In situ analysis reveals that CFTR is expressed in only a small minority of β-cells in normal adult human pancreas

Michael G White[1], Rashmi R Maheshwari[1], Scott J Anderson[1], Rolando Berlinguer-Palmini[2]
Claire Jones[3], Sarah J Richardson[4], Pavana G Rotti[5], Sarah L Armour[1], Yuchun Ding[6],
Natalio Krasnogor[6], John F Engelhardt[5], Mike A Gray[7], Noel G Morgan[4], James AM
Shaw[1,8]*

[1]Diabetes Regenerative Medicine Research Group, Institute of Cellular Medicine, Newcastle
University Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK.

[2]Bio-imaging Unit, Newcastle University, Newcastle Upon Tyne, NE2 4HH, UK.

[3]Molecular Pathology Node Proximity Laboratory, Royal Victoria Infirmary, Queen Victoria
road, Newcastle upon Tyne NE1 4LP

[4]Institute of Biomedical and Clinical Sciences, University of Exeter Medical School, Exeter,
UK

[5]Fraternal Order of Eagles Diabetes Research Center, University of Iowa, Iowa City, Iowa

 52242

[6]Interdisciplinary Computing and Complex Biosystems (ICOS) Research Group, School of
Computing, Newcastle University, Urban Sciences Building, 1 Science Square, Newcastle
Helix, Newcastle upon Tyne NE4 5TG, UK.

[7]Epithelial Research Group, Institute for Cell and Molecular Biosciences, Faculty of Medical
Sciences, Newcastle University, Newcastle upon Tyne, UK

[8]Newcastle Diabetes Centre, Newcastle upon Tyne NHS Foundation Trust, Newcastle upon
Tyne NE4 6XH, UK

*Corresponding Author and Lead Contact: James AM Shaw, Institute of Cellular Medicine,
Newcastle University, Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH.
Email: jim.shaw@ncl.ac.uk Tel: +44 191 208 7019. ORCID ID: 0000-0002-2893-3211

Abstract

Context: Although diabetes affects 40-50% of adults with cystic fibrosis, remarkably little is known regarding the underlying mechanisms leading to impaired pancreatic β-cell insulin secretion. Efforts towards improving the functional β-cell deficit in cystic fibrosis related diabetes (CFRD) have been hampered by an incomplete understanding of whether β-cell function is intrinsically regulated by CFTR. Definitively excluding meaningful CFTR expression in human β-cells *in situ* would contribute significantly to the understanding of CFRD pathogenesis.

Objective: To determine CFTR mRNA and protein expression within β-cells *in situ* in the unmanipulated human pancreas of donors without any known pancreatic pathology.

Design: *In situ* hybridisation for CFTR mRNA expression in parallel with insulin immunohistochemical staining; and immunofluorescence co-localisation of CFTR with insulin and the ductal marker, Keratin-7, were undertaken in pancreatic tissue blocks from 10 normal adult, non-obese deceased organ donors over a wide age range (23-71 years) with quantitative image analysis.

Results: CFTR mRNA was detectable in 0.45 (0.17-0.83)% (mean(range)) of insulin-positive cells. CFTR protein expression was co-localised with Keratin-7. 100% of insulin-positive cells were immunonegative for CFTR.

Conclusions: For the first time, *in situ* CFTR mRNA expression in the unmanipulated pancreas has been shown to be present in only a very small minority (<1%) of normal adult β-cells. These data signal a need to move away from studying endocrine-intrinsic mechanisms and focus on elucidation of exocrine-endocrine interactions in human cystic fibrosis.

2

Introduction

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. This encodes an important chloride channel, with loss of protein function leading to impaired ion and water transport across epithelial membranes, causing the production of viscous mucus in multiple organs, including the lungs, intestines and pancreas. As life expectancy for individuals affected by CF has increased, the prevalence of cystic fibrosis-related diabetes (CFRD) has risen dramatically, such that it now affects approximately 50% of adults (1). Although CF is associated with varying degrees of insulin resistance, CFRD is primarily caused by reduced β-cell function leading to insufficient insulin secretion (2-3).

The mechanisms through which mutations in CFTR lead to impaired insulin secretion in humans remain contentious, with recent reports providing conflicting evidence (reviewed in Norris et al., 2019) (4). CFTR expression has been reported in cultured rodent β-cell lines and primary rodent islets with impaired CFTR function associated with reduced glucose stimulated insulin secretion (5-7). Similarly, CFTR expression has been inferred in human islet β-cells, with a small CFTR current detected following whole cell patch clamp studies (5). This is supported by CFTR inhibitor studies in which GlyH-101 and CFTRinh-172 were reported to cause disruptions in human β-cell stimulus-secretion coupling, suggesting that CFTR directly regulates insulin secretion through a β-cell intrinsic mechanism (5). Conversely, studies in a ferret CF model have indicated that CFTR is absent from β-cells, providing evidence that insulin secretory defects and progression to CFRD can be caused by pancreatic exocrine derived pro-inflammatory mediators (8).

3

Absence of CFTR protein immunostaining in β-cells has been reported in four young human organ donors without CF (aged 1 day to 4 years) (9). Recent high quality studies have detected significant CFTR gene expression in <5% of β-cells following human islet isolation, dissociation and single cell RNA sequencing (9-11). Nevertheless unresolved contention remains regarding whether or not the CFTR gene is expressed in unmanipulated pancreatic β-cells *in situ*. To date, definitive resolution has been precluded by the limited availability of suitable human tissue (8), problematic antibodies (9,12), the absence of optimal antibody controls (5), and reliance on dissociated pancreatic cells (8,10-11) with the potential for misinterpretation arising from islet processing leading to phenotypic alterations (13). To address this, we have assessed β-cell CFTR mRNA expression *in situ* in pancreata from 10 adult deceased organ donors without any known pancreatic pathology using an *in situ* hybridisation (CFTR) and immunohistochemistry (insulin) dual-labelling approach. Appropriately controlled CFTR/insulin immunohistochemical staining studies were conducted in parallel. Our findings strongly suggest that insulin secretion in humans is not directly regulated by β-cell intrinsic CFTR expression.

Materials and Methods

Donors and sample processing

Pancreata were procured from deceased organ donors within accredited facilities by the UK National Organ Retrieval Service. Absence of diabetes and other history of pancreatic disease was confirmed through the NHS Blood and Transplant Organ Donation and Transplantation Electronic Offering System. LDIS174 was a donor following circulatory death (DCD) with functional warm ischaemic time of 32 minutes. All others donors followed brainstem death (DBD). Pancreata were shipped by licensed organ courier, and the pancreatic tissue was biopsied on receipt. Biopsies for LDIS072 – LDIS101 were fixed in 4% formaldehyde (made

4

in house) and biopsies for LDIS121 – LDIS204 were fixed in 10% neutral buffered formalin (Sigma cat# HT5011-1) at room temperature and were processed for paraffin embedding and sectioning (4 μm). Tissue processing and sectioning was undertaken within the ISO accredited Newcastle Cellular Pathology department (ISO15189:2012).

CFTR knockout ferret pancreas tissue collection and tissue processing

A ferret *CFTR* exon-10 disruption model (*CFTR* knockout) was used to confirm the specificity of CFTR protein and mRNA expression in the pancreas (14). This *CFTR* knockout (CFTR KO) model develops spontaneous lung (15) and pancreatic disease (16). Heterozygotes for the *CFTR* gene disruption were mated in Marshall Farms (North Rose, NY, US) and pregnant jills gave birth at the University of Iowa (Iowa City, IA, US). Genotypes of litters were confirmed soon after birth. Whole pancreata from newborn *CFTR*+/+ (non-CF) and *CFTR*-/- (CF) littermates were harvested immediately after euthanasia. The tissues were fixed in 10% neutral buffered formalin for 72 hours at room temperature and routinely processed for paraffin sectioning at 4 μm. The unbaked sections were stored at room temperature until use.

In Situ Hybridisation and Immunohistochemistry (IHC)

Tissue sections (4 μm) from formalin fixed paraffin embedded pancreas samples were evaluated for CFTR RNA expression by *in situ* hybridisation (ISH) (Advanced Cell Diagnostics (ACD); Probe: 503569). CFTR ISH alone was performed on five donors (LDIS072, LDIS152, LDIS155, LDIS174 and LDIS204) using RNAscope 2.5 VS reagent kit (brown; ACD, 322200) on the Ventana Discovery Ultra according to the manufacturer's instructions including Haematoxylin counter-stain. Dual CFTR ISH (Advanced Cell Diagnostics (ACD); Probe: 503569) and insulin (Agilent Antibody; A056401-2; Guinea Pig

5

Polyclonal; Dilution 1:100) IHC (Ventana Discovery Red Kit; 07425333001) was performed on all ten donors using RNAscope 2.5 VS reagent kit (brown; ACD, 322200) on the Ventana Discovery Ultra according to the manufacturer's instructions. All studies were performed within the Newcastle MRC Molecular Pathology Node.

Slide digitalisation and automated quantification

For automated determination of CFTR mRNA and insulin protein expression in human pancreas sections, slides were scanned (x40 magnification) and digitalised using the Leica SCN400 slide scanner. Images were accessed through the Slidepath (Leica Biosystems) DIH system and evaluated using the software's 'Measure Stained Cells' algorithm. Details on development of this automated algorithm can be found at: https://prd-medweb-cdn.s3.amazonaws.com/documents/dhsr/files/Tissue_IA_2-0_Training_Presentation.pdf. Briefly, pixel inclusion of insulin (red) and CFTR RNA (brown) was determined using the colour definition function and inputted in to the algorithm. Prior to whole section analysis, 5 random fields of view (20x) were assessed in each donor to confirm algorithm accuracy. The Tissue IA function was used for image analysis of whole pancreas sections, and data was exported to Microsoft Excel. The overall percentage of insulin/CFTR co-expressing cells was calculated by dividing the total number of insulin/CFTR-positive cells by the total number of insulin-positive cells identified in the whole tissue section of each donor. These data are summarised in Table 1. All image files are stored and made available on the Newcastle University data repository (17-18).

Immunofluorescence

Tissue sections (4 μm) from formalin fixed paraffin embedded samples were evaluated for insulin and CFTR immunolocalisation by immunofluorescence staining in all ten donors.

6

Sections were deparaffinised and rehydrated followed by heat-mediated antigen retrieval (HIER) performed in 10 mM citrate buffer (pH 6) in a pressure cooker. Following blocking in 10% goat serum, sections were incubated with mouse anti-CFTR (596) (Cystic Fibrosis Foundation, Chapel Hill, NC; CFTR 596 (A4); 1:2000) primary antibody overnight at 4°C. Secondary antibody staining was undertaken with the anti-mouse Alexa Fluor 488 Tyramide SuperBoost Kit (ThermoFisher, UK; B40912), according to manufacturer's instructions. Sections were then blocked as described and incubated with either guinea pig anti-insulin (Dako, Carpinteria CA, USA; A0564;1:200) or rabbit anti-Keratin-7 (KRT7) (Abcam, Cambridge, UK; ab181598; 1:1000) overnight at 4 °C. Primary antibody binding was detected by Alexa Fluor 647 conjugated anti-guinea pig or anti-rabbit secondary antibodies (Life technologies, CA, USA; A-21450; 1:500). Sections were counterstained with DAPI (ThermoFisher, UK; 62248) before being mounted with Vectashield Mounting medium (Vector Laboratories, CA, USA; H-1000).

Image acquisition and processing

Confocal images were acquired on a Leica SP8 point scanning confocal microscope with white light super continuum lasers at 40x using a 40x/1.3NA HC PL APO CS2 oil immersion lens at Nyquist rate (voxel size: X,Y 46 nm, Z 165 nm) applying 2x line average and acquiring each channel sequentially. Images were deconvolved using the Huygens Essential (Scientific Volume Imaging, SVI) deconvolution express (standard) algorithm. All images were acquired and processed at the Newcastle University Bioimaging Unit.

Statistics and image analysis

Data are reported as mean (range) or mean ±SEM, calculated in Prism 8.0.1 for Mac (Prisms, GraphPad Software, Inc.). Colocalization analysis was performed using Manders

7

Colocalization Coefficients (MCC) as previously described (19). This was undertaken

following optimized background estimation process from SVI (www.svi.nl) based on the

Costes method (20), but without the assumption that the ideal background threshold

combination is on the regression line.

Study approval

The study was approved by the National Research Ethical Committee for Wales and written

informed consent was obtained from donor relatives before any study-related work.

Results

The study group comprised optimally procured non-obese adult deceased organ donors across

a wide age range (23-71 years) with no history of diabetes or other pancreatic pathology.

Demographics are summarised in Table 1, confirming blood glucose level below the

diagnostic threshold for diabetes in all donors.

To determine whether pancreatic β-cells express CFTR, we investigated CFTR mRNA

localisation within human pancreas sections (n=5 donors). Initially we undertook *in situ*

hybridisation (ISH) for CFTR alone, which revealed expression in a heterogeneous

distribution throughout the exocrine pancreas (Figure 1a, c). Morphological appearances on

haematoxylin counter-staining were in keeping with previously published CFTR protein

staining predominantly in small intra-lobular pancreatic ducts with sparing of islets (Figure

1b,d) (21). Representative images are presented in Figure 1, and whole slide scans are

available digitally (17).

We next employed an ISH (CFTR) and immunohistochemistry (IHC) (insulin) dual-labelling approach to assess CFTR mRNA expression in β-cells in human pancreatic tissue sections (n=10 donors). Assessment by transmission light microscopy indicated an absence of detectable CFTR mRNA expression in the majority of insulin-containing cells, although rare co-expressing cells were detected (Figure 2). Whole slide scans are available digitally (18). Automated quantification of digitalised pancreatic sections enabled assessment of 48,416 β-cells, and only 214 of these had detectable CFTR mRNA (Table 2). CFTR mRNA was thus detectable in a mean of 0.45% of insulin-positive cells, with range from the donor with lowest proportion to the donor with the highest proportion of 0.17-0.83%.

Evaluation of CFTR protein expression and localisation *in situ* in human pancreas has previously been limited by the small number of available human donors (9). Studies have also been hampered by a lack of optimal antibody controls (5) and detailed image analysis and quantification (5,9). Given concerns regarding CFTR antibody suitability, particularly for use in IHC, we initially assessed the specificity of the anti-CFTR (596) monoclonal antibody in pancreatic tissue from newborn wild type (WT) and CFTR knock-out (KO) ferrets. This antibody is provided by the Cystic Fibrosis Foundation and has previously been validated for use in a range of immunodetection techniques (12). Using immunofluorescence staining, we confirmed CFTR expression in WT but not CFTR KO animals (Figure 3). Dual labelling for CFTR and insulin demonstrated that CFTR was localised to the exocrine pancreas but was not expressed in β-cells which were present as developing islet clusters and as single cells dispersed within the exocrine pancreas in WT animals (Figure 3). Having confirmed specificity of the CFTR (596) antibody, co-staining with the ductal protein, Keratin 7 (KRT7) was undertaken in two human donors. CFTR expression was in keeping with previously published localisation to the apical (luminal-facing) domain of small intralobular ducts, with

9

larger ducts expressing Keratin-7 alone (21). Following image acquisition, deconvolution and signal thresholding, the Manders coefficient for CFTR and KRT7 were 34±2% and 48±2% for M1 and M2 respectively, confirming significant co-localisation and thus expression of CFTR in human pancreatic ductal cells. Representative images are shown in Figure 4a. Using this validated antibody and detection method, we then assessed 116 islets across 10 donors for insulin and CFTR expression and found no evidence of co-localisation of CFTR and insulin i.e M1=0 and M2=0, indicating that CFTR protein cannot be detected by IHC in normal human β-cells. Representative images from two donors are shown in Figure 4 b, c.

Discussion

Using sensitive (dual ISH/IHC) and unbiased (software quantification) methodologies, CFTR mRNA expression was detectable in <1% of β-cells *in situ* in 10 adult deceased organ donors (aged 23-71 years) without known pancreatic pathology. Following IHC and co-localisation analysis using a validated antibody, CFTR protein expression could not be detected in islet β-cells. We conclude that CFTR is not expressed in most human β-cells and is thus unlikely to play a significant intrinsic role in normal human β-cell function.

Our findings are supported by the sensitive and complementary approach of single cell sequencing following tissue dissociation which has found CFTR transcripts at low levels in only a small population of sorted β-cells with the majority being devoid of such transcripts (9-11). Given that alterations in β-cell gene expression may occur following pancreas processing and islet isolation (13), it was important to extend these studies by conducting *in situ* mRNA localisation. Our analysis revealed that co-expression of insulin protein and CFTR mRNA is rare, occurring in only 0.45% of the β-cell population examined in 10 donors. These findings are consistent with the conclusions of a recent CFTR ISH study in

10

isolated human β-cells, supporting the absence of meaningful β-cell CFTR mRNA expression (8).

In support of our ISH interpretations, we failed to detect the presence of CFTR protein in insulin-positive cells following the detailed examination of islets from all 10 donors. These observations are in contrast to previous studies reporting CFTR localisation in single human β-cells following immunofluorescent staining with the MATG-1061 CFTR antibody (5). However, given the recently reported lack of specificity of certain CFTR antibodies (9,12), and the omission of a cell control known not to express CFTR, false positivity using MATG-1061 cannot be excluded. Also, subsequent immunohistochemical staining of human pancreas sections using this antibody failed to demonstrate the expected presence of CFTR in ductal cells (22).

Here, we assessed CFTR protein localisation using the Cystic Fibrosis Foundation 596 monoclonal antibody, which has previously been reported as suitable for detection of wild type CFTR by immunofluorescence (12). The specificity of this antibody was confirmed by the presence of positive CFTR staining in pancreatic tissue samples from wild type ferrets and its absence from CFTR knockout animals. As previously reported, insulin-positive cells were distributed throughout the pancreatic parenchyma as well as in developing islet clusters in the newborn ferret (23). No insulin$^+$ / CFTR$^+$ cells were seen. Co-localisation of CFTR with the ductal cell marker, Keratin7, was confirmed in human pancreas. We conclude that CFTR protein is not expressed in human β-cells, consistent with the Human Protein Atlas (24). While paediatric samples were not included in the current study, absence of detectable CFTR protein within β-cells in infancy and early childhood has recently been reported (9).

11

These observations lead us to propose that human β-cell dysfunction and progression to CFRD is likely to be mediated by factors extrinsic to the pancreatic β-cell. Whilst contrasting reports have proposed a direct, intrinsic functional role for CFTR in human insulin secretion (5) these conclusions were based predominately on *in vitro* assessments of human islets following treatment with the CFTR inhibitors, CFTR (inh)-172 and GlyH-101. However, both these compounds have been reported to inhibit activity of other chloride channels (25) at the concentrations used by Edlund et al (5), highlighting the potential limitations in using CFTR inhibitors to determine an intrinsic role for CFTR in insulin secretion. This is supported by reports that CFTR(inh)-172 (20 μM) negatively affects glucose-stimulated insulin secretion in islets from CFTR KO in addition to WT ferrets (8), suggesting inhibition through a non-CFTR mediated pathway.

Although CFTR protein expression was not detectable in β-cells by IHC, in keeping with absence of significant translation even in cells with detectable CFTR mRNA, we cannot exclude the technical limitation that the method is not sufficiently sensitive to detect very low level CFTR protein expression. Thus, whilst our data suggest that CFTR is not expressed in a sufficient number of β-cells at sufficient levels to play an important role in controlling β-cell function through stimulus-secretion coupling or other intrinsic mechanisms, it remains possible that such a minority population may have functional consequences if, for example, they were highly electrically active, such as the hub β-cells that have recently been described (26). Alternatively, rare insulin-positive cells expressing CFTR mRNA may represent ductal derived neo-endocrine cells in which CFTR protein has been downregulated despite mRNA expression persisting. Such extrainsular cells with endocrine differentiation potential have been described, however, the presence of CFTR in such cells has not been reported (27). Whilst studies in fixed pancreatic tissue preclude determination of the origin or functionality

12

of 'neo-endocrine' cells, more definitive phenotyping *in situ* in unmanipulated pancreas is merited.

Clinical studies in CF patients treated with the CFTR potentiator, Ivacaftor, have demonstrated improved insulin secretion (28). Our data suggest that this is mediated indirectly through improvements in CF pathophysiology in the exocrine pancreas and other tissues. In support of this, a direct effect of Ivacaftor on β-cell secretory function was disputed in a recent human islet study (9).

Our data exclude CFTR expression in the majority of human β-cells within the intact minimally-manipulated pancreas. We propose that further translational research efforts should focus on understanding the role of exocrine-endocrine interactions in CFRD pathogenesis, and particularly the role of inflammatory mediators, with the goal of diabetes remission / prevention through a combinatorial approach limiting pancreatic destruction and reversing dysfunction in remaining β-cells. It is hoped that deeper understanding of the mechanisms through which extrinsic metabolic stress and proinflammatory factors can indirectly impair beta-cell function will facilitate progress towards new therapeutic approaches for more prevalent type 2 and type 1 diabetes.

Acknowledgements

13

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15

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17

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Figure legends

Figure 1. CFTR mRNA localisation in human pancreas (a-d) CFTR mRNA (brown) was detected by ISH in pancreas sections of donors without diabetes (n=5). Duct-like structures (dotted black lines) and islet-like structures (red arrows) were identified morphologically. Boxes (a and c) indicate regions imaged at higher magnification (b and d). Scale bars represent 100 μm (a and c) and 30 μm (b and d).

Figure 2. CFTR mRNA is absent from most human β-cells (a-f) CFTR mRNA (brown) and insulin protein (red) were detected using a combination of ISH and IHC in pancreas sections from donors without diabetes (n=10). Arrows (e-f) indicate rare insulin-positive cells co-expressing CFTR mRNA. Scale bars represent 30 μm.

Figure 3. CFTR expression in newborn WT and CFTR KO ferrets Immunofluorescence staining for CFTR and insulin protein in pancreas sections from newborn WT (a-c) (n=2) and CFTR KO (d-f) ferrets (n=2). Boxes (a and d) indicate the regions imaged at higher magnification (b-c and e-f). Scale bars represent 50 μm (a and d) and 25 μm (b and e).

Figure 4. CFTR protein is undetectable in human β-cells (a-c) Immunofluorescence staining for CFTR with Keratin-7 (KRT7) (A) (n=2 donors: LDIS101 and LDIS155.) and insulin protein (b-c) (n=10 donors) in pancreas sections from donors without diabetes. Cytofluorograms illustrate presence and absence of CFTR colocalization with KRT7 (a) and insulin (b-c) respectively. Areas highlighted by white dotted lines indicate islets. Scale bars represent 25 μm.
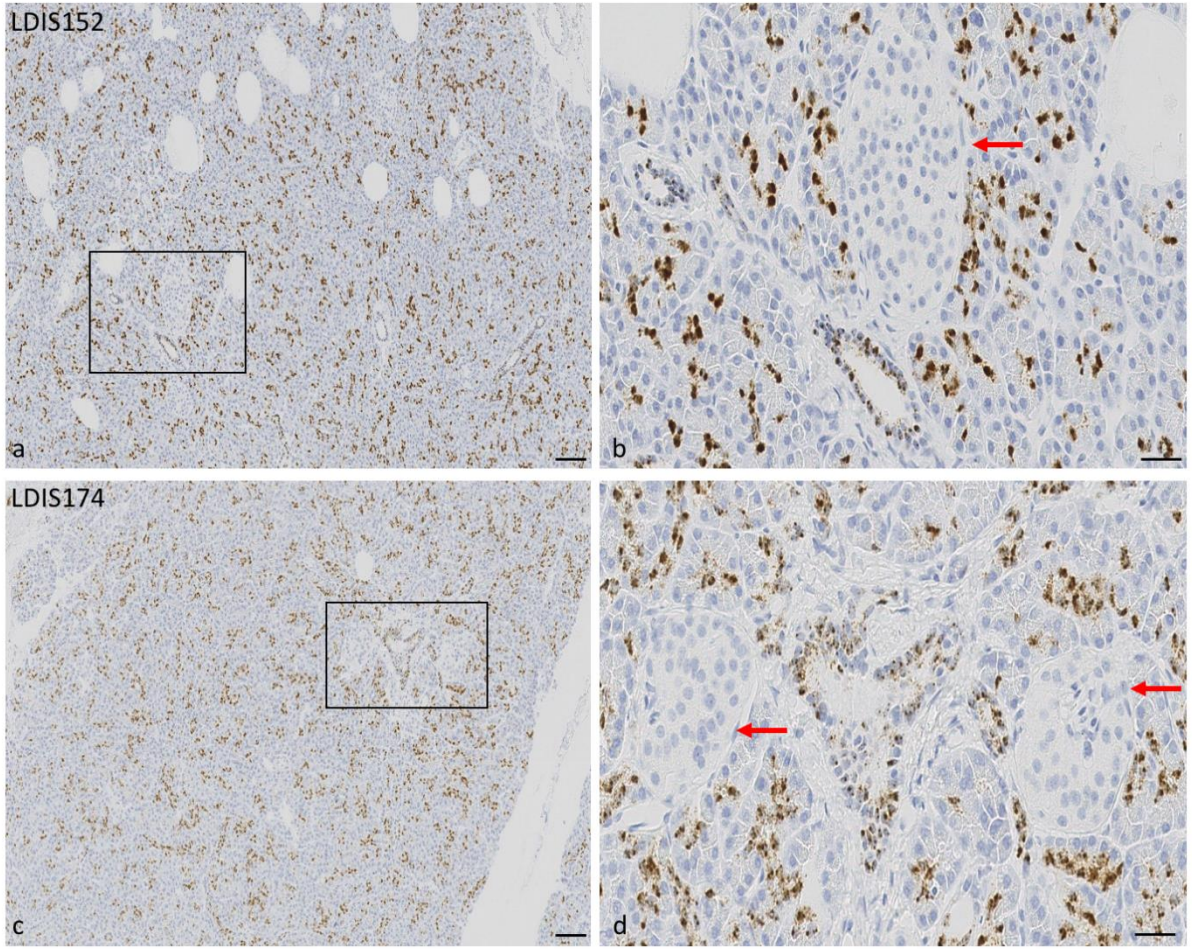
21

Donor ID	Age (years)	Sex	BMI (kg/m^2)	DBD/ DCD	CIT (hours)	Blood glucose (mmol/L)
LDIS072	24	F	25.95	DBD	27	9.4
LDIS087	35	M	26.26	DBD	22	5.5
LDIS101	50	M	27.17	DBD	72	n/a
LDIS121	58	F	21.72	DBD	22.7	6.5
LDIS124	57	F	23.83	DBD	22.2	7.6
LDIS152	49	M	25.39	DBD	8.5	6.5
LDIS155	48	F	22.91	DBD	13.1	6.4
LDIS156	23	M	21.13	DBD	31.2	7.4
LDIS174	71	M	26.73	DCD	11.4	7.4
LDIS204	56	F	26.9	DBD	13.6	9.0

Table 1. Donor demographics

22

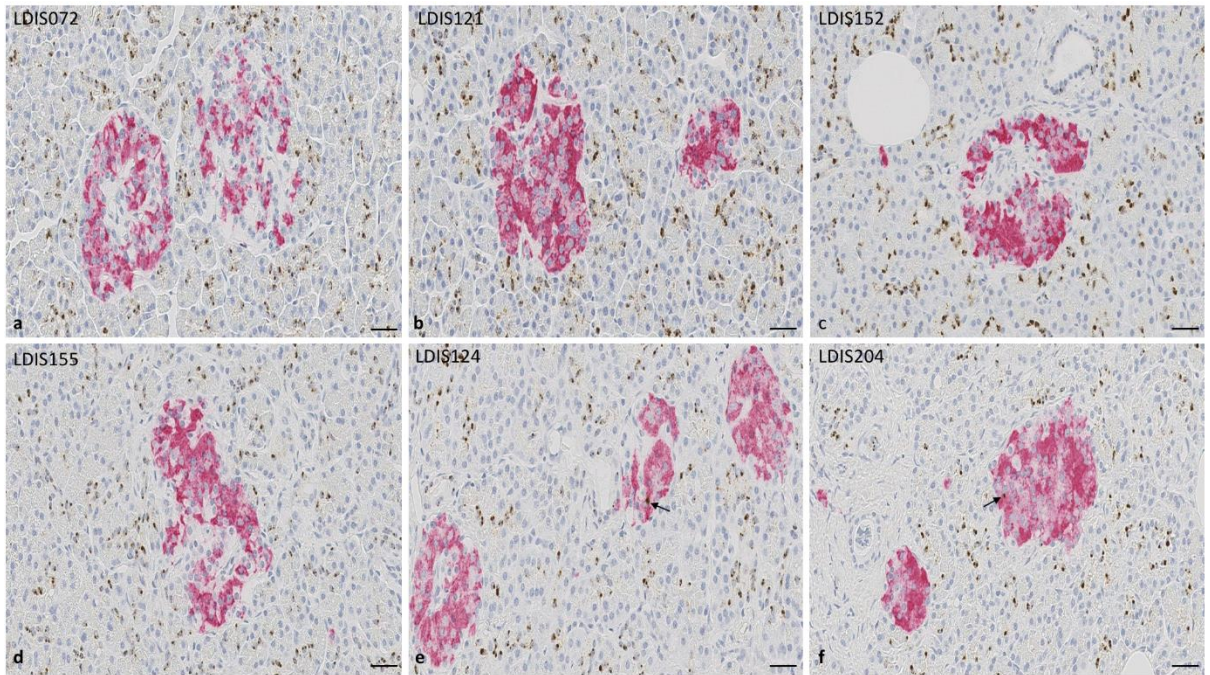| Donor ID	CFTR Cell no	CFTR Cells/ mm²	Insulin Cell no	Insulin Cells /mm²	CFTR+ /Insulin+ Cell no	% CFTR+/ Insulin+ (of total insulin cells)
LDIS072	87,778	2,300.87	6,607	173.2	23	0.35
LDIS087	71,750	1,228.39	8,156	139.6	32	0.39
LDIS101	98,214	1,880.41	6,924	132.6	58	0.83
LDIS121	86,607	1,976.88	5,950	135.8	12	0.20
LDIS124	87,184	2,398.46	8,764	241.1	15	0.17
LDIS152	44,570	3,947.74	3,237	286.7	18	0.55
LDIS155	27,548	1,538.14	1,871	104.5	9	0.48
LDIS156	36,578	1,486.91	1,526	62.0	7	0.46
LDIS174	36,180	2,528.30	1,139	79.6	2	0.18
LDIS204	33,319	2,524.17	4,242	321.4	38	0.89
Mean	60,973	2,181	4,842	167.6	21.4	0.45
SEM	8,773	243.9	891.4	27.7	5.4	0.08

Table 2. Number of cells expressing CFTR (ISH) and insulin (IHC) in a single pancreatic section from 10 human donors
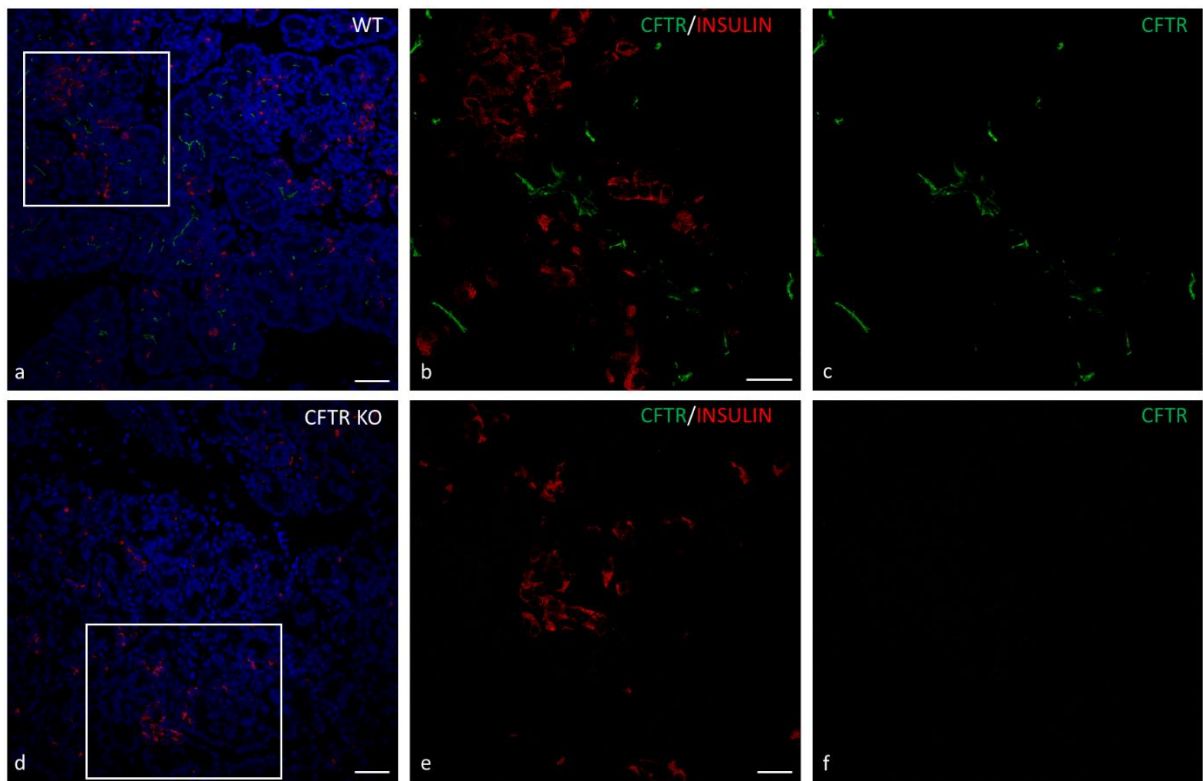
23
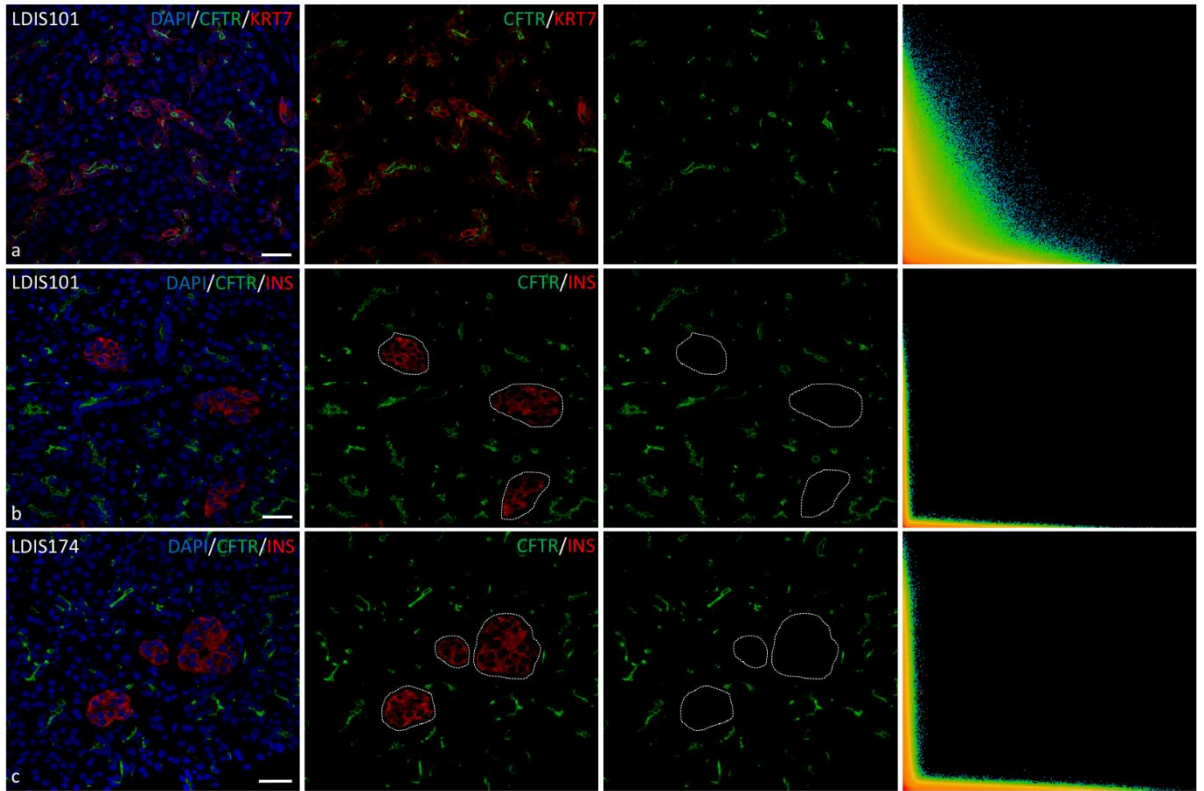
Figure 1. CFTR mRNA localisation in human pancreas

Figure 2. CFTR mRNA is absent from most human β-cells

Figure 3. CFTR expression in newborn WT and CFTR KO ferrets

Figure 4. CFTR protein is undetectable in human β-cells