ANIMAL MODELS

Pancreatic and Islet Remodeling in Cystic Fibrosis Transmembrane Conductance Regulator (*CFTR*) Knockout Ferrets

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Accepted for publication
December 28, 2017.

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In cystic fibrosis (CF), there is early destruction of the exocrine pancreas, and this results in a unique form of diabetes that affects approximately half of adult CF individuals. An animal model of cystic fibrosis—related diabetes has been developed in the ferret, which progresses through phases of glycemic abnormalities because of islet remodeling during and after exocrine destruction. Herein, we quantified the pancreatic histopathological changes that occur during these phases. There was an increase in percentage ductal, fat, and islet area in CF ferrets over time compared with age-matched wild-type controls. We also quantified islet size, shape, islet cell composition, cell proliferation (Ki-67), and expression of remodeling markers (matrix metalloprotease-7, desmin, and α -smooth muscle actin). Pancreatic ducts were dilated with scattered proliferating cells and were surrounded by activated stellate cells, indicative of tissue remodeling. The timing of islet and duct proliferation, stellate cell activation, and matrix remodeling coincided with the previously published stages of glycemic crisis and inflammation. This mapping of remodeling events in the CF ferret pancreas provides insights into early changes that control glycemic intolerance and subsequent recovery during the evolution of CF pancreatic disease. (*Am J Pathol* 2018, 188: 876–890; <https://doi.org/10.1016/j.ajpath.2017.12.015>)

The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride and bicarbonate channel expressed in the pancreatic ductal epithelium. CFTR-mediated bicarbonate transport into the ductal lumen plays an important role in maintaining pH. Luminal pH regulation is critical to prevent premature activation of digestive enzymes secreted by pancreatic acinar cells.¹ Loss-of-function mutations in the *CFTR* gene and the ensuing decrease in pH in the pancreatic ductal lumen lead to pancreatic tissue destruction, cystic duct dilation, and fibrosis of the pancreas classically described in CF patients. There are six classes of mutations (I to VI) in the *CFTR* gene that vary the functionality of CFTR, and four of these mutations (specifically, I to IV) lead to exocrine pancreatic insufficiency.² Exocrine pancreatic insufficiency is caused by reduced digestive enzyme production, primarily because of substantial loss of acinar cells in the pancreas.^{3,4}

Exocrine pancreatic insufficiency in CF patients is associated with a decline in β -cell function and with the development of cystic fibrosis—related diabetes (CFRD). It has been reported that 19% of adolescents and 50% of adults with CF have CFRD.⁵ Abnormal glucose tolerance before development of diabetes is common and occurs primarily in CF children <5 years of age.⁶ Interestingly, approximately 55% of CF children aged 2 to 4.6 years have abnormal

Supported by NIH R24 grants DK096518 (J.F.E.), HL123482 (J.F.E.), and R01 DK097820 (A.U. and A.W.N.); a Fraternal Order of Eagles Diabetes Research Center scholar award (A.W.N.); the University of Iowa Center for Gene Therapy grant DK54759 (J.F.E.); the Carver Chair in Molecular Medicine award (J.F.E.); and the UK Cystic Fibrosis Trust grant SRC007 (J.F.E.).

Disclosures: None declared.

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glucose tolerance, and this frequency declines to approximately 20% by 5 to 6 years of age, suggesting a recovery of endocrine pancreas function after destruction of the exocrine pancreas.⁶ CFRD is associated with reduced first-phase insulin secretion during oral glucose tolerance tests,⁷ and insulin secretion is impaired to a greater extent in CF patients with exocrine pancreatic insufficiency.⁸ CF patients who develop CFRD have a more rapid decline in pulmonary function and increased morbidity and mortality than patients without CFRD.^{2,9,10}

Although clinical markers for onset of CFRD and its progression are currently available, there is little known regarding pathogenic processes that influence the occurrence and progression of this disease.² CF animal models have begun to shed light on the pathogenesis of CFRD, with some limitations.² CF mice lack exocrine pancreatic pathology, likely because of the presence of alternative anion channel(s) that compensate for the lack of CFTR.^{11,12} By contrast, CF pigs have extensive exocrine pancreatic pathology that is initiated in utero¹³ and are born with insulin secretion deficits¹⁴ not yet observed in CF infants <2 years of age.⁶ Thus, newborn CF pigs more closely represent pancreatic pathology seen in older CF children and/or adolescents. By contrast, CF ferrets are born with relatively minor pancreatic pathology¹⁵ and with age recapitulate the pancreatic phenotype seen in CF humans.¹⁶ CF ferrets exhibit mild glucose intolerance and reduced first-phase insulin secretion at birth,¹⁷ but rapidly progress through phases of glycemic disturbances and recovery as the exocrine pancreas is destroyed and the endocrine pancreas is remodeled.¹⁸ Four phases of glycemic regulation have been characterized in CF ferrets based on age.¹⁸ Phase I (3 to 4 weeks of age) is characterized by normal glucose tolerance, early pancreatic fibrosis, and mild inflammation. Phase II (1 to 2 months of age) is characterized by a significant increase in pancreatic inflammation, significant glycemic instability, and a decline in endocrine pancreas function. During phase III (2 to 4 months of age), there is a transient recovery in glucose tolerance and a rapid decline in inflammation that correlate with a recovery in pancreatic endocrine function. Recovery during this period was associated with activation of an adipogenic program driven by increased pancreatic peroxisome proliferator-activated receptor- γ and adiponectin expression.¹⁸ A subsequent period of worsening glucose tolerance was noted in ferrets >4 months of age and was classified as phase IV. Phase IV and beyond likely represents the most well-studied stage of developing CFRD in CF patients >10 years of age, whereas phase II is similar to the early glycemic disturbances observed in 2- to 4-year-old CF children.⁶

The structural changes in the endocrine and exocrine pancreas during these phases of varying glycemic tolerance have not been fully characterized. Such studies would not be feasible in human samples, but the CF ferret model provides the opportunity to characterize these events. Given that pancreatic tissue architecture in end-stage CF patients is comparable to CF ferrets >4 months,¹⁶ we sought to

characterize changes in islet composition and structure during the first three CF phases encompassing functional decline and recovery of the CF islet. Furthermore, we have evaluated features of pancreatic remodeling in these phases that lead to functional reorganization of the CF islet.

Materials and Methods

Ferret Pancreas Specimens

All animal experiments were approved by the University of Iowa Institutional Animal Care and Use Committee. The previously described CFTR exon 10 disrupted ferret model was used for these studies.¹⁹ CFTR knockout kits were paired with a non-CF littermate at birth and reared, as previously described,²⁰ with the following exception: all kits were reared on the antibiotics metronidazole (20 mg/kg, 2 $\times$ daily) and piperacillin/tazobactam (4.0 mg/kg, 2 $\times$ daily) from birth, and enrofloxacin (10 mg/kg, 2 $\times$ daily) was initiated at 5 days of age. These antibiotics were maintained throughout life to avoid lethal lung infection. No changes were made to the diet before sacrifice. Pancreatic tissue was harvested after euthanasia, fixed in 4% paraformaldehyde, routinely processed, embedded, and cut into sections (5 μ m thick). Both male and female animals were included in the studies, and animal numbers and sexes are included in each figure legend when appropriate.

Human Pancreas Specimens

Human pancreas tissue was obtained from the National Disease Research Interchange (Philadelphia, PA) as fixed samples in neutral-buffered formalin. Samples were processed and sectioned in paraffin. The use of deidentified human tissues in the study was approved by the Institutional Review Board at the University of Iowa.

Immunostaining

Fixed paraffin-embedded sections (5 μ m thick, one per animal) were stained with the following primary antibodies for immunofluorescence (all diluted at 1:500) (Table 1): polyclonal guinea pig anti-porcine insulin, mouse monoclonal anti-human glucagon, polyclonal goat anti-somatostatin, polyclonal rabbit anti-matrix metalloproteinase-7 (MMP-7), polyclonal rabbit anti-smooth muscle actin, and monoclonal mouse anti-desmin. Immunofluorescence staining was performed by incubating primary antibodies overnight at 4°C. The primary antibodies were detected using a combination of DyLight 488-, 549-, and 649-conjugated secondary antibodies. The secondary antibodies were incubated at room temperature for 1 hour. Dual immunohistochemistry was also performed on paraffin-embedded sections (5 μ m thick, one per animal), and the sections were antigen retrieved at a pH of 6.0 using citrate buffer at 110°C for 15 minutes. The primary antibody for Ki-67, monoclonal mouse anti-Ki-67 (M7240;

Table 1 Antibody Vendor Information

Peptide name	Antibody name	Catalog no.	Vendor (location)	Species
Insulin	Guinea pig anti-porcine	08651041	MP Biomedicals (Santa Ana, CA)	Guinea pig
Glucagon	Monoclonal anti-glucagon antibody	G2654	Sigma-Aldrich (St. Louis, MO)	Mouse
Somatostatin	Somatostatin antibody (D-20)	sc-7819	Santa Cruz Biotechnology (Dallas, TX)	Goat
MMP-7	Anti-MMP-7 antibody	ab5706	Abcam (Cambridge, MA)	Rabbit
α -SMA	Anti- α -SMA antibody	ab5694	Abcam	Rabbit
Desmin	Desmin Novocastra	DES-DERII-L-CE	Leica Biosystems (Wetzlar, Germany)	Mouse
Ki-67 (IHC)	Ki-67 antigen	M7240	Dako (Carpenteria, CA)	Mouse
Insulin (IHC)	Insulin polyclonal antibody	65104	MP Biomedicals	Guinea pig

IHC, immunohistochemistry; MMP-7, matrix metalloprotease-7; α -SMA, α -smooth muscle actin.

Dako, Carpenteria, CA) was diluted at 1:500 and incubated for 2 hours at room temperature, followed by application of horseradish peroxidase and diaminobenzidine. After multiple washing steps, the primary antibody for insulin (65104; MP Biomedicals, Santa Ana, CA) was applied at a dilution of 1:2000 for 2 hours at room temperature, followed by application of a rabbit anti-guinea pig horseradish peroxidase at 1:500 for 30 minutes, and finally the Rabbit Envision from Dako. 3-amino-9-ethylcarbazole (AEC) red was used for detection of insulin, and slides were counterstained with hematoxylin.

Microscopy and Computing Platforms

Hematoxylin and eosin and immunohistochemistry images were acquired using an Olympus BX51 with attached microscope camera (Olympus DP71) and Olympus CellSens software version 1.9 (Olympus, Melville, NY). Immunofluorescence images were acquired using Olympus IX8 DSU spinning disk confocal microscope, Olympus CellSens, and Zeiss 700-point scanning confocal microscope (Zeiss, Oberkochen, Germany). Immunofluorescence image processing and analyses were performed using a previously published method, described in detail.²¹ To briefly describe the process, custom-written scripts with watershed segmentation algorithms on Fiji/ImageJ software version 2.0.0 (NIH, Bethesda, MD; <http://imagej.nih.gov/ij>) were used to identify the islet cell types and morphometric features of the islet, including Feret diameter and circularity index. MATLAB (MathWorks, Natick, MA) was used to analyze data obtained from Fiji/ImageJ and to generate graphs of the data.

Quantification of Islet, Adipose, and Ductal Areas

The areas of the entire pancreas, fat tissue, islet cells, and ducts were separately calculated using custom-written scripts and watershed segmentation algorithm in Fiji/ImageJ.²¹ Pancreatic area without fat was calculated by subtracting the previously calculated fat area from the entire pancreatic tissue area. The duct area and the islet area (sum of area of each islet cell type) were normalized to the pancreatic area without fat, whereas the fat area was normalized to the entire pancreatic tissue area. This was

performed because fatty replacement of the pancreas during CF can be extensive, and in advanced disease, it is impossible to differentiate fatty replacement from resident peripancreatic fat. These values were used to calculate the percentage fat, islet, and duct area. To quantify the different cell types in islets, individual channel images of ferret pancreatic sections were immunostained with insulin (INS), glucagon (GCG), somatostatin (SST), and DAPI and were captured using an automated whole-slide virtual slices capture technique.²¹ Customized image analysis script was used to identify individual islets, cell types, and the nucleus, which belongs to each cell type.²¹ The analysis script computed the area of each islet, area of each cell type, islet circularity, and islet Feret diameter, and generated Excel spreadsheets for every section. Customized MATLAB data analysis scripts used the Excel spreadsheets to generate graphs depicting the relationship between islet area, circularity, and Feret diameter, as previously described.²¹

Statistical Analysis

Mann-Whitney tests were used to calculate significance. Statistics for islet area, circularity, and Feret diameter were calculated using the analysis of variance function in R version 3.2.3 (The R Foundation for Statistical Computing Platform; <https://www.r-project.org>). To account for the correlated nature of these three variables, when regressing for significance for each, the other two variables were accounted for before considering genotype or phase. Analysis of covariance formula for calculating the significance of islet area, circularity, and Feret diameter was as follows:

$$\begin{aligned} \text{Area} &\sim \text{Circularity} + \text{Feret} + \text{Genotype} * \text{Phase}; \\ \text{Circularity} &\sim \text{Area} + \text{Feret} + \text{Genotype} * \text{Phase}; \\ \text{Feret} &\sim \text{Circularity} + \text{Area} + \text{Genotype} * \text{Phase}. \end{aligned}$$

The Tukey honestly significant difference function was used for calculated post-hoc significance across individual groups. MMP-7 immunostaining was quantified using MetaMorph (Molecular Devices, San Jose, CA). Dual-stained insulin and Ki-67 slides were imaged at a magnification of $\times 40$. Ten separate fields of view were imaged, and then INS-positive cells and dual-positive cells were counted using ImageJ software

Figure 1 Pancreatic pathology in phase II cystic fibrosis (CF) ferrets. **A–J:** Representative images of pancreatic sections, from wild-type (WT) ferret pancreas and CF ferret pancreas both 40 days of age, stained with hematoxylin and eosin. **A–E:** Wild-type ferret pancreas presented at multiple magnifications is characterized with abundant exocrine pancreatic tissue (**gray arrowhead**), multiple pancreatic ducts (**white arrows**), and interspersed islets of Langerhans (**dotted circles**). **F–J:** CF ferret pancreas that exhibits moderate-to-marked loss of exocrine pancreatic tissue and replacement by fibrous connective tissue (**black arrowhead**) and to a lesser extent by adipose tissue (**white arrowheads**), dilation of pancreatic ducts (**white arrows**), and clumping of islets (**dotted circles**). **J:** Most islets are surrounded by loose connective tissue and appear less discrete compared with wild type. **K–M:** The percentages fat, duct, and islet cell areas were quantified using FIJI/ImageJ software version 2.0.0. Although no significant difference is observed in the percentage islets area and percentage fat area, an approximately 10-fold increase in the percentage duct area is seen in phase II CF ferrets. Data are expressed as means $\pm$ SEM (**L–M**). $n = 4$ WT (1 male and 3 female), $n = 4$ CF (4 female) (numbers are animals per group). * $P < 0.05$ (Mann-Whitney test in GraphPad Prism software version 7.0a was used to calculate the P value). Original magnifications: $\times 2$ (**A** and **F**); $\times 4$ (**B**); $\times 10$ (**C** and **G–I**); $\times 20$ (**D**); $\times 40$ (**E** and **J**).

version 1.51. Each field of view was averaged per animal, and the percentage dual-positive islet cells was calculated by dividing dual-positive cells by INS-only positive cells. Statistics were performed using Mann-Whitney tests in GraphPad Prism software version 7.0a (GraphPad Prism, Inc., La Jolla, CA).

Results

Onset of Loss of Pancreatic Exocrine Tissue and Increase in Duct Area in Phase II CF Ferrets

In adult CF patients in whom pancreatic histopathology has been well described, findings include almost complete loss of

the exocrine acini, with presence of adipose and fibrous connective tissue in place of the bulk of the pancreas (**Supplemental Figure S1**). To understand the onset and evolution of this CF-related pancreatic pathology, the CF ferret model of disease was used. Paraffin-embedded pancreatic tissues from age-matched CF and non-CF ferrets from newborn and phases I to III¹⁸ were analyzed by a board-certified veterinary pathologist (K.N.G.-C.). Newborn CF ferret pancreas had mild pathology and displayed little evidence of inflammation or fibrosis (**Supplemental Figure S2**). Phase I CF ferrets exhibited mild inflammation and dilation of acini compared with the age-matched wild-type (WT) controls (**Supplemental Figure S3**). Onset of inflammation

Figure 2 Pancreatic pathology in phase III cystic fibrosis (CF) ferrets. **A–F:** Representative images of pancreatic sections from wild-type (WT) ferret pancreas and CF ferret pancreas, both 104 days of age, were stained with hematoxylin and eosin. **A–C:** Wild-type ferret pancreas has abundant exocrine pancreatic tissue (gray arrowhead), multiple pancreatic ducts (white arrows), and interspersed islets of Langerhans (dotted circles). **D–F:** The CF ferret pancreas is characterized by almost complete loss of exocrine pancreatic tissue, having only scattered dilated ducts (white arrows) remaining, whereas the remaining tissue is composed of dense fibrous connective tissue (black arrowheads) or fat (white arrowhead). Endocrine tissue is clumped haphazardly and surrounded by dense connective tissue (dotted circles). **G–I:** The percentages fat, duct, and islet cell areas were quantified by using FIJI/ImageJ software version 2.0.0. **G:** A 10-fold increase in percentage fat area is seen in phase III CF ferrets. **H:** The presence of dilated ducts produces a 10-fold increase in the percentage duct area in phase III CF animals compared with age-matched WT. **I:** A sixfold increase is seen in percentage islet cell area in phase III CF animals relative to WT. Data are expressed as means $\pm$ SEM (**G–I**). Phase II: $n = 4$ WT [1 male (M) and 3 female (F)], $n = 4$ CF (4 F); phase III: $n = 5$ WT (2 M and 3 F), $n = 5$ CF (5 F) (numbers are animals per group). * $P < 0.05$ (Mann-Whitney post-hoc test in GraphPad Prism software version 7.0a was used to calculate the P value). Original magnifications: $\times 2$ (**A** and **D**); $\times 10$ (**B**); $\times 20$ (**C**); $\times 40$ (**E** and **F**).

was visually noted in phase I (Supplemental Figure S3) and preceded the loss of exocrine acini and ductal dilation. CF ferrets in phase II exhibited more marked pancreatic pathology, including extensive loss of exocrine tissue (Figure 1, A, D, E–J), presence of extensive fibrous connective tissue, and, to a lesser extent, presence of adipose tissue (Figure 1F). In addition, multifocal mildly to moderately dilated ducts were also noted (Figure 1, B and I). Islets were present in large variably shaped clumps, frequently surrounded by dense fibrotic connective tissue (Figure 1, C, E, H, and J). A trend toward increased percentage fat area was observed (Figure 1K). On quantification, a 10-fold increase ($P < 0.05$) in percentage duct area relative to the age-matched WT ferrets was seen (Figure 1L). No significant difference in percentage islet cell area was present during phase II (Figure 1M), but this calculation excluded adipose and ductal luminal area.

Continued Adipose Replacement of the Exocrine Pancreas Is Associated with Increased Islet Area in the Phase III CF Ferret Pancreas

Pancreatic sections of phase III CF and age-matched WT ferrets were analyzed to study progression of CF-related pancreatic disease (Figure 2). This phase was of interest because it marks a stage of postprandial glycemic recovery.¹⁸ There was almost complete replacement of exocrine tissue with fat or fibrosis in phase III CF ferrets (Figure 2, A and D). Phase III CF islets were composed of larger clusters of cells, similar to that observed during phase II (Figure 2, B–F). CF animals had significantly higher (10-fold, $P < 0.05$) percentage fat area in the pancreas compared with WT ferrets (Figure 2G). The percentage duct area was approximately 10-fold greater ($P < 0.05$) in phase III CF

Figure 3 Changes in pancreatic islet cells in wild-type (WT) and cystic fibrosis (CF) ferrets. **A–H:** Representative images of wild-type (**A–D**) and CF (**E–H**) ferrets from newborn (NB) to phase III, immunostained for insulin (INS; green), glucagon (GCG; gray), somatostatin (SST; red), and nuclei (blue). **Insets:** Fluorescent signals of the **yellow boxed areas** from the main images. **A–D:** In WT ferrets, during phases I to III, the dispersed islet cells coalesce and form the typical islet structure of an INS cell core surrounded by GCG cells. **E–H:** CF ferret stained pancreatic sections. **F and I:** In CF phase I, there is a decrease in INS cells, followed by the appearance of dispersed INS cells in phase II. **H and I:** A drastic increase in INS cells is observed in phase III, where islets appear as a large mass in certain regions of the intact pancreas and lack the representative islet structure seen in WT ferrets. **I–L:** Frequency of occurrence of INS, GCG, and SST cells and islets in the pancreatic tissue was calculated by normalizing the area of INS-, GCG-, and SST-positive regions and their sum to the pancreatic tissue area without fat, respectively. **I–L:** The quantified frequencies of INS (**I**), GCG (**J**), and SST (**K**) producing cells and whole islets (**L**) in the pancreas of WT and CF ferrets indicate a significant increase in islet cell types and whole islets in CF phase III ferrets. One section (5 μ m thick) was evaluated per animal. Data are expressed as means $\pm$ SEM (**I–L**). $n = 9$ WT, $n = 10$ CF (newborn; **A, E**, and **I–L**); $n = 8$ WT [1 male (M) and 7 female (F)], $n = 6$ CF (1 M and 5 F) (phase I; **B, F**, and **I–L**); $n = 4$ WT (1 M and 3 F), $n = 4$ CF (4 F) (phase II; **C, G**, **I–L**); $n = 5$ WT (2 M and 3 F), $n = 5$ CF (5 F) (phase III; **D, H**, and **I–L**); numbers are animals per group. * $P < 0.05$ (Mann-Whitney test in GraphPad Prism 7.0a) was used to calculate the P value). Original magnification, $\times 10$ (**A–H**).

ferrets compared with WT animals (Figure 2H), and similarly the percentage islet cell area was approximately sixfold greater ($P < 0.05$) in CF animals during this phase (Figure 2I). The transition from phase II to III in CF animals was associated with an approximately twofold ($P < 0.05$) increase in percentage fat area and an approximately sixfold increase ($P < 0.05$) in percentage islet cell area; duct area remained unchanged between these phases (Supplemental Figure S4).

Aging CF Ferrets Exhibit Atypical Islet Organization

Previous work by our laboratory has demonstrated distinct phases of glucose tolerance in CF ferrets as they age.¹⁸ Diabetic level fasted and postprandial hyperglycemia were observed during phase II in CF animals, and this rapidly normalized during phase III with a resurgence of islet hormone transcripts in the CF pancreas.¹⁸ Alterations in islet structure, including vascularization, innervation, and/or

composition of islet cell types that affects intra-islet paracrine signaling, may all be causes of dysfunctional insulin-producing cells during phase II. To begin to address this hypothesis, pancreatic islets were analyzed in WT and CF ferrets across these phases of CF-related pancreatic disease. Three islet cell types were first quantified (Figure 3). Paraffin-embedded sections of ferret pancreas were stained with the islet hormones INS, GCG, and SST to evaluate the changes in islet cell composition. The area of INS, GCG, and SST positivity on the stained pancreatic sections was normalized to the total pancreatic area without fat to gauge the frequency of islet cell types. The frequency of these three islet cell types in newborn WT and CF pancreas was largely indistinguishable, with smaller irregularly shaped islets and dispersed single islet hormone-expressing cells in both genotypes during this phase (Figure 3, A, E, and I–L). With age, islet cells coalesced to form larger islets in WT ferrets with a higher percentage of INS-producing cells and a lower percentage of GCG- and SST-producing cells in each discrete

Figure 4 Relative frequency of insulin (INS), glucagon (GCG), and somatostatin (SST) cells correlates to size of the islet in cystic fibrosis (CF) and wild-type (WT) ferrets. **A–H:** The relative frequencies of INS (green), GCG (red), and SST (blue) cells in individual islets were calculated using custom-written MATLAB scripts. **A–H:** Discrete islet area, not normalized to pancreatic tissue area, was plotted against discrete islet size, as seen in WT (**A–D**) and CF (**E–H**) ferrets. The effective diameter of islets (in micrometers) is correlated with the number of cells in the islet. The frequency of occurrence of islets of a particular size and cell number is represented in gray bars. **A–C** and **E–G:** A consistent higher frequency of INS cells and corresponding lower frequency of GCG and SST cells is seen in newborn, phase I, and phase II WT (**A–C**) and CF (**E–G**) islets. **I–L:** Quantification of the average relative frequency of INS, GCG, and SST cells in discrete islets of WT and CF ferrets from newborns to phase III shows no significant changes between WT and CF animals. Data are expressed as means $\pm$ SEM (**I–L**). $n = 9$ WT, $n = 10$ CF (newborn; **A, E**, and **I**); $n = 8$ WT [1 male (M) and 7 female (F)], $n = 6$ CF (1 M and 5 F) (phase I; **B, F**, and **J**); $n = 4$ WT (1 M and 3 F), $n = 4$ CF (4 F) (phase II; **C, G**, and **K**); $n = 5$ WT (2 M and 3 F), $n = 5$ CF (5 F) (phase III; **D, H**, and **L**; numbers are animals per group).

islet (Figure 3, A–D and I–K). Consistent with a previous report describing a decline in insulin-producing cell mass in CF ferrets during phase I preceding the glycemic crisis,¹⁸ there was a decline in the frequency of all three islet cell types in the CF pancreas compared with WT animals (Figure 3, B, F, and I–L). No significant difference in the frequency of the islet cell types was observed in phase II CF and WT animals (Figure 3, C, G, and I–K). However, the phase II to III transition in CF ferrets was accompanied by a 6.1-fold increase ($P < 0.05$) in INS-positive cells, consistent with improved glycemia during this phase (Figure 3, H–L). The frequency of GCG- and SST-positive cells also significantly increased (7.5- and 12.2-fold, respectively; both $P < 0.05$) during phase III, and they were found surrounding a small cluster of INS-producing cells contained within these

larger islet aggregates. Overall, the difference in development of islets in CF compared with WT ferrets may be attributable to the influence of congruent exocrine pancreatic pathology in phases I, II, and III.

Islet Cell Populations Dynamically Change in Aging CF Ferrets

A normal human islet contains >40% INS, approximately 30% GCG, and approximately 10% SST cells, with fewer pancreatic polypeptide producing cells.²² Changes in relative percentages of islet cells attributable to age and body mass index in human donors imply the presence of dynamic islet populations in response to systemic metabolic needs.²³ Murine studies on variations in relative islet cell composition of

Figure 5 Islet shape and distribution in wild-type (WT) and cystic fibrosis (CF) ferrets. **A** and **B**: Three-dimensional visualization of islet size (area) and shape (circularity and Feret diameter) distribution for WT (**A**) and CF (**B**) ferrets, calculated using custom-written MATLAB script. Each dot represents a single islet/cluster. Islet area is divided by the single-cell area ($178 \mu\text{m}^2$) to represent the number of cells in a given islet area. The conversion between logarithmic islet area (logarithmic) and effective diameter (μm) is shown in the figure. Regions identified as islets are indicated by **white arrows**. Custom MATLAB scripts enclose identified islet regions with dotted yellow boundaries to calculate islet area, circularity index, and Feret diameter. The density of islets is color coded from sparse to dense. **C–E**: Quantification of islet area (**C**), circularity (**D**), and Feret diameter (**E**) for various phases of CF and WT ferrets. Graphs show box-and-whisker plots for various groups, and analysis of covariance in R version 3.2.3 was used to quantify the significance in individual nonnormalized islet area, circularity, and Feret diameter. A significant difference in Feret diameter is observed between WT and CF phase III ferrets. Data are expressed as means (**C–E**). $n = 9$ WT, $n = 10$ CF (newborn); $n = 8$ WT [1 male (M) and 7 female (F)], $n = 6$ CF (1 M and 5 F) (phase I); $n = 4$ WT (1 M and 3 F), $n = 4$ CF (4 F) (phase II); $n = 5$ WT (2 M and 3 F), $n = 5$ CF (5 F) (phase III; numbers are animals per group). *** $P < 0.001$. Original magnification, $\times 10$ (**A** and **B**). GCG, glucagon; INS, insulin; SST, somatostatin.

islets in type 2 diabetes have reported an increase in GCG, SST, and pancreatic polypeptide cells and a decrease in INS cells in nonobese diabetic mice.^{24,25} Given the phases of hyperglycemia in CF ferrets, it was analyzed how islet size and the relative percentage of individual islet cells varied at each stage of disease. Paraffin sections of the pancreas stained with INS, GCG, and SST were imaged and analyzed using custom-written scripts in MATLAB.²¹ Custom-written macros identified islets in the pancreas and calculated the area of individual identified islets. Islet area was divided by the

average single-cell area ($170 \mu\text{m}^2$). These calculations were not normalized but instead are presented to provide an estimate of number of cells present in each discrete islet.²¹ The size of the islets was correlated with the percentage of islet cell populations.²¹ The presence of a wide range of islet size, varying from single islet cells to islets approximately $600 \mu\text{m}$ diameter, required the representation of islet sizes in logarithmic scale.²¹ The conversion scale to correlate diameter of the islet to the number of cells is shown in Figure 4. The frequencies of individual nonnormalized islet sizes was

Figure 6 Expression of matrix metalloprotease-7 (MMP-7) in cystic fibrosis (CF) ferret pancreas. **A–H:** Wild-type (WT) and CF ferret pancreas sections from phase I to III and older than phase III were immunostained with antibodies against insulin (INS) and MMP-7; MMP-7 colocalizes only in the islet cells. **Insets:** Fluorescent signals of the **yellow boxed areas** from the main images. **I–K:** Intensity of MMP-7 immunoreactivity was quantified using Fiji. **J:** Increased immunoreactivity of MMP-7 is observed in phase II CF ferrets when compared with age-matched WT. Data are expressed as means $\pm$ SEM (**I–K**). $n = 5$ WT [1 male (M) and 4 female (F)], $n = 6$ CF (2 M and 4 F) (phase I; **A**, **C**, and **I**); $n = 5$ WT (5 F), $n = 6$ CF (2 M and 4 F) (phase II; **B**, **D**, and **J**); $n = 4$ WT (2 M and 2 F), $n = 4$ CF (1 M and 3 F) (phase III; **E**, **G**, and **K**); $n = 3$ WT (2 F and 1 M), $n = 3$ CF (1 F and 2 M) (more than phase III; **F**, **H**, and **K**; numbers are animals per group). * $P < 0.05$ (Mann-Whitney test in GraphPad Prism 7.0a was used to calculate the P value). Original magnification, $\times 20$ (**A–H**).

overlaid with the relative frequency of INS, GCG, and SST cells in islets associated with their respective size bins (Figure 4, A–H). On average, approximately 60% to 80% of the cells in the islets throughout the size range were INS cells in both WT and CF animals in newborns and phases I to III. GCG and SST cells contributed to, on average, approximately 10% to 20% and approximately 20% to 40% of the

cells in the islet, respectively, across all stages of WT and CF ferrets. The pattern of cellular composition across various sized islets was similar in newborn CF and WT animals (Figure 4, A and E). In both newborn genotypes, the smallest islets were predominately composed of INS and SST cells, whereas with increasing islet size, GCG cells increased at the expense of SST cells. Subtle changes in these trends emerged

Figure 7 Expression of Ki-67 in phase II and phase III cystic fibrosis (CF) ferrets. **A–L:** Proliferation in phase II (**A–F**) and phase III (**G–L**) wild-type (WT) and CF ferret pancreas was analyzed by immunohistochemical staining for Ki-67 (brown). The sections were costained with insulin (INS; red) to analyze proliferation specifically in insulin-producing cells. **B, C, H, and I:** Ductal epithelium in the WT phase II and phase III ferrets does not have Ki-67–positive cells (**C** and **I**), and rare scattered Ki-67–positive cells are found within or around the insulin-producing cells (**B** and **H**). **F** and **L:** Conversely, proliferating cells, positive for Ki-67, are found in the ductal epithelium of phase II and phase III CF ferrets. **E** and **K:** In addition, Ki-67–positive (**thick short arrow**) and INS-positive (**thin short arrow**) cells are found in the phase II and phase III CF ferrets, indicating the presence of proliferating insulin-producing cells. **M** and **N:** Quantification of percentage Ki-67– and insulin-positive cells in WT and CF animals from both phase II (**M**) and phase III (**N**) indicates a significant increase in proliferating INS cells. Data are expressed as means $\pm$ SEM (**M** and **N**). $n = 4$ WT [4 female (**F**)], $n = 4$ CF [2 male (**M**) and 2F] (phase II; **A–F** and **M**); $n = 4$ WT (3 M and 1 F), $n = 4$ CF (1 M and 3 F) (phase III; **G–L** and **N**; numbers are animals per group). * $P < 0.05$, ** $P < 0.001$ (Mann-Whitney test in GraphPad Prism 7.0a was used to calculate the P values). Original magnifications: $\times 2$ (**A** and **G**); $\times 40$ (**B, C, F**, and **H**); $\times 10$ (**D** and **J**); $\times 20$ (**E**); $\times 60$ (**K** and **L**).

with age. For example, islets with effective diameter $>85 \mu\text{m}$ in phase I CF ferrets had a gradual increase in the α cell frequency that was less pronounced in age-matched WT ferrets (Figure 4, B and F). During phase II, islets $>240 \mu\text{m}$ in diameter had near 100% INS cells in CF animals (Figure 4G). Phase III WT and CF ferrets had larger islets than younger ferrets. During this phase, GCG and SST cells were in near equal proportions across various sizes of islets in WT animals (Figure 4D). By contrast, phase III CF islets had a greater percentage of SST cells than GCG cells across all islet sizes (Figure 4H). On quantification of the average frequency of INS, GCG, and SST cells in discrete islets, no significant difference was found between WT and CF ferrets (Figure 4, I–L). These findings suggest that the cellular

composition of CF islets dynamically changes with islet size and phase of disease.

Changes in Islet Structure after Exocrine Pancreas Destruction in CF Ferrets

Previously published morphometric analyses²¹ were used to quantify the changes in shape and size of islets across the phases of exocrine/endocrine disease and correlate these changes with the density of islets. Custom-written macros identified islets in the pancreas and calculated the area of individual islets. The calculated area of the islets was not normalized to the pancreatic area to analyze changes in discrete islet area. The shape of islets was analyzed for the

Figure 8 Stellate cell activation in cystic fibrosis (CF) ferrets. **A–D:** Phase II and phase III wild-type (WT; **A** and **C**) and CF (**B** and **D**) ferret pancreatic sections were immunostained with antibodies against α -smooth muscle actin (α -SMA) and desmin to analyze stellate cell activation. Activated stellate cells are defined by colocalization of α -SMA and desmin. **B** and **D:** Activated stellate cells encircle the ductal epithelium in CF ferrets. **A–D:** Immunofluorescence signal in activated stellate cells around ducts is noted in CF phase II and phase III ferrets, but is absent in the age-matched WT ferrets (**B** and **D** compared with **A** and **C**, respectively). **Insets** show fluorescent signals of the **yellow boxed areas** from the main images. $n = 3$ WT (2 F and 1 M), $n = 3$ CF (1 F and 2 M) (phase II; **A** and **B**); $n = 3$ WT (2 F and 1 M), $n = 3$ CF (3 F) (phase III; **C** and **D**; numbers are animals per group). Original magnification, $\times 20$ (**A–D**).

degree of circularity represented as a range from 0.0 to 1.0, wherein 1.0 is a perfect circle. The longest diameter of the islet, called the Feret diameter, was calculated for WT and CF ferrets and correlated to the circularity and islet density using a three-dimensional scatter plot (Figure 5, A and B). A gradual increase in larger islets was seen in WT ferrets as they age. Larger islets typically were low on the circularity scale and less densely distributed, whereas smaller islets were more densely distributed and relatively higher on the circularity scale. In CF animals, there was a greater proportion of larger, less dense, and less circular islets during phase II and phase III (Figure 5B), compared with the age-matched WT (Figure 5A). The transient improvement in glucose tolerance in phase III CF ferrets, previously reported,¹⁸ coincided at the time during which these islet structural changes occurred.

Feret diameter, circularity, and area were quantified after regressing out the dependencies between the three variables using analysis of covariance to analyze the difference between genotypes (Figure 5, C–E). A significant increase in the Feret diameter and a trend toward increase in discrete islet area were observed in phase III CF ferrets when compared with age-matched WT ferrets (Figure 5, C and E).

MMP-7 Is Up-Regulated in β Cells during CF Islet Remodeling

Matrix metalloproteases play important roles in tissue remodeling after injury through their ability to modify the extracellular matrix.^{26,27} MMP-7 has been studied for its role in pancreatic ductal adenocarcinoma, intraepithelial

Figure 9 Schematic of coinciding pancreatic pathology-related processes in aging cystic fibrosis (CF) ferrets. **A:** The chronological changes in the CF ferret pancreas contributing to the remodeling are presented in the context of previously reported changes in glucose tolerance and inflammation. **A:** Phases I and II are characterized by pancreatic inflammation, followed by activation of stellate cells and abnormal glucose tolerance (indicated by islet function). In phase III, much of the exocrine pancreas is replaced by adipocytes with less inflammation and a recovery in islet function, and an increase in islet frequency is seen. **B:** The presence of inflammation in the pancreas results in activation of stellate cells. We hypothesize the stellate cells differentiate into adipose tissue, causing the significant increase in fat in the CF pancreas. Chronic inflammation surrounding the islets results in remodeling by metalloproteases. We hypothesize the metalloprotease-induced remodeling of the islets can result in islet neogenesis and trans-differentiation of ductal epithelial cells into islet cells. α -SMA, α -smooth muscle actin.

neoplasia, and metaplasia,²⁸ and it has been shown to be up-regulated in islets from type 2 diabetes patients.²⁹ Therefore, the expression profile of MMP-7 was investigated across various phases of disease in the CF pancreas (Figure 6, A–H). Insulin was used to distinguish the endocrine from the exocrine pancreas. Immunoreactivity of MMP-7 in insulin-expressing INS cells significantly increased during phase II in CF ferrets (Figure 6, I–K). These findings suggest that MMP-7 expression within CF ferret islets during phase II may play a role in remodeling during and after exocrine pancreas destruction.

Remodeling in Phase II and Phase III Includes Islet Cell and Ductal Hyperplasia and Activated Stellate Cells

The large increase in islet frequency between CF phases II and III suggests that islet neogenesis may be occurring.³⁰ Previous studies have demonstrated in various injury models that ducts may contribute progenitor cells during islet neogenesis.^{31,32} We, therefore, evaluated cellular proliferation across phases II and III using Ki-67 as a marker (Figure 7). Interestingly, Ki-67 labeled the nuclei of the ductal epithelium and insulin-expressing cells in both phase II and phase III of the CF pancreas (Figure 7, D–F and J–L), whereas there was little ductal cell proliferation in WT animals (Figure 7, A–C and G–I). INS immunoreactive cell clusters juxtaposed to ducts were observed that appeared to be an extension of the ductal epithelium (Figure 7, D and F). There was a significant increase in proliferating islet cells during phases II and III, indicating

that islet neogenesis is occurring (Figure 7, M and N). Because of the presence of fibrotic tissue surrounding the proliferating ductal epithelium, pancreatic sections were stained for α -smooth muscle actin and desmin to detect the presence of activated stellate cells (Figure 8). Activated stellate cells surrounded the ductal epithelium in phase II and phase III CF ferrets (Figure 8, B and D), but not in age-matched WT ferrets (Figure 8, A and C).

Discussion

The pathogenesis of CFRD remains poorly understood, with two major underlying hypotheses about how CFTR defects lead to β -cell dysfunction. The first claims that CFTR functions directly within islet cells. The second posits that islet dysfunction is caused by exocrine pancreas damage that invokes functional changes to the endocrine pancreas. Although these two hypotheses are not mutually exclusive, a more complete understanding of how the CF pancreas is remodeled during and after exocrine destruction is needed to define the state of the endocrine pancreas. The development of the CF ferret model has provided opportunities to address early events in pancreatic disease that may influence progression to CFRD. The findings that CF ferrets and young CF children undergo phases of glycemic disturbances and recovery^{6,18} attributable to exocrine pancreas destruction prompted the current study to identify architectural remodeling of the CF pancreas. Although these studies provide a descriptive framework for exocrine and endocrine

pancreas remodeling in the CF ferret, they also provide clues regarding remodeling factors that may control progenitors involved in reestablishment of functional islets. A full understanding of these factors may aid in developing regenerative therapies for CFRD patients after functional decline of the insulin-producing cell.

Islet frequency and islet cell composition were not significantly different between newborn CF and WT ferrets. However, during the first month of life (phase I), the percentage frequency of INS, GCG, and SST cells and islets was significantly lower in CF compared with WT animals (Figure 3). Although there were no significant changes in islet cell or islet frequency between phase I and II in either genotype, the phase II to III transition was marked by significant genotypic differences that may be of physiological relevance. Although all islet cell frequencies increased significantly in CF across this transition, SST cells increased to the greatest extent (12.2-fold), nearly twice the increase in frequency of GCG and INS cells (approximately 6- to 7.5-fold) (Figure 3, I–K). These changes appeared to be CF dependent, because GCG cell frequency increased the greatest in WT animals (threefold), with SST cells increasing only 1.3-fold (Figure 3, I–K). SST cells secrete somatostatin, and this has been shown to inhibit insulin and glucagon secretion.³³ Thus, this alteration to the composition of the CF ferret islet may affect the ability of INS cells to properly function longer term.

We have previously reported that peroxisome proliferator-activated receptor- γ and adiponectin increase significantly during the glycemic recovery phase (phase III) when there is a sharp decrease in inflammatory mediators in the CF pancreas.¹⁸ This was interpreted as the transition when adipogenesis occurs. Indeed, there was significant increase in fat and islet frequency in phase III CF ferrets compared with age-matched WT and phase II CF ferrets (Figures 1 and 2). However, our findings that demonstrate that fat content was also greater in phase II CF versus WT animals suggest that the adipogenic program initiates during the inflammatory phase of injury. Although fat has been primarily thought to be a source of proinflammatory cytokines in diabetes, recent lineage-tracing studies in mice have begun to show that fat can play a key role in regeneration through transdifferentiation of myofibroblasts into adipocytes.³⁴ Given that α -smooth muscle actin, a marker of myofibroblasts, is highly expressed in fibrotic regions of the CF pancreas during phase II and III, these cells may be the source of adipocytes (Figure 8). Furthermore, the pancreatic stellate cell is a myofibroblast-like cell type³⁵ and may, thus, be the source of myofibroblasts during CF pancreatic remodeling. Interestingly, quiescent pancreatic stellate cells store fat in the pancreas; however, when activated, they lose fat droplets.³⁵

MMPs play important roles in tissue remodeling. As extracellular proteases, they can modulate cell surface receptors and cleave extracellular matrix to enable cell migration, replication, and the release of growth factors sequestered by the extracellular matrix.²⁶ MMPs were

recently identified as a marker for islet morphogenesis and neogenesis.³⁶ MMP-7, in particular, is studied for its role in remodeling the extracellular matrix in response to inflammation. For example, MMP-7 cleaves syndecan-1 to release CXCL1, which attracts neutrophils.³⁷ MMP-7 immunoreactivity was strongest in the CF ferret pancreas during the stage of glycemic crisis and peak inflammation (phase II) (Figure 6).¹⁸ The activation of MMP-7 preceding (phases I and II) and during (phase III) the stage of glycemic recovery in CF ferrets may play an important role in islet neogenesis. For example, MMP-7 expression was observed in CF pancreatic acinar cells during phase I. MMP-7 has been previously shown to be required for activation of the Notch signaling pathway controlling acinar-to-ductal cell transdifferentiation²⁷ and as a metaplastic precursor state of pancreatic ductal adenocarcinoma.³⁸ Enhanced *MMP7* gene expression in human β -cell-enriched type 2 diabetes pancreatic samples, compared with normal controls, coincides with the induction of several regenerating islet genes and ductal marker *Sox9*.²⁹ Whether activation of these genes reflects duct cell to islet cell regenerative pathways in type 2 diabetes patients remains unclear; however, lineage tracing studies of duct cells in mice support the notion that ductal progenitors can serve as a facultative stem cell for islets.³⁹ Similar to this study in mice, the formation of insulin-expressing islets was observed in close proximity to ducts harboring Ki-67⁺ proliferating cells. As in the previously described mouse ductal ligation model,³⁹ islet-like structures in CF ferret pancreas were observed that appeared to be budding from the ducts (Figure 7, D and F).

In our attempt to gauge concurrent processes in aging CF ferrets, remodeling events in both endocrine and exocrine pancreas were investigated in reference to previously studied trends in glycemic tolerance and inflammation.¹⁸ Our findings are summarized in Figure 9. CF ferrets with near normal glycemic tolerance in phase I (<1 month old) have mild inflammation that continues to increase into phase II (approximately 2 months old). This heightened inflammation in CF pancreas significantly affects both the exocrine and endocrine pancreas. We hypothesize that the inflammation activates the stellate cell myofibroblasts, which may play a role in adipogenesis (Figure 9B). Hence, as CF ferrets near 3 months of age, almost complete replacement of acinar tissue by fat is seen (Figure 9B). The endocrine pancreas also undergoes remodeling in response to inflammation during phase II. Expression of metalloproteases, like MMP-7, suggests remodeling of the endocrine pancreas and may enhance islet neogenesis. In addition, the remodeling marker MMP-7 may induce expression of regenerative genes and influence transdifferentiation of ducts to islet cells (Figure 9B). We hypothesize that a combination of islet proliferation and transdifferentiation from ducts contributes to the increase in islet frequency seen in phase III CF pancreas. Increases in islet frequency would, thus, help normalize hyperglycemia as CF ferrets near 3 months of age (Figure 9A).¹⁸

The CF ferret model provides the opportunity to study both onset and evolution of the various synchronous pathways that control these pancreatic remodeling events. Questions arising from this study include adipose-induced remodeling of the pancreas in the presence of inflammation, loss of exocrine tissue, compensatory increase in islet frequency in the pancreas, induction of ductal proliferation and transdifferentiation to islet cells, and stellate cell activation. Further mechanistic analysis of these events is needed to understand the pathogenesis of CFRD and to develop potential therapies.

Acknowledgment

We thank the Comparative Pathology Laboratory at The University of Iowa for technical expertise.

Supplemental Data

Supplemental material for this article can be found at <https://doi.org/10.1016/j.ajpath.2017.12.015>.

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