the comparison of PDEC marker (*CFTR, SOX9, AQP1, KRT7,* and *KRT19*) mRNA expression between HPd1+ and HPd3+ samples on day of isolation. (D) Box and whisker plots showing the comparison of the acinar markers (*AMY2A, CPA1,* and *PNLIP*) mRNA expression between input and HPd3+ samples on day of isolation. (E) Box and whisker plots showing the comparison of the islet markers (*INS* and *GCG*) mRNA expression between input and HPd3+ samples on day of isolation. Samples were normalized to input mRNA expression. $n=5$

A





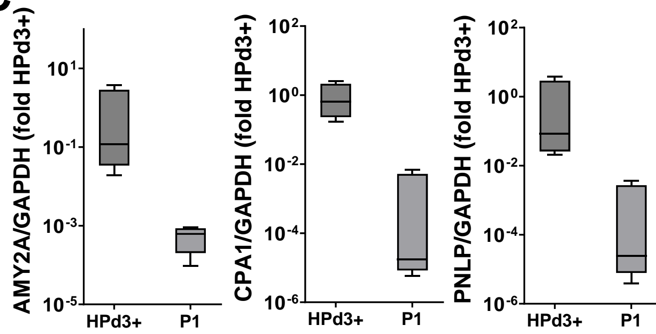


Figure 3: Morphology of HPd3+ cells in culture and assessment of PDEC and acinar cell markers in short-term culture (A) Image of HPd3+ cells in short-term culture at 10x magnification using a bright field microscope. (B) Box and whisker plot of showing the comparison of PDEC markers (*CFTR, SOX9, AQP1, KRT7,* and *KRT19*) mRNA expression between day of isolation HPd3+ samples and HPd3+ passage 1 "P1" cells. (C) Box and whisker plot showing the comparison of acinar cell markers (*AMY2A, CPA1,* and *PNLIP*) mRNA expression between day of isolation HPd3+ samples and HPd3+ passage 1 "P1" cells. Samples were normalized relative to HPd3+ samples on the day of isolation. *n=4*; *p<0.05 and **p<0.01

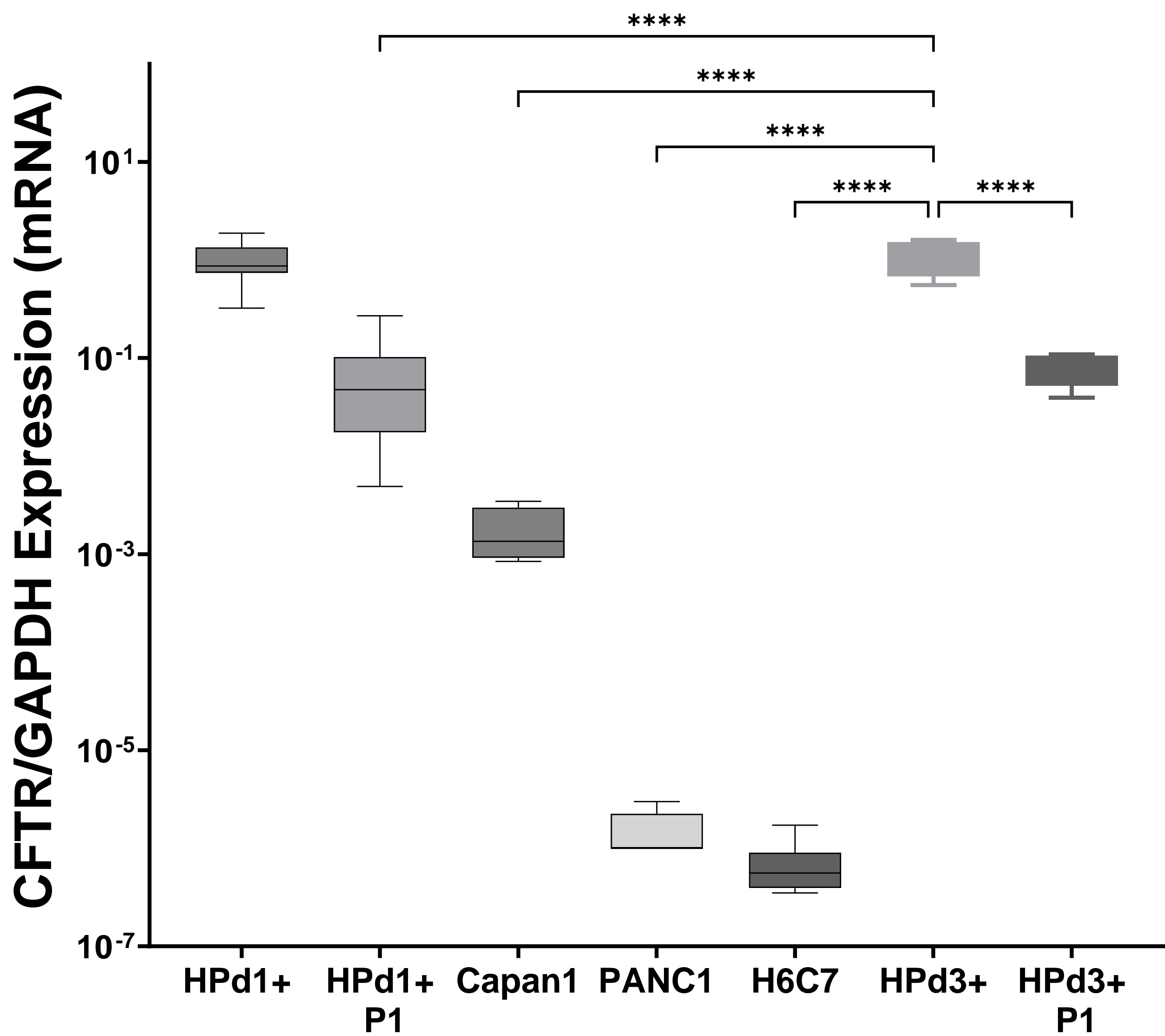


Figure 4: Comparison of fold change in CFTR mRNA expression between HPd samples and commercially available PDEC lines. Box and whisker plot demonstrating the comparison of CFTR mRNA expression between day of isolation HPd1+ samples, HPd1+ passage 1 “P1” cells, Capan1, PANC1, H6C7, day of isolation HPd3+ samples, and HPd3+ passage 1 “P1” cells. Samples were measured by qPCR with endogenous controls (*GAPDH*) and normalized to HPd1+ cells on the day of isolation**.** HPd1+ and HPd1+ *n=9*, Capan1 *n=4*, PANC1 *n=6*, H6C7 *n=6*, HPd3+ and HPd3+ P1 *n=4.* ****p<0.005

Chapter 4. CLOSING SUMMARY

In this thesis, I first reviewed the current understanding of the pathological changes that manifest in the pancreas/islet microenvironment due to cystic fibrosis. I discussed the global changes in CF pancreas which may contribute to CFRD which occurs in 40-50% of adults with CF. While the mechanisms of the loss of β cell function remain unknown, the pathological changes in CF pancreas provide clues to these mechanisms that result in insufficient insulin release from β cells. Loss-of-function mutations in *CFTR* in the pancreatic duct remains the core of CF pancreas pathology. The ductal pathology observed in CF pancreas disease commences by ductal plugging and subsequent obliteration of the ducts and results in the ruination of the exocrine pancreas which leads to fibrosis and fatty replacement. The clinical consequence of this destruction is pancreatic insufficiency with about 85% of people with CF subscribing to lifelong pancreatic enzyme replacement therapy.

Despite these changes in the pancreas/islet microenvironment, the islets persist. From the limited work that has been done to understand the PDEC-islet axis, data from animal models have suggested that crosstalk between PDECs and islets not only allow for islet survival in transplantation but could also be responsible for detrimental paracrine signals to the islet. This prompted our hypothesis, that PDECs may secrete factors that may impair islet function observed in CFRD.

In summary, chapter 2 is a collection of experiments that were dedicated to building a model of CF pancreas disease using primary human PDECs isolated from cadaveric islet-depleted human pancreas tissue. In this chapter, I explored methods to isolate primary human PDECs using HPd1 antibody and verified PDEC gene expression *ex vivo* in HPd1+ samples on the day of isolation (n=17) as well in cultured HPd1+ cells (n=9). Moreover, I experimented with different molecular tools to modulate *CFTR* expression in these primary human PDECs with success using a LentiCRISPR2-*CFTR* construct. Chapter 3 contains experiments designed to improve our method of acquiring primary human PDECs. In this chapter, I compare isolation efficiencies of HPd1 and HPd3 antibodies and determine that HPd3 is capable of yielding 2x number of *CFTR* expressing cells from cadaveric islet-depleted human pancreas tissue. Additionally, in this chapter, I determined that cells isolated using HPd3 are capable of being propagated in culture. While these cells isolated with either HPd1 or HPd3, lose *CFTR* expression between day of isolation and

passage 1, the ability to scale experiments for downstream analysis using HPd3 may prove useful for developing PDECs ± CFTR as a model of CF pancreas disease.

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