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Adult Human Pancreatic Islet Beta-Cells Display Limited Turnover and Long Lifespan as Determined by In-Vivo Thymidine Analog Incorporation and Radiocarbon Dating

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Abstract

Aims: Diabetes mellitus results from an absolute or relative deficiency of insulin producing pancreatic beta-cells. The adult human beta-cell's turnover rate remains unknown. We employed novel techniques to examine adult human islet beta-cell turnover and longevity in vivo.

Methods: Subjects enrolled in NIH clinical trials received thymidine analogues [iododeoxyuridine (IdU) or bromodeoxyuridine (BrdU)] 8-days to 4-years prior to death. Archival autopsy samples from ten patients (aged 17-74 years) were employed to assess beta-cell turnover by scoring nuclear analog labeling within insulin staining cells. Human adult beta-cell longevity was determined by estimating the cells' genomic DNA integration of atmospheric carbon-14 (^{14}C). DNA was purified from pancreatic islets isolated from cadaveric donors; whole islet prep DNA was obtained from a 15 year old donor, and purified beta-cell DNA was obtained from two donors (age 48 and 80 years). ^{14}C levels were then determined using accelerator mass spectrometry (AMS). Cellular "birth date" was determined by comparing the subject's DNA ^{14}C content relative to a well-established ^{14}C atmospheric prevalence curve.

Results: In the two subjects less than age 20 years,1-2% of the beta-cell nuclei co-stained for BrdU/IdU. No beta-cell nuclei co-stained in the eight patients more than 30 years old. Consistent with the BrdU/IdU turnover data, beta-cell DNA ^{14}C content indicated the cells' "birth date" occurred within the subject's first 30 years of life.

Conclusions: Under typical circumstances, adult human beta-cells and their cellular precursors are established by young adulthood.

Introduction

Beta-cell turnover has been extensively explored in rodents, yet studies exploring human beta-cell turnover are more difficult and limited. Some studies have reported indirect evidence suggesting continued human beta-cell turnover [1]. In contrast, while the technique is not well established, a prolonged human beta-cell lifespan has recently been suggested by quantifying lipofucin accumulation [2]. In-vitro, human beta-cells have a restricted capacity for proliferation compared to rodents [3.]. In the present work, we used two novel techniques to study human beta-cell turnover in vivo.

Materials and Methods

Immuno-histochemistry and proliferation analysis

IRB exemption was obtained to analyze samples from deceased patients who had received thymidine analogs during previous cancer-related clinical trials. Five micron longitudinal sections of the paraffin embedded pancreata (NIH pathology and autopsy archives) were rehydrated with xylene, followed by decreasing concentrations of ethanol, microwaved in 0.01 mol/L sodium citrate (pH 6.0) for 26 minutes, and permeabilized with 1% Triton X-100 in phosphate buffered saline (PBS) before incubation with primary antisera (guinea pig anti-insulin, Zymed Laboratories Inc., South San Francisco, CA; and rat anti-BrdU [BU1/75], Accurate Chemical, Westbury, NY). Secondary antibodies were highly cross-adsorbed and labeled with Cyanine 2 or Cyanine 3 (Jackson ImmunoResearch Laboratories, West Grove, PA) as previously described [4]. Nuclear staining was performed with 4,6 diamino-2-phenylindole (DAPI, Molecular Probes, Eugene, OR). Triple-labeled DAPI/insulin/BrdU images were acquired (Carl Zeiss

MicroImaging, Thornwood, NY). All islets on a single section were counted to determine the number of cells that co-stained for BrdU (or IdU) and insulin relative to the number of cells that stained for insulin alone.

Carbon 14 (^{14}C) dating of beta-cells

Using previously described techniques, islets from brain dead donors were obtained from the Islet Cell Consortium or in the lab under a protocol approved by the Institutional Review Board of the National Institute of Diabetes, Digestive, and Kidney Diseases (www.clinicaltrials.gov, NCT00006505). Islets were then dispersed into single cells by 20-minute incubation at 37ºC in PBS (without calcium and magnesium) with 0.5mM EDTA, followed by repeated pipetting for 2 minutes. Cells were fixed in 4% paraformaldehyde for 30 minutes at room temperature, filtered through a 40-micron filter, permeabilized with 0.1% saponin in 2% bovine serum albumin (BSA) in PBS, stained with 1 microgram of phycoerythrin (PE) conjugated pro-insulin antibody per $1x10^6$ beta-cells (R&D systems, Minneapolis, MN; custom conjugated by Invitrogen, Carlsbad, CA), and incubated on ice for 30 minutes. Cells were washed once with saponin/BSA/PBS and resuspended in PBS/BSA. Single beta-cells were sorted using a FACS Aria (BD Biosciences, San Jose, CA). Single cells were gated on PE positivity and side scatter width/area.

DNA was isolated as previously described and purity assessed using a Nanodrop spectrophotometer (NanoDrop, Wilmington, DE). DNA with a 260/280 ratio >1.85 and 230/260 >2.0 was considered pure and was subsequently analyzed at the Center for

Accelerator Mass Spectrometry (AMS), Lawrence Livermore National Laboratory using previously described methods [5].

Results

Tissues from 10 subjects were analyzed for insulin and thymidine analog co-staining (Figure 1A). The pancreas from the 20 year-old subject who received IdU for 12 consecutive days revealed 2.0% of beta-cells labeled with the analog (Figure 1B, left panel and Figure 1C), from which we can estimate that 60% of the beta-cells would divide over the course of a year. Similarly, the pancreas from the 18 year old subject who received thymidine analog for 14 days displayed 0.97% beta-cell turnover (estimated annual turnover of 25%). In contrast, analysis of the 45 year-old subject's pancreas revealed many IdU labeled pancreatic exocrine cells, but no IdU staining beta-cells (Figure 1B, middle panel). We quantified the number of cells that co-stained for insulin and thymidine analog in the pancreatic sections. Islets from all other subjects (ages 31 to 74) who received thymidine analog for 1-20 days showed no thymidine analog positive beta-cells (Figure 1C). Further, among the older subjects' thymidine analog positive islets, other endocrine markers (glucagon, pancreatic polypeptide, and somatostatin) were also negative (Figure 1B, right panel).

We attempted to apply the ^{14}C dating method[5] using 20 brain dead donor islet preparations, each with ≥100,000 islet equivalents. However, ^{14}C dating requires 60 mcg of pure DNA since contamination with other cells, or even minute amounts of solvent may substantially affect the dating. Multi-gate sorting was performed to exclude cell aggregates and minimize non-beta cell contamination. Post-sort analysis confirmed the

beta-cell fraction was 90-95% pure (data not shown). Using such stringent purity criteria however, only 2 of the 20 islet preparations contained sufficient DNA quantity and beta-cell purity for accurate AMS ^{14}C dating. Results were plotted along the established atmospheric ^{14}C curve (Figure 2A) [5]. Granulocytes served as a control, and showed a cellular "birth date" close to the time of granulocyte collection (data not shown). In contrast, the first subject, born in 1926 (blue lines, Figure 2B), had a beta-cell ^{14}C level corresponding to 1955 or earlier. This subject's beta-cells' ^{14}C content was lower than that found on earth since 1955, ruling out significant beta-cell turnover in the last 50 years of his life. The second subject, born in 1957 (green lines, Figure 2B), had average beta-cell ^{14}C levels corresponding to 1958 or 1988. The third subject, born in 1990, had whole islet ^{14}C content corresponding to 2001, consistent with 20% annual turnover. This rate is equivalent to 40% annual cell division, since only half of the cells' carbon is renewed when cells divide (Figure 2C and 2D). These data collectively indicated that the beta-cells' average "birthdate" occurred within each subject's first 30 years of life.

Discussion

Data evaluating human beta-cell turnover are limited, relying heavily on indirect measurements of beta-cell function. The most direct method reported to study human beta-cell replication has been to quantify Ki-67 positivity in autopsy or surgical samples. Ki-67 is a cell-cycle protein expressed at all stages except G_0. Co-expression of Ki-67 and insulin in the same cell suggests active division, but studies using this technique have reached contradictory conclusions. One report described the pancreas of an 89 year-old man with T1D and reported Ki-67 beta-cell staining [6]. Another report from the same

group evaluated nine patients (ages 12-38) who died of diabetic ketoacidosis and found no beta-cell Ki-67 staining [7]. The authors suggested that deleterious effects associated with DKA may have explained the differences

Since thymidine analogs are commonly applied to study in vivo cell turnover, we relied on historical clinical trials that administered thymidine analogs to patients with cancer to assess their beta-cell replication. The two youngest subjects, ages 18 and 20 years, demonstrated that 1-2% of their beta-cells had divided over the 14 and 12 days of labeling, corresponding to an annual turnover rate of 30-60%. No thymidine analog was observed in the beta-cells from the eight older subjects (age 31-74 years old), suggesting no beta-cell division. Ki-67 staining detects only beta-cell replication from existing beta-cells and may underestimate turnover rate if actively dividing cells are more apt to apoptose. Thymidine analogs have the advantage of detecting beta-cells formed by neogenesis from any progenitors, as long as the progenitors have divided while the analog was administered. Limitations of the thymidine analog technique include DNA repair being interpreted as proliferation and potential direct toxicity resulting from these agents [7]. For instance, one report suggested a modest 16-25% decrease in islet cell proliferation in rats treated with thymidine analogs compared to rats treated with heavy water, while another report showed that BrdU in the drinking water did not reduce beta-cell proliferation as measured by Ki-67 incorporation[8]. Regardless, toxicity does not likely explain the absent beta-cell proliferation observed in our subjects age 31 years and older since other tissues from the same subjects and the beta-cells from the younger subjects all demonstrated thymidine labeling. Given that approximately 1,000 beta-cells were evaluated among the 30 to 50 islets sampled, a single positive beta-cell would give

an annual turnover rate of 2.6% (assuming 2 weeks of in vivo labeling per cell turnover, 26 weeks x 1/1000 = 2.6%). Since we saw no positive beta-cells among the 8 subjects older than age 30 years, our results suggest an annual turnover rate of less than 2.6%.

Biological sample DNA ^{14}C dating has recently been employed to address replication in neurons, adipocytes, and cardiomyocytes. While the method's sensitivity and limitations are discussed thoroughly elsewhere [5], it can detect about 0.5% new cells within a population of old cells. Our ^{14}C data suggest that overall beta-cell turnover is limited to the first three decades of life. While we were able to date only a small number of the samples given the requirement for large amounts of high purity DNA, the 80 year old subject's beta-cell DNA content is lower than that found on earth since the 1950s, and argues strongly that cell turnover was very limited for the last 50 years of the subject's life. The findings also reinforce that we excluded exogenous carbon contamination during the DNA isolation. The consistency between the ^{14}C data and the thymidine analog data minimize concerns that the subjects' primary illness (cancer and its treatment) diminished the subjects' beta-cell turnover.

The observed minimal beta-cell turnover in adult humans has significant practical implications as therapies are developed to expand beta-cell mass. Our results suggest that strategies should be tested using more predictive animal models such as aged adult mice[4] or mature non-human primates.

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Figure legends:

Figure 1. In vivo thymidine analog labeling of human pancreas.

(A) Clinical information on 10 subjects who received thymidine analogs on clinical studies.

(B) Co-staining of thymidine analog and pancreatic hormones. These images were obtained using an Axioskop 2 microscope with a 10X objective and a 0.63X converter, and captured with a Hamamatsu Orca ER digital camera using Open Lab 5.2 software.
(Left panel) Pancreatic islet from a 20 year-old subject, stained with IdU (green), insulin (red), and DAPI for nucleus (blue). The two white arrows show 2 nuclei positive for IdU, completely surrounded with insulin, providing evidence for β-cells turnover (20X magnification).
(Middle panel) Pancreatic islets from a 45 year-old subject. There were no IdU positive β-cells. The two white arrows represent cells that have divided outside of pancreatic islets (10x magnification).
(Right panel) Pancreatic islet from the same 20 year-old subject as in left panel. Many proliferating cells within the islets are not mature endocrine cells: IdU (green); somatostatin, glucagon, or pancreatic polypeptide (blue); and insulin (red).

(C) Dosing details of thymidine analogs and the results of in vivo insulin and thymidine analog labeling.

Figure 2. ^{14}C dating of human beta-cells.

(A) Explanation of ^{14}C curve: The black curve in all graphs shows the atmospheric concentrations of ^{14}C over the decades since 1920. The measured ^{14}C concentration plotted on the Y-axis (1) is related to the atmospheric ^{14}C concentration on the established atmospheric ^{14}C bomb curve (2). The average birth date of the cell population can be inferred by where the data point intersects on the X-axis (3). $F^{14}C$ notation represents the $^{14}C/C$ ratio associated with the ^{14}C bomb pulse where $F^{14}C$ =1 occurred in the late 19th century before burning fossil fuels started to depress the $^{14}C/C$ ratio of atmospheric CO_2 [9].

(B) ^{14}C concentration in the DNA of beta and non-beta cell fractions. Vertical lines mark the birth year, square symbols with the dashed lines represent non-beta cell fractions, and the circles with dotted lines represent the beta-cell fraction. The first subject was born in 1926 (blue lines) and was 80 years-old at the time of death from head trauma with a HbA1c of 5.7%. His beta-cell ^{14}C level corresponded to 1956 on the black curve. The second subject was born in 1957 (green lines) and was 48 years-old at the time of death from a subarachnoid

hemorrhage with a HbA1c of 5.5%. That subject's beta-cell ^{14}C levels corresponded to 1958 or 1988. The data from these 2 subjects suggest beta-cells' birthdates do not exceed 30 years after the subjects' actual birthdate.

(C) ^{14}C concentrations in the DNA from whole islets (red circle) and spleen cells (yellow square) of a subject born in 1990 (15 year-old, healthy, died in a car accident).

(D) Expanded view of panel C from 1980 to 2010. The ^{14}C concentrations in DNA from whole islets and spleen cells of the same subject born in 1990. The ^{14}C levels in the spleen (yellow square) follow the bomb curve, suggesting 100% annual turnover of DNA. The islets (red circle and line) have a slower then atmospheric decline in ^{14}C, suggesting a 20% annual ^{14}C turnover, which corresponds to 40% annual cell turnover, since only half of the cell's carbon is renewed when cells divide .

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Figure 1A. Clinical information of 10 subjects who received thymidine analogs on clinical studies.

Age (years)	**Gender**	**Diagnosis**	**Type 2 Diabetes**	**Weight** (kg)	**Height** (cm)	**Fasting blood glucose** (mg/dL)
18	M	Osteosarcoma	No	52	171	84
20	M	Astrocytoma	No	64	181	90
31	F	Soft tissue sarcoma	No	58	160	86
45	M	Mesothelioma	No	74	188	88
53	M	Glioblastoma multiforme	No	75	180	90
55	M	Glioblastoma multiforme	Yes	68	183	132
70	M	Pancreatic cancer	Yes	83	175	N/A
70	F	Esophageal cancer	No	47	162	87
71	M	Esophageal cancer	No	82	187	83
74	M	Mesothelioma	No	75	170	83

N/A, not available.

Figure 1B. Co-staining of thymidine analog and pancreatic hormones.

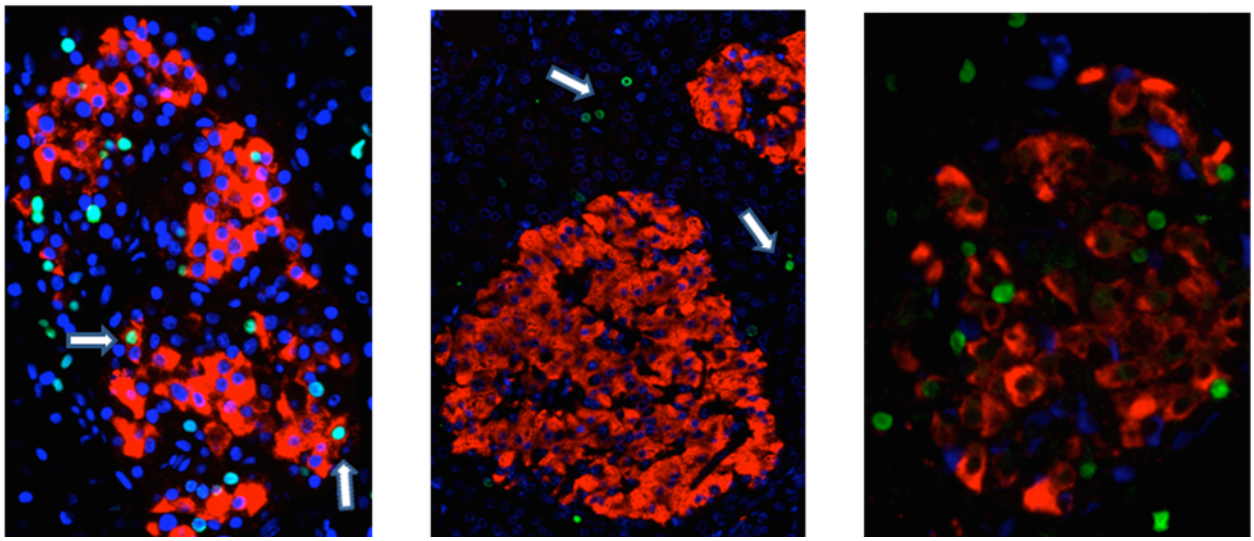
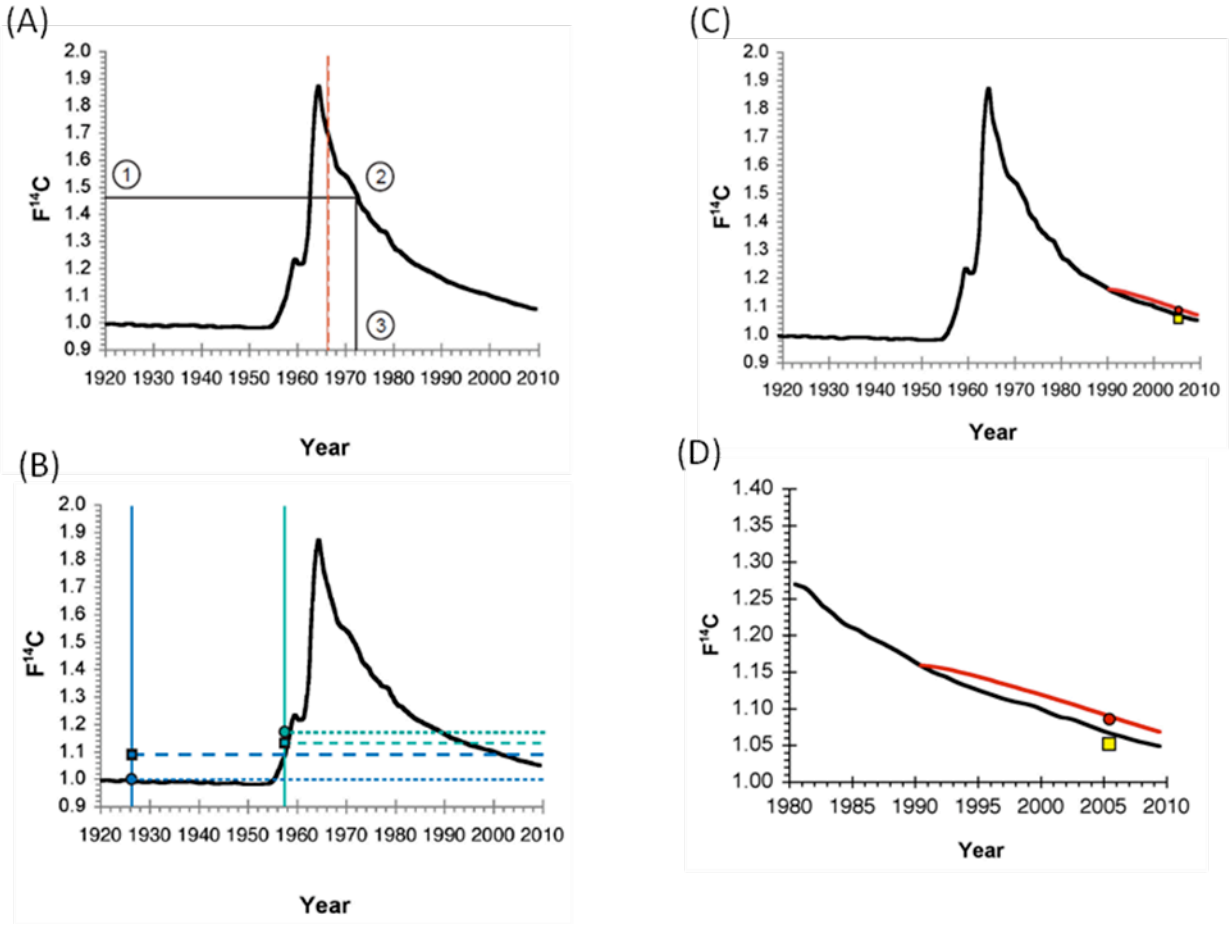


Figure 1C. Dosing details of thymidine analogs and the results of in vivo insulin and thymidine analog labeling

Age (years)	**Last dose of thymidine analog to autopsy** (days)	**Number of doses***	**Number of islets**	**Double positive for insulin and thymidine analog**
18	20	14	30	10 of 1029 β-cells (0.97%)
20	8	12	30	15 of 753 β-cells (2.0%)
31	55	19	40	0
45	71	19	31	0
53	548	20	51	0
55	8	10	30	0
70	10	3	50	0
70	315	10	33	0
71	1460	9	52	0
74	2	1	55	0

* Each dose of thymidine analog is 1000mg/m2 given over 24 hour by continuous intravenous infusion.

Figure 2. ^{14}C dating of human beta-cells.



Supplemental figure.

Pronsulin positive β-cells by flow cytometry.
The left panel indicates a representative pancreatic islet cell preparation, analyzed by cell size (forward scatter, X-axis) and cellular complexity (side scatter, Y-axis). The right panel shows a distinct and sortable proinsulin positive islet cell population. The middle panel shows unstained control preparation.
SSC-A – Side Scatter Area, FSC-A –Forward Scatter Area, PE- Phycoerythring