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### Title

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### Permalink

<https://escholarship.org/uc/item/4600q42k>

### Journal

The Journal of Clinical Endocrinology & Metabolism, 101(9)

### ISSN

0021-972X

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### Publication Date

2016-09-01

### DOI

10.1210/jc.2016-1350

Peer reviewed

## Increased hormone-negative endocrine cells in the pancreas in type 1 diabetes

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**Context and objective:** Type 1 diabetes (T1D) is characterized by a beta-cell deficit due to auto-immune inflammatory mediated beta-cell destruction. It has been proposed the deficit in beta-cell mass in T1D may be in part due to beta-cell degranulation to chromogranin positive hormone negative (CPHN) cells.

**Design, setting and participants:** We investigated the frequency and distribution of CPHN cells in pancreas of 15 individuals with T1D, 17 autoantibody positive non diabetics and 17 non-diabetic controls.

**Results:** CPHN cells were present at a low frequency in pancreas from non-diabetic and autoantibody positive brain dead organ donors, but are more frequently found in pancreas from donors with type 1 diabetes. (Islets:  $1.11 \pm 0.20$  vs  $0.26 \pm 0.06$  vs  $0.27 \pm 0.10\%$  of islet endocrine cells, T1D vs AA+ vs ND; T1D vs AA+ and ND  $p < 0.001$ ). CPHN cells are most commonly found in the single cells and small clusters of endocrine cells rather than within established islets, (Clusters:  $18.99 \pm 2.09$  vs  $9.67 \pm 1.49$  vs  $7.42 \pm 1.26\%$  of clustered endocrine cells, T1D vs AA+ vs ND; T1D vs AA+ and ND  $p < 0.0001$ ) mimicking the distribution present in neonatal pancreas.

**Conclusions:** From these observations, we conclude that CPHN cells are more frequent in T1D and, as in (type 2 diabetes) T2D, are distributed in a pattern comparable to neonatal pancreas, implying possible attempted regeneration. In contrast to rodents, CPHN cells are insufficient to account for loss of beta-cell mass in T1D.

**T**ype 1 diabetes results from insufficient  $\beta$ -cell mass due to auto-immune destruction, and afflicts approximately 1 million people in the United States. While the concept that  $\beta$ -cell loss in type 1 diabetes is a consequence of auto-immunity remains well accepted (1, 2), interesting new concepts have arisen to suggest loss of  $\beta$ -cells in type 1 and 2 diabetes may be in part due to degranulation of  $\beta$ -cells and or transdifferentiation of  $\beta$ -cells to other cell types (3–5). An alternative explanation for the increased presence of hormone negative endocrine cells in the pancreas in diabetes is that they represent ongoing attempted  $\beta$ -cell regeneration. Support for this possibility arises from the comparable pattern and distribution of such cells in late gestation and early infancy in humans (6).

To further probe these possibilities, in the present study we examined pancreas of brain dead organ donors with type 1 diabetes vs nondiabetic controls. It has already been established that there is ongoing  $\beta$ -cell turnover in adults with long standing type 1 diabetes, although the origin of these cells is unknown (7–9). We reasoned that if we identified in humans with type 1 diabetes a comparable pattern of hormone negative pancreatic endocrine cells to that which we noted in the developing human endocrine pancreas, this would be consistent with the possibility that there are newly forming  $\beta$ -cells in type 1 diabetes.

Therefore, we sought to address the question, is there an increase in the frequency of nonhormone expressing endocrine cells in the pancreas in humans with type 1

ISSN Print 0021-972X ISSN Online 1945-7197

Printed in USA

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Received February 10, 2016. Accepted June 8, 2016.

Abbreviations: CPHN, Chromogranin positive hormone negative. AA<sup>+</sup>, auto-antibody positive. ChrgA, Chromogranin A. T1D, type 1 diabetes. ND, nondiabetic.

diabetes, and if so does the pattern mimic that observed in infancy, with a preponderance of distribution as scattered cells within the exocrine pancreas? We were able to approach this issue because of the outstanding repository of human pancreas assembled by the nPOD consortium based at the University of Florida.

## Materials and Methods

### Study subjects

**Design and Case Selection.** All pancreata from the type 1 diabetic, autoantibody positive and the nondiabetic donors were procured from brain-dead organ donors by the JDRF Network for Pancreatic Organ Donors with Diabetes (nPOD), a program coordinated by the University of Florida in Gainesville, Florida (10). All procedures were in accordance with federal guidelines for organ donation and the University of Florida Institutional Review Board.

**Case Characteristics (Supplemental Table 1).** Pancreata were procured from 15 adult donors with type 1 diabetes (T1D), 17 autoantibody positive (AA+) donors and 17 nondiabetic (ND) donors matched for age ( $42.9 \pm 5.6$  vs  $36.4 \pm 3.5$  vs  $38.7 \pm 4.4$  years, T1D vs AA+ vs ND,  $p=ns$ ) (Supplemental Figure 1A, 1B), sex (T1D group: 10 males, 5 females; AA+ group: 10 males, 7 females; ND group: 11 males, 6 females) and BMI ( $24.4 \pm 1.1$  vs  $25.3 \pm 1.2$  vs  $25.8 \pm 1.1$  kg/m<sup>2</sup>, T1D vs AA+ vs ND,  $p=ns$ ) (Supplemental Figure 1C, 1D).

Pancreas weight was decreased in the T1D donors and, to a lesser degree, in the AA+ donors, compared with the ND donors, in accord with previous observations (11) ( $38.1 \pm 4.9$  vs  $73.8 \pm 5.7$  vs  $90.5 \pm 6.5$  g, T1D vs AA+ vs ND,  $P < .0001$  T1D vs AA+ and T1D vs ND) (Supplemental Figure 1E, 1F).

### Pancreas acquisition and processing

nPOD utilizes a standardized preparation procedure for pancreata recovered from cadaveric organ donors (12). The pancreas is divided into three main regions (head, body, and tail), followed by serial transverse sections throughout the medial to lateral axis, allowing for sampling of the entire pancreas organ while maintaining anatomical orientation. Because preparation is completed within 2 hours, tissue integrity is maintained. Tissues intended for paraffin blocks are trimmed to pieces no larger than  $1.5 \times 1.5$  cm and fixed in 10% neutral buffered formalin for  $24 \pm 8$  hours. Fixation is terminated by transfer to 70% ethanol, and samples are subsequently processed and embedded in paraffin. Mounted transverse sections from the paraffin-embedded tissue blocks were obtained from the body of pancreas in most cases; where blocks of pancreas body were unavailable, sections from the head of pancreas were used.

In all type 1 diabetic and autoantibody positive donors, and in most nondiabetic donors, the whole pancreas was weighed at the time of procurement. Pancreas from five nondiabetic organ donors was procured before the routine weighing of pancreas was instituted.

## Immunostaining

### For assessment of presence and frequency of CPHN cells

All staining was performed at the University of California, Los Angeles. Paraffin tissue sections from each subject were stained for Chromogranin A, insulin, glucagon, somatostatin, pancreatic polypeptide, and ghrelin. Standard immuno-histochemistry protocol was used for fluorescent immuno-detection of various proteins in pancreatic sections (1). Briefly, slides were incubated at 4°C overnight with a cocktail of primary antibodies prepared in blocking solution (3% BSA in TBST) at the following dilutions: rabbit anti-Chromogranin A (1:200, Novus Biologicals NB120–15 160, Littleton, CO); mouse antiglucagon (1:1000, Sigma-Aldrich G2654-.2ML; St. Louis, MO); guinea-pig anti-insulin (1:200; Abcam7842; Cambridge, MA), rat antisomatostatin (1:300, EMD Millipore MAB354; Billerica, MA), goat antipancreatic polypeptide (1:3000, Everest Biotech; Ramona, CA), rat antighrelin (1:50, R&D Systems MAB8200, Minneapolis, MN). The primary antibodies were detected by a cocktail of appropriate secondary antibodies (Jackson ImmunoResearch, Westgrove, PA) conjugated to Cy3 (1:200, for ChrgA), FITC (1:200 each, to detect glucagon, somatostatin, pancreatic polypeptide, and ghrelin) or Cy5 (1:100, to detect insulin). Slides were counterstained to mark the nuclei using a mounting medium containing DAPI (Vectashield, Vector Labs, Burlingame, CA), and viewed using a Leica DM6000 microscope (Leica Microsystems, Deerfield, IL) and images were acquired using the 20x objective (200x magnification) using a Hamamatsu Orca-ER camera (C4742–80-12AG, Indigo Scientific, Bridgewater, NJ) and Openlab software (Improvision, Lexington, MA).

### Assessment of Endocrine hormones with endocrine transcription factors / replicating markers / islet inflammatory markers and chromogranin A

It is of great interest to know whether the CPHN cells are replicating and if they express endocrine transcription factors or are surrounded by an inflammatory infiltrate. To demonstrate this, we developed and utilized a new immunohistochemical staining technique. The new strategy involves monovalent F(ab')<sub>2</sub> fragments to distinguish between the two mouse primary antibodies. Here is a brief description of the protocol. After antigen retrieval of the paraffin sections of pancreas of nondiabetic or T1D cases (by following standard antigen retrieval procedure using citrate buffer), tissues were then blocked with blocking buffer (3% BSA, 0.2% Triton X-100) for 1hr at room temperature followed by incubation with first primary antibodies prepared in antibody buffer (3% BSA in TBST) at the following dilutions (mouse anti Ki67 [1:50, DAKO M7240; Carpinteria, CA], mouse anti-Nkx6.1 [1:300, DSHB F55A10; Iowa city, IA], mouse anti-Nkx2.2 [1:50, DSHB 74.5A5; Iowa city, IA]), mouse anti-CD45 [1:500, M0701; Carpinteria, CA]) in separate sections at 4°C for overnight. First primary Ab was detected by Cy3 conjugated donkey antimouse IgG (1:100, Jackson Immuno research 715–166–150; Westgrove, PA). Slides were then incubated sequentially with mouse serum (5% V/V, Jackson ImmunoResearch; 015–000–120) and unconjugated F(ab')<sub>2</sub> fragment of donkey antimouse IgG (40 μg/ml, Jackson Immunoresearch, 715–007–003) for 1hr at room temperature. After each incu-

bation slides were washed with 1xTBST and 1xTBS (10 minutes each). After that slides were incubated with second primary antibody (mouse antiglucagon, 1:2000, Sigma-Aldrich G2654-.2ML; St. Louis, MO) at 4°C for overnight. Second primary antibody was detected with donkey antimouse Alexa 647 (1:100, Jackson ImmunoResearch; 715-606-151). Finally slides were incubated at 4°C overnight with a cocktail of third primary antibodies prepared at the following dilutions: guinea-pig anti-insulin (1:100; Abcam7842; Cambridge, MA), rat antisomatostatin (1:100, EMD Millipore MAB354; Billerica, MA), goat antipancreatic polypeptide (1:3000, Everest Biotech; Ramona, CA), rat antighrelin (1:50, R&D Systems MAB8200, Minneapolis, MN); rabbit anti-Chromogranin A (1:200, Novus Biologicals NB120-15 160, Littleton, CO). The third primary antibodies were detected by a cocktail of secondary antibodies [F(ab')<sub>2</sub> fragments]; donkey antiguinea pig Alexa 647 (1:100, Jackson ImmunoResearch; 706-606-148, for insulin), donkey antirat Alexa 647 (1:100, Jackson ImmunoResearch; 712-606-153, for ghrelin and somatostatin), donkey antigoat Alexa 647 (1:100, Jackson ImmunoResearch; 705-606-147 for PP) and donkey antirabbit FITC (1:100, Jackson ImmunoResearch; 711-096-152 for ChrgA). Slides were counterstained to mark the nuclei using a mounting medium containing DAPI (Vectashield, Vector Labs, Burlingame, CA), and viewed using a Leica DM6000 microscope (Leica Microsystems, Deerfield, IL) and images were acquired using the 20x objective (200x magnification) using a Hamamatsu Orca-ER camera (C4742-80-12AG, Indigo Scientific, Bridgewater, NJ) and Openlab software (Improvision, Lexington, MA).

### Morphometric Analysis

One section of body of pancreas per subject was stained, except in 5 cases of the nondiabetic cohort (6012, 6015, 6017, 6021 and 6022) where pancreas body was unavailable; in these cases a section of pancreas tail was stained. Fifty islets per subject were imaged at 20x magnification. An islet was defined as a grouping of four or more endocrine cells. A cluster was defined as a grouping of three or fewer chromogranin positive cells. Islets were selected by starting at the top left corner of the pancreatic tissue section and working across the tissue from left to right and back again in a serpentine fashion, imaging all islets in this systematic excursion across the tissue section. Analysis was performed in a blinded fashion (ASM, AEB and CS), and all chromogranin positive hormone negative (CPHN) cells identified were confirmed by a second observer. The endocrine cells contained within each islet were manually counted and recorded as follows: 1. The number of cells staining for chromogranin A, 2. The number of cells staining for the endocrine hormone cocktail, 3. The number of cells staining for insulin. Thus, cells staining for chromogranin A but not the other known pancreatic hormones (insulin, glucagon, somatostatin, pancreatic polypeptide or ghrelin) were noted.

At 200x magnification, using the Leica DM6000 with Hamamatsu Orca-ER camera and a 0.7x C-mount, each field of view was calculated to be 0.292 mm<sup>2</sup>. Within the fields imaged to obtain the fifty islets per subject, all single endocrine cells and clusters of endocrine cells (two or three adjacent endocrine cells) were counted and recorded as outlined above.

The mean number of endocrine cells counted within islets for the type 1 diabetic group was 1719 ± 192 cells per donor, for the autoantibody positive group was 2363 ± 247 cells per donor and

for the nondiabetic group was 2380 ± 266 cells per donor. The mean number of cells counted in clusters for the type 1 diabetic group was 298 ± 32 cells per donor, for the autoantibody positive group was 90 ± 7 cells per donor and for the nondiabetic group was 122 ± 16 cells per donor. The mean number of CPHN cells counted in islets for the type 1 diabetic group was 19.3 ± 4.1 cells per donor, for the autoantibody positive group was 4.9 ± 0.9 cells per donor and for the nondiabetic group was 5.7 ± 2.3 per donor. The mean number of chromogranin positive hormone negative [CPHN] cells per individual identified in clusters for the type 1 diabetic group was 58.1 ± 9.4 cells per donor, for the autoantibody positive group was 8.2 ± 1.3 cells per donor and for the nondiabetic group was 11.4 ± 2.2 cells per donor.

### Statistical Analysis

Statistical analysis was performed using the Student t test or ANOVA, where appropriate, with GraphPad Prism 5.0 software (GraphPad Software, La Jolla, CA). Data in graphs and tables are presented as means ± SEM. Findings were assumed statistically significant at  $P < .05$ .

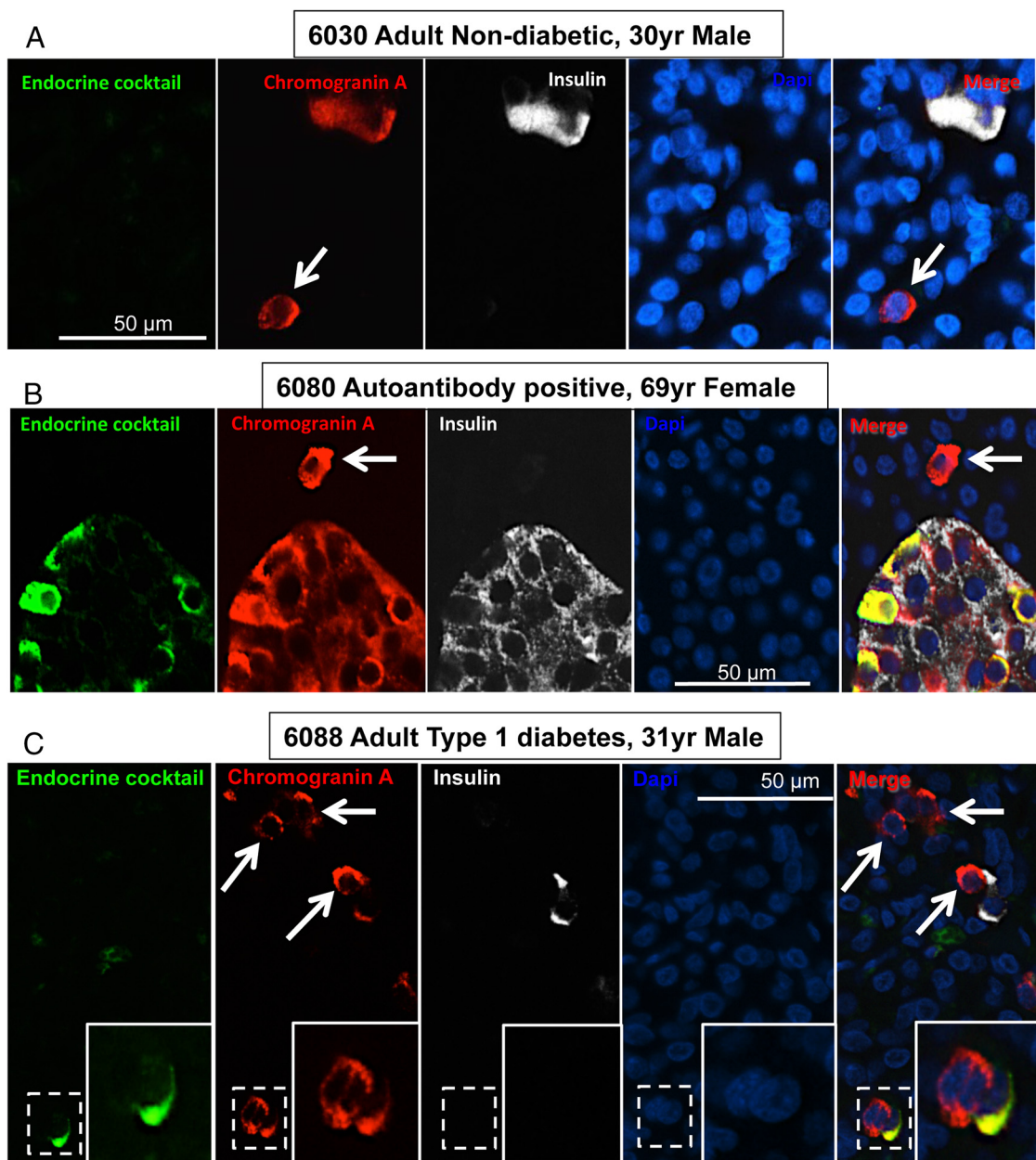
### Results

*Non hormone expressing endocrine cells are more frequent in type 1 diabetes.* Endocrine cells identified by immunoreactivity for chromogranin A but that did not express any known pancreatic islet hormone could be identified in islets and among scattered endocrine cells or as individual cells in the exocrine pancreas in type 1 diabetes and nondiabetic controls (Figure 1). When quantified, these chromogranin positive, hormone negative [CPHN] cells, while infrequent, were more abundant in the islets of donors with type 1 diabetes compared to nondiabetic individuals (1.11 ± 0.20 vs. 0.27 ± 0.10% of islet endocrine cells, T1D vs. ND,  $P < .001$ ) (Figure 2A, 2B). CPHN cells were also more frequent in scattered endocrine cell clusters (18.99 ± 2.09 vs. 7.42 ± 1.26% of all endocrine cells occurring in clusters, T1D vs. ND,  $P < .0001$ ) (Figure 2C, 2D), and as single endocrine cells (21.27 ± 2.63 vs. 8.90 ± 1.43% of all endocrine cells occurring as single cells, T1D vs. ND,  $P < .001$ ).

To determine if the increase in CPHN cells precedes the onset of type 1 diabetes, we also quantified their abundance in islet auto-antibody positive nondiabetic organ donors. In contrast to established type 1 diabetes, we found no increase in CPHN cells in the islet auto-antibody group compared to nondiabetic controls in islets (0.26 ± 0.06 vs. 0.27 ± 0.10% of all islet endocrine cells, AA<sup>+</sup> vs. ND,  $p=ns$ ) (Figure 2A, 2B), scattered endocrine clusters (9.67 ± 1.49 vs. 7.42 ± 1.26% of all endocrine cells occurring in clusters, AA<sup>+</sup> vs. ND,  $p=ns$ ) (Figure 2C, 2D) or individual cells (10.36 ± 1.65 vs. 8.90 ± 1.43% of all endocrine cells occurring as single cells, AA<sup>+</sup> vs. ND,  $p=ns$ ).

The frequency of the scattered CPHN cells within endocrine cell clusters and present as single cells were also increased in type 1 diabetes when expressed per unit area of pancreas but, again, were not increased in pancreas from islet autoantibody positive donors (Clusters:  $10.17 \pm 1.59$  vs  $1.06 \pm 0.16$  vs  $2.23 \pm 0.50$  cells/mm<sup>2</sup> pancreas, T1D vs AA+ vs ND,  $P < .0001$  T1D vs AA+ and T1D vs ND; Single cells:  $7.91 \pm 1.28$  vs  $0.78 \pm 0.13$  vs  $1.88 \pm 0.42$  single cells/mm<sup>2</sup> pancreas, T1D vs AA+ vs ND,  $P < .0001$  T1D vs AA+ and T1D vs ND) (Figure 2E, 2F).

**Potential contribution of non hormone expressing  $\beta$ -cells to the  $\beta$ -cell deficit in type 1 diabetes.** It has been reported in NOD mice that approximately 50% of the apparent  $\beta$ -cell deficit may be due to degranulation of  $\beta$ -cells, raising the question of whether the  $\beta$ -cell deficit in humans with type 1 diabetes may be in part due to degranulation rather than loss of  $\beta$ -cells (5). The  $\beta$ -cell deficit per islet section in type 1 diabetes in the present study was 95% ( $1.31 \pm 0.72$  vs  $26.51 \pm 3.03$   $\beta$ -cells/islet section, T1D vs ND,  $P < .0001$ ). If all the CPHN cells/islet ( $0.40 \pm 0.09$  vs  $0.11 \pm 0.05$  CPHN cells/islet, T1D vs ND,  $P < .005$ )



**Figure 1.** Examples of Chromogranin Positive Hormone Negative [CPHN] cells in pancreas from an adult nondiabetic donor (1A), an adult autoantibody positive donor (1B) and an adult type 1 diabetic donor (1C). Individual layers stained for Endocrine cocktail (Glucagon, Somatostatin, Pancreatic Polypeptide and Ghrelin) [green], Chromogranin A [red], Insulin [white] and Dapi [blue] are shown along with the merged image. Arrows indicate Chromogranin Positive Hormone Negative [CPHN] cells. Scale bar is 50 microns.

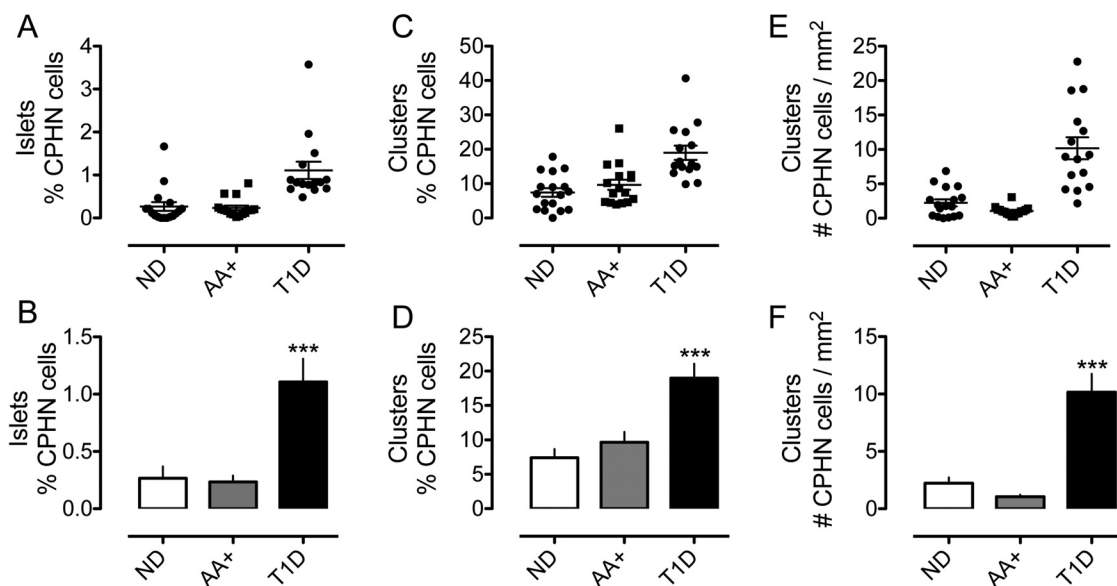
were degranulated  $\beta$ -cells, then the  $\beta$ -cell deficit per islet would be reduced from 95% to 94%. (Figure 3A, 3B). Therefore, in contrast to the NOD mouse model, we conclude that  $\beta$ -cell degranulation does not contribute significantly to the evaluated  $\beta$ -cell loss in type 1 diabetes in humans.

**Potential contribution of transdifferentiation of  $\beta$ -cells to other cell types in type 1 diabetes.** Recent interest has focused on the capacity for pancreatic endocrine cells to transdifferentiate to other endocrine cell types, and in the case of type 2 diabetes, this has been postulated as a mechanism of  $\beta$ -cell loss, while in type 1 diabetes it has been postulated as a potential source of  $\beta$ -cells (13, 14). This has arisen from the apparent increase in  $\alpha$ -cells per islet vs loss of  $\beta$ -cells per islet in type 2 diabetes. When examined in this manner, there is also a  $\sim$ 50% increase in other nonbeta endocrine cells per islet in type 1 diabetes ( $33.0 \pm 4.0$  vs  $20.87 \pm 2.74$  endocrine cocktail cells/islet, T1D vs ND,  $P < .05$ ) (Figure 3B). As in type 2 diabetes, the  $\beta$ -cell deficit in type 1 diabetes ( $1.31 \pm 0.72$  vs  $26.51 \pm 3.03$   $\beta$ -cells/islet, T1D vs ND,  $P < .0001$ ) cannot be explained by conversion of  $\beta$ -cells to other endocrine cells. Moreover, the apparent increase in other nonbeta endocrine cells per islet is likely an ascertainment bias, since sections of residual islets (after near complete  $\beta$ -cell loss) would be expected to be relatively enriched in other endocrine cells.

**The presence of detectable insulin expressing cells in type 1 diabetes does not predict the frequency of nonhormone ex-**

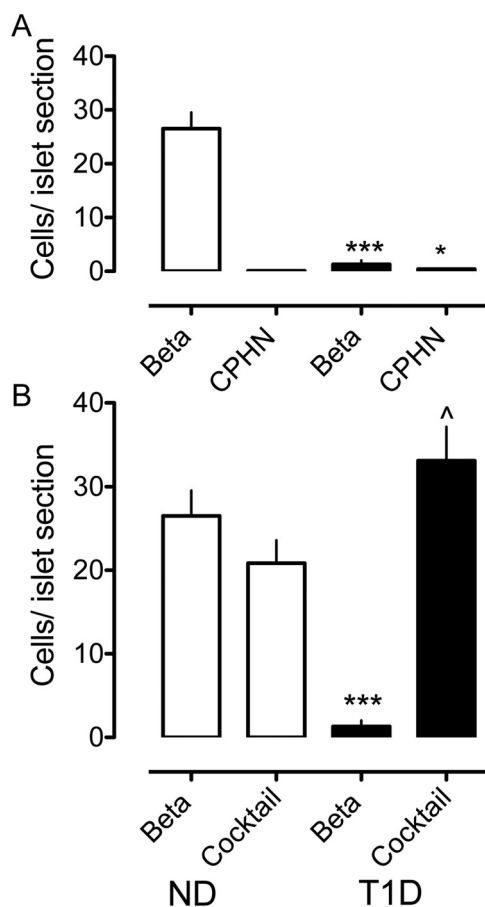
**pressing endocrine cells.** In type 1 diabetes, occasional insulin expressing  $\beta$ -cells are commonly identified in small numbers, particularly as scattered cells in the exocrine pancreas (15, 16). Given the possibility that nonhormone expressing endocrine cells may be precursors of newly forming  $\beta$ -cells, we hypothesized that scattered nonhormone expressing endocrine cells would be more frequent in those individuals with detectable insulin expressing  $\beta$ -cells. We identified insulin expressing  $\beta$ -cells in 7 of the 15 individuals with type 1 diabetes. Contrary to our hypothesis, CPHN cells in islets were more frequently found in T1D subjects in whom no residual insulin positive cells were present ( $0.56 \pm 0.14$  vs.  $0.21 \pm 0.03$  CPHN cells/islet, No residual insulin vs. Residual insulin,  $P < .05$ ). There was no difference in the number of scattered CPHN cells between those T1D subjects in whom insulin positive cells were still present in pancreatic sections and those subjects in whom no residual insulin positive cells were detected ( $10.31 \pm 2.55$  vs.  $10.00 \pm 1.97$  CPHN cells in clusters/ $\text{mm}^2$  pancreas, No residual insulin vs. Residual insulin,  $p = \text{ns}$ ) (Figure 4A-4D). In conclusion, the CPHN cells do not predict the presence or extent of  $\beta$ -cells in type 1 diabetes.

**The relationship between age, BMI and duration of diabetes on the frequency of nonhormone expressing endocrine cells (Figure 5).** To further investigate the potential origins of nonhormone expressing endocrine cells we examined the relationship of age, BMI and duration of diabetes on



**Figure 2.** The frequency of nonhormone expressing endocrine cells was indeed increased in islets from donors with type 1 diabetes ( $1.11 \pm 0.20$  vs  $0.26 \pm 0.06$  vs  $0.27 \pm 0.10$  of islet endocrine cells, T1D vs AA+ vs ND,  $P < .0001$  T1D vs AA+ and T1D vs ND) (2A and 2B) and in scattered clusters of endocrine cells in pancreas from type 1 diabetic donors ( $18.99 \pm 2.09$  vs  $9.67 \pm 1.49$  vs  $7.42 \pm 1.26$  of scattered endocrine cells T1D vs AA+ vs ND,  $P < .0001$  T1D vs AA+ and T1D vs ND) (2C and 2D). The frequency of scattered nonhormone expressing endocrine cells, expressed per unit area of the pancreas section, was increased in pancreas sections from donors with type 1 diabetes (2E and 2F) ( $10.17 \pm 1.59$  vs  $1.06 \pm 0.16$  vs  $2.23 \pm 0.50$  cells/ $\text{mm}^2$  pancreas, T1D vs AA+ vs ND,  $P < .0001$  T1D vs AA+ and T1D vs ND).

the abundance of these cells. There was a positive linear relationship between the frequency of the nonhormone expressing endocrine cells and both age ( $r = 0.44$ ,  $P < .05$ ) (Figure 5A) and BMI ( $r = 0.66$ ,  $P < .005$ ) (Figure 5B) in the nondiabetic donors. No relationship with age or BMI was present in either the type 1 diabetic donors or the autoantibody positive donors. There was no relationship with duration of disease in the type 1 diabetic donors (Figure 5C).



**Figure 3.** The impact of nonhormone expressing endocrine cells on the deficit in beta cell mass in type 1 diabetes is minimal. The number of cells/islet section of the CPHN cell fraction is very small when compared with the number of beta cells/islet section in the ND, and the deficit in beta cells in the T1D donors cannot be accounted for by the increase in CPHN cells in type 1 diabetes. A. There is an approximate 95% deficit in beta cells in the islets from the subjects with type 1 diabetes ( $1.31 \pm 0.72$  vs  $26.51 \pm 3.03$  beta cells/islet, T1D vs ND,  $***P < .0001$ ). The number of CPHN cells/islet section is increased in type 1 diabetes ( $0.40 \pm 0.09$  vs  $0.11 \pm 0.05$  CPHN cells/islet section, T1D vs ND,  $P < .005$ ) but this increase does not account for the beta cell deficit seen in type 1 diabetes. B. Islet endocrine cells expressing islet hormones other than insulin are increased in type 1 diabetes ( $33.10 \pm 4.06$  vs  $20.87 \pm 2.74$  endocrine cocktail cells/islet section, T1D vs ND,  $P < .05$ ). However, the beta cell deficit in type 1 diabetes ( $1.31 \pm 0.72$  vs  $26.51 \pm 3.03$  beta cells/islet section, T1D vs ND,  $***P < .0001$ ) cannot be explained by conversion of beta cells to other endocrine cells. White bars = Non-Diabetic [ND] donors; Black bars = type 1 Diabetic [T1D] donors

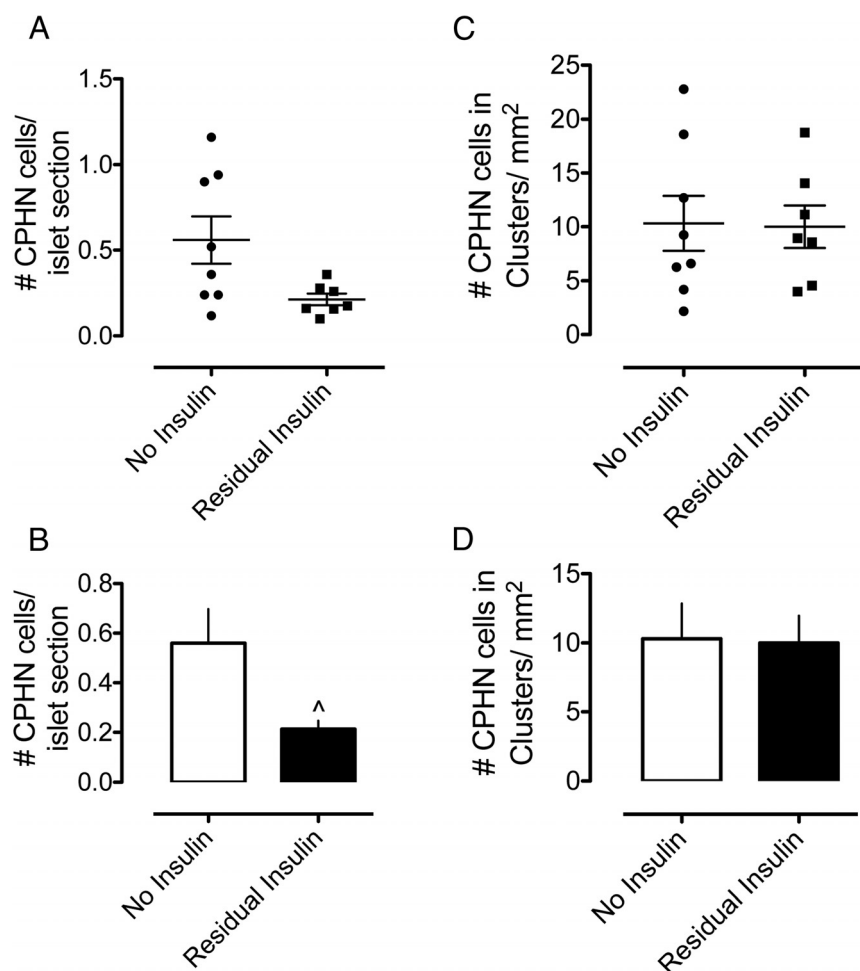
**There are more scattered endocrine cells in type 1 diabetes.** Having already established that there are more nonhormone expressing endocrine cells scattered in pancreas in type 1 diabetes, we sought to establish if this was true of all endocrine cells (the sum of all scattered chromogranin positive cells per unit area of pancreas section, whether expressing hormones or not). There is indeed an increase in the total number of scattered endocrine cells in the pancreas in type 1 diabetes ( $52.26 \pm 5.47$  vs  $11.30 \pm 1.04$  vs  $24.53 \pm 5.13$  cells/mm<sup>2</sup>, T1D vs AA+ vs ND,  $P < .0001$  T1D vs AA+ and T1D vs ND) (Figure 6A, 6B). This raised the possibility that the apparent increase in hormone-negative endocrine cells in type 1 diabetes might be simply a reflection of the decreased exocrine pancreas size in type 1 diabetes.

To address this we examined the relationship between the frequency of nonhormone expressing endocrine cells and all endocrine cells per unit of pancreas area (Figure 6C). While there was a linear relationship between these parameters in both groups, the slope of the increase was greater in type 1 diabetes ( $r = 0.73$ ,  $P < .01$ ) than in nondiabetic controls ( $r = 0.89$ ,  $P < .0001$ ), implying an excess of nonhormone expressing endocrine cells independent of pancreas size. This impression was also corroborated by examining the relationship between the frequency of nonhormone expressing endocrine cells and pancreatic weight in both groups (Figure 6D). While the pancreas weight in individuals with type 1 diabetes was on average decreased compared to nondiabetic and autoantibody positive donors, the increase in nonhormone expressing endocrine cells in individuals with type 1 diabetes was not related to pancreas size.

### Characterization of CPHN cells

**Replication.** Since CPHN cells were scattered in foci reminiscent of neonatal pancreas, we questioned if these cells are replicating. To address this, because of the number of antibodies required, we applied a newly developed immunohistochemical technique in pancreatic sections of T1D and ND ( $n = 4$  each group) with anti-Ki67 and antiendocrine cocktail (containing antibodies against all the islet endocrine hormones and anti-ChrgA). The 4 T1D cases were chosen as having the highest frequency of CPHN cells (6138, 6051, 6076 and 6050) and the ND cases matched as closely as possible for age and BMI (6015, 6021, 6029 and 6134). While we again successfully identified increased CPHN cells in the T1D sections, none of these cells were positive for Ki67 (Supplemental Figure 2A and 2B). We therefore conclude that the CPHN cells in T1D are not actively replicating.

**CPHN cells have endocrine identity.** In pancreatic sections



**Figure 4.** Graphs compare the number of CPHN cells in type 1 diabetic subjects, grouped according to those in whom insulin positive cells were still present in pancreatic sections and those subjects in whom no residual insulin positive cells were detected in pancreas sections. (Panels A and B) The number of Chromogranin A positive cells per islet. CPHN cells were identified more frequently in T1D subjects in whom no residual insulin positive cells were present ( $0.56 \pm 0.14$  vs.  $0.21 \pm 0.03$  CPHN cells/islet section, No residual insulin vs. Residual insulin,  $P < .05$ ). (Panels C and D) The number of CPHN cells found in endocrine clusters per  $\text{mm}^2$  of pancreas. There was no difference in the number of CPHN cells between those T1D subjects in whom insulin positive cells were still present in pancreatic sections and those subjects in whom no residual insulin positive cells were detected ( $10.31 \pm 2.55$  vs.  $10.00 \pm 1.97$  CPHN cells in clusters/ $\text{mm}^2$  pancreas, No residual insulin vs. Residual insulin,  $p = \text{ns}$ ).

from the same 4 T1D and 4 ND as above, we deployed the same novel immunohistochemistry approach to establish if CPHN cells conform to an islet endocrine lineage, and if so a  $\beta$ -cell lineage by staining for Nkx6.1 (Supplemental Figure 3A and 3B) and Nkx2.2 (Supplemental Figure 4A and 4B). Between 50 and 60 20x fields were imaged and analyzed in each section for each transcription factor. For Nkx2.2, CPHN cells were identified in both T1D and ND pancreas sections and 100% of CPHN cells were also positive for Nkx2.2. For Nkx6.1, again CPHN cells were identified in both T1D and ND pancreas sections, and between 10%–20% of CPHN were positive for Nkx6.1, with no difference between T1D and ND cases. The pancreatic CPHN cells are therefore confirmed to be of an islet lineage, and of these ~10%–20% are of a beta cell lineage.

**CPHN and inflammation.** T1D is associated with invasion of inflammatory cells in or around islets. We questioned whether CPHN cells are also targets of the inflammatory process. To address this, we stained both ND and T1D pancreatic sections with anti-CD45, antiendocrine cocktail (that includes all the endocrine hormones) and anti-ChrgA. As expected, we identified a few CD45 positive cells in or around islets in the T1D cases (and more infrequently in ND islets) but in none of the cases were CPHN cells surrounded by CD45 positive cells. The same cases (4 T1D, 4 ND) were used as for assessment of replication.

## Discussion

We report that there is an increased frequency of endocrine cells that express no known islet hormone in type 1 diabetes, both within islets and, more prominently, scattered in clusters and individually in the exocrine pancreas. There is also an increase in scattered endocrine cells that express known islet hormones in the exocrine pancreas in type 1 diabetes. While the abundance of these cells is insufficient to make a meaningful contribution to the loss of  $\beta$ -cell mass in type 1 diabetes, their increased presence in this setting is comparable to that seen in humans

with type 2 diabetes.

There has been recent interest in the concept that apparent loss of  $\beta$ -cell mass and  $\beta$ -cell failure in type 2 diabetes may be due in part to either degranulation of  $\beta$ -cells and/or a change in endocrine cell identity to other islet endocrine cell types (4). It is not clear if these changes precede diabetes and are important in the pathogenesis of diabetes, or whether the changes are secondary to diabetes. Furthermore, it is unknown if the nonhormone-expressing endocrine cells are due to a loss of identity or are newly forming endocrine cells. The pattern of distribution of endocrine cells that express no known hormones in type 2 diabetes shares some of the characteristics of the newly forming endocrine pancreas in late gestation and early



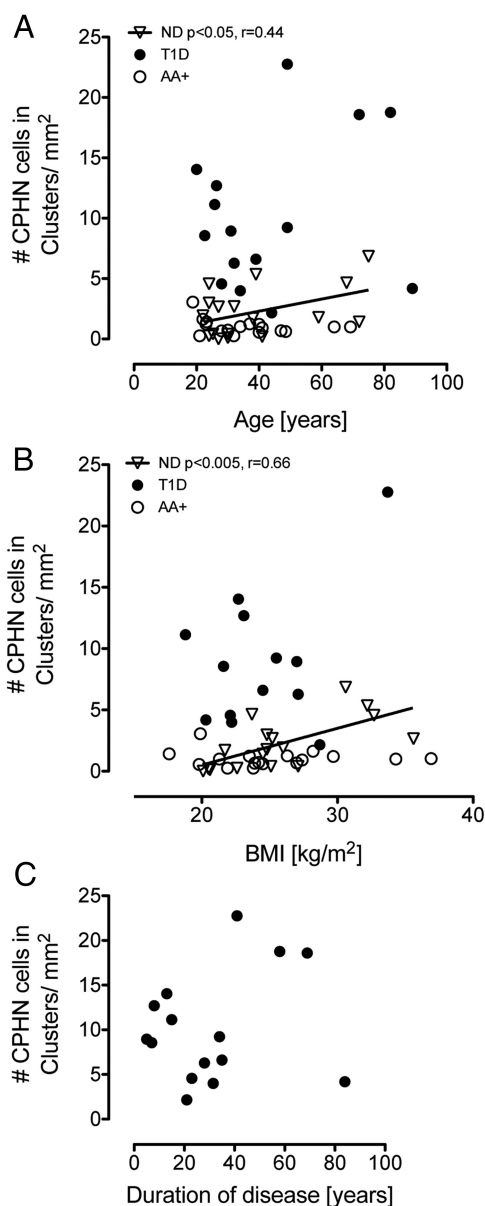
infancy (6). The finding reported here that the abundance and pattern of distribution of these cells is comparable in type 1 and 2 diabetes has several implications.

First, the relatively high abundance of degranulated  $\beta$ -cells ( $\sim 50\%$  of  $\beta$ -cells) reported in mouse models of type 1 and 2 diabetes far exceeds that in humans with type 1 or 2 diabetes (3%) (17, 18). Therefore, the proposal that  $\beta$ -cell loss in type 1 and type 2 diabetes may be in large part an artifact due to degranulation of  $\beta$ -cells may be valid in mice, but is not the case for humans. Second, the fact that

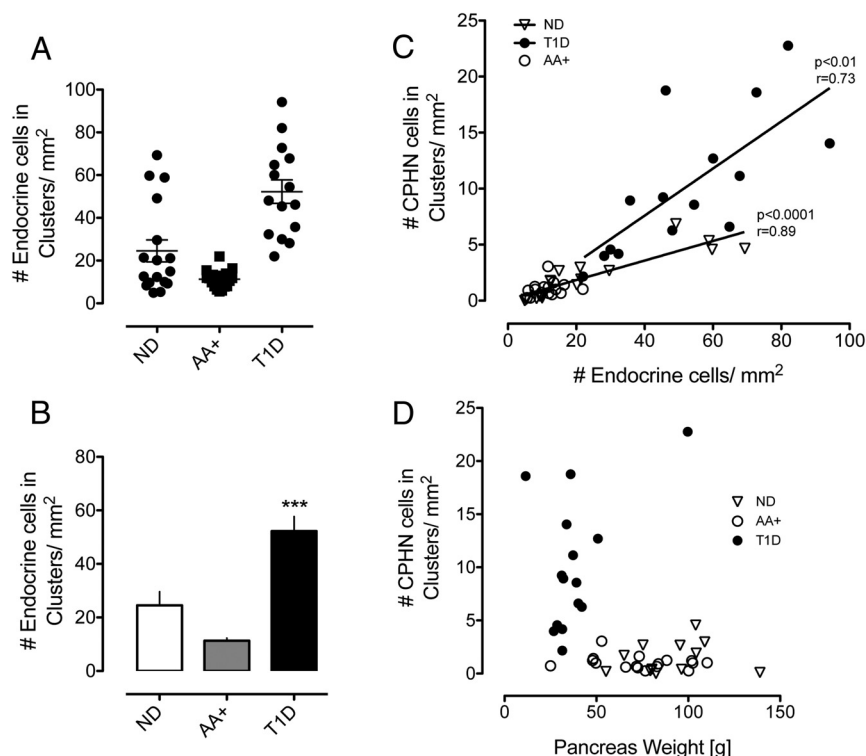
changes in  $\beta$ -cell identity (degranulation and mixed identity) have been reported in both type 1 and 2 diabetes suggests that these changes are secondary to diabetes rather than primary drivers of  $\beta$ -cell dysfunction (18, 19). In both type 1 and 2 diabetes there are well characterized and specific inducers of  $\beta$ -cell stress, cytokines delivered by autoreactive immune cells and misfolded islet amyloid polypeptide toxic oligomers respectively (20, 21).

Stress pathways induced by both cytokines and misfolded protein toxic oligomers are known to both alter cell identity and to induce local regeneration programs (22, 23). Studies in other pathological contexts, such as the renal tubule, suggest that in response to acute injury the tissue can dedifferentiate to permit replication of viable cells towards regeneration while clearing out nonviable cells through apoptosis (24). Ideally, to establish the sequence of events leading to defective  $\beta$ -cell function and mass in diabetes, pancreas would be evaluated from prediabetic individuals. We tried to approach this by use of the nPOD autoantibody positive non diabetic pancreas repository. We did not identify any increase in hormone negative endocrine cells in this group. However, most these pancreases were secured from individuals that were autoreactive for only one autoantibody, and as such it is not surprising that they have no loss of  $\beta$ -cell mass (25) or identified islet inflammation, since such individuals are unlikely to develop diabetes (26). If pancreas from triple antibody positive prediabetic individuals was to become available, this would be more relevant to the pattern of endocrine cell identity alteration in prediabetes.

We recently noted the similarity between the pattern of nonhormone expressing endocrine cells in early human development and in pancreas of individuals with type 2 diabetes, implying that this might be due to attempted  $\beta$ -cell regeneration (6). That we see the same pattern in humans with type 1 diabetes in who there have been prior studies (7, 22, 27) suggesting ongoing attempted  $\beta$ -cell regeneration, lends further credence to this possibility. The origin of the putative new  $\beta$ -cells in adult human pancreas, for example in type 1 diabetes, remains to be resolved. While the frequency of replication is very low in adult human  $\beta$ -cells, it has been reported to increase in islets of new onset type 1 diabetes in association with an autoreactive-inflammatory infiltrate (22). However it is of note that the CPHN cells evaluated here do not show increased replication. The cells scattered in the exocrine pancreas have been considered as derivatives of endocrine cell neogenesis previously (28), but this remains controversial, and its resolution is beyond the reach of investigation in human pancreas in the absence of lineage tracing. Furthermore, although the CPHN cells are reported here as of islet lineage since they express Nkx2.2, and a subgroup are



**Figure 5.** The relationship of age, BMI and duration of diabetes on the abundance of CPHN cells in pancreas. There was a positive linear relationship between the frequency of the nonhormone expressing endocrine cells and both age ( $r = 0.44, P < .05$ ) (Figure 5A) and BMI ( $r = 0.66, P < .005$ ) (Figure 5B) in the nondiabetic donors. No relationship with age or BMI was present in either the type 1 diabetic donors or the autoantibody positive donors. There was no relationship with duration of disease in the type 1 diabetic donors (Figure 5C).



**Figure 6.** Endocrine cells occurring in scattered clusters were increased in pancreas sections from donors with type 1 diabetes (6A and 6B) ( $52.26 \pm 5.47$  vs  $11.30 \pm 1.04$  vs  $24.53 \pm 5.13$  cells/mm<sup>2</sup> pancreas, T1D vs AA+ vs ND,  $P < .0001$  T1D vs AA+ and T1D vs ND). The relationship between the frequency of nonhormone expressing endocrine cells and all endocrine cells per unit of pancreas area (6C). While there was a linear relationship between these parameters in both groups, the slope of the increase was greater in type 1 diabetes ( $r = 0.73$ ,  $P < .01$ ) than in nondiabetic controls ( $r = 0.89$ ,  $P < .0001$ ), implying an excess of nonhormone expressing endocrine cells independent of pancreas size. The relationship between the frequency of nonhormone expressing endocrine cells and pancreatic weight in both groups (D). While the pancreas weight in individuals with type 1 diabetes was on average decreased compared to nondiabetic and autoantibody positive donors, the increase in nonhormone expressing endocrine cells in individuals with type 1 diabetes was not related to pancreas size.

of potential  $\beta$ -cell lineage expressing Nkx6.1, given the single time point that is available from human pancreas procured at death, it is impossible to know if these cells are advancing toward a differentiated endocrine fate, dedifferentiating from a mature endocrine fate or static.

In summary, hormone-negative endocrine cells are present in the pancreas of individuals with type 1 diabetes at an increased frequency, and with an abundance and distribution comparable to that observed in type 2 diabetes. The number of these cells is insufficient to explain  $\beta$ -cell loss in type 1 (or type 2) diabetes, through putative degranulation. On the other hand the distribution of these cells, being predominately scattered in the exocrine pancreas, mimics that present in the developing human endocrine pancreas and is consistent with the possibility that there is ongoing attempted, all be it insufficient,  $\beta$ -cell regeneration in both forms of diabetes.

## Acknowledgments

We appreciate the editorial assistance of Bonnie Lui from the Larry L. Hillblom Islet Research Center at UCLA. This research was performed with the support of the Network for Pancreatic Organ Donors with Diabetes (nPOD), a collaborative type 1 diabetes research project sponsored by the Juvenile Diabetes Research Foundation International (JDRF). Organ Procurement Organizations (OPO) partnering with nPOD to provide research resources are listed at [www.jdrfnpod.org/our-partners.php](http://www.jdrfnpod.org/our-partners.php). Funding for these studies was from NIH/NIDDK Grant #DK077967, the Larry Hillblom Foundation Grant #2014-D-001-NET and the Network for Pancreatic Organ Donors with Diabetes through the Helmsley Charitable Trust George S. Eisenbarth nPOD Award for Tetam Science Grant ##2015PG-T1D052, Subaward #664215.

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Disclosure Summary: The authors have nothing to disclose.

### AUTHOR CONTRIBUTIONS:

ASM, AEB, MC and CS performed the studies, undertook the microscopy and performed the morphological analysis. ASM, AEB, SD, PCB researched data, wrote the manuscript, reviewed the manuscript, edited the manuscript, and contributed to the discussion.

This work was supported by **Funding:** NIH/NIDDK Grant #DK077967 and Larry Hillblom Foundation Grant #2014-D-001-NET to PCB; The Helmsley Charitable Trust #2015PG-T1D052, Subaward #664215 to AEB.

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