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# Decrease in Ins<sup>+</sup>Glut2<sup>LO</sup> β-cells with advancing age in mouse and human pancreas

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## Abstract

The presence and location of resident pancreatic β-cell progenitors is controversial. A subpopulation of insulin-expressing but glucose transporter-2-low (Ins<sup>+</sup>Glut2<sup>LO</sup>) cells may represent multipotent pancreatic progenitors in adult mouse and in human islets, and they are enriched in small, extra-islet β-cell clusters (<5 β cells) in mice. Here, we sought to identify and compare the ontogeny of these cells in mouse and human pancreata throughout life. Mouse pancreata were collected at postnatal days 7, 14, 21, 28, and at 3, 6, 12, and 18 months of age, and in the first 28 days after β-cell mass depletion following streptozotocin (STZ) administration. Samples of human pancreas were examined during fetal life (22–30 weeks gestation), infancy (0–1 year), childhood (2–9), adolescence (10–17), and adulthood (18–80). Tissues were analyzed by immunohistochemistry for the expression and location of insulin, GLUT2 and Ki67. The proportion of β cells within clusters relative to that in islets was higher in pancreas of human than of mouse at all ages examined, and decreased significantly at adolescence. In mice, the total number of Ins<sup>+</sup>Glut2<sup>LO</sup> cells decreased after 7 days concurrent with the proportion of clusters. These cells were more abundant in clusters than in islets in both species. A positive association existed between the appearance of new β cells after the STZ treatment of young mice, particularly in clusters and smaller islets, and an increased proportional presence of Ins<sup>+</sup>Glut2<sup>LO</sup> cells during early β-cell regeneration. These data suggest that Ins<sup>+</sup>Glut2<sup>LO</sup> cells are preferentially located within β-cell clusters throughout life in pancreas of mouse and human, and may represent a source of β-cell plasticity.

## Key Words

- ▶ aging
- ▶ β-cell
- ▶ Glut2
- ▶ islet
- ▶ regeneration

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## Introduction

Insulin-producing β cells to be utilized for the reversal of diabetes may be theoretically derived from endogenous pancreatic β-cell progenitors. However, the presence of resident β-cell progenitors is controversial (Dor *et al.* 2004, Seaberg *et al.* 2004, Nir *et al.* 2007, Teta *et al.* 2007, Xu *et al.*

2008, Van de Casteele *et al.* 2013), especially as the tissue origins of new β cells may differ with age (Solar *et al.* 2009, Salpeter *et al.* 2010, Stolovich-Rain *et al.* 2012), pregnancy (Parsons *et al.* 1992, Sorenson & Brelje 1997), obesity and impaired glucose tolerance (Porat *et al.* 2011,

Chen *et al.* 2014) and experimental pancreatic injury (Bonner-Weir *et al.* 2008, Inada *et al.* 2008). There is evidence for rare pancreatic progenitor cells in human and mouse islets capable of multi-lineage differentiation and which express insulin (Smukler *et al.* 2011). Importantly, these cells demonstrate low/absent glucose-transporter 2 (GLUT2), exhibit poor glucose-stimulated insulin release, have a higher endogenous rate of proliferation than mature  $\beta$  cells, and express transcription factors normally associated with early endocrine development (Smukler *et al.* 2011). Recently, we have demonstrated that Ins<sup>+</sup>Glut2<sup>LO</sup> cells were enriched within the small extra-islet pancreatic  $\beta$ -cell clusters (<5  $\beta$  cells) as compared to prototypical islets (Beamish *et al.* 2016). Small  $\beta$ -cell clusters have been previously assumed to be of minor physiological importance. The relative abundance of the cells capable of multi-lineage differentiation ranged from 1/1500  $\beta$ -cells within 7 days mouse islets as found by us (Beamish *et al.* 2016), to 1/10,000 in the adult human and mouse islet as found by others *in vitro* (Smukler *et al.* 2011). On performing immunohistochemistry of pancreas sections, it was found that up to 3.5% of all  $\beta$  cells present in the 7-day mouse pancreas express insulin but not GLUT2, the majority of them being located outside of the islets. However, only 1/200 of such cells were capable of differentiation into non- $\beta$ -cell lineages. The non-lineage plastic Ins<sup>+</sup>GLUT2<sup>LO</sup> cells then possibly representing immature and/or glucose-unresponsive  $\beta$ -cells in the developing postnatal pancreas. It was further demonstrated that the total relative proportion of these cells had decreased in adult mice. The present study therefore sought to explore the anatomical location, proliferation rate and change in abundance of these Ins<sup>+</sup>Glut2<sup>LO</sup> cells across the lifespan in both mouse and human pancreas, and the possible involvement of these cells during compensatory  $\beta$ -cell regeneration in the young mouse with diabetes.

## Materials and methods

### Human and mouse pancreas sample collection

Twenty-seven samples of human pancreas were assessed, obtained as formalin-fixed and paraffin-embedded tissue sections from the Department of Pathology at Western University, Schulich School of Medicine (London, Ontario, Canada), and the University of Chicago (Chicago, Illinois, USA), with approval from the Western University Research Ethics Board (#103167), the Institutional Review Board at the University of Chicago,

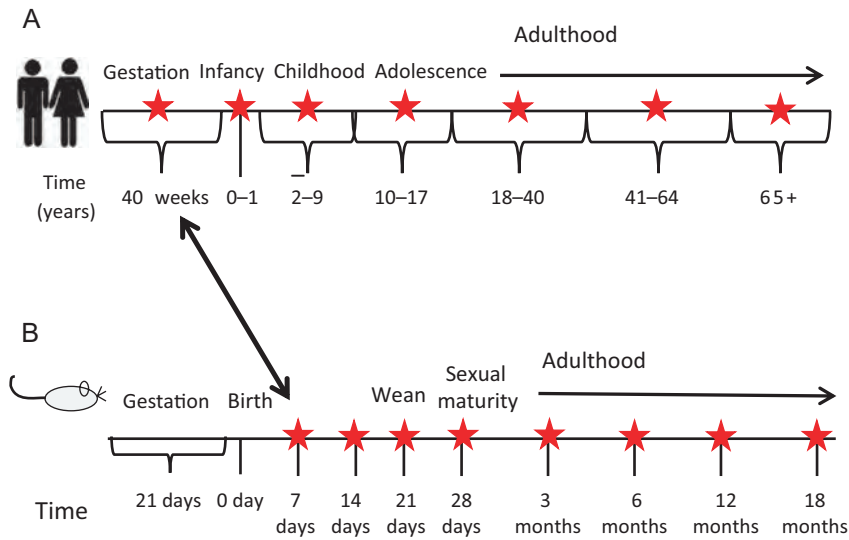
and Lawson Health Research Institute (R-12-501). Criteria for inclusion were: pancreas samples should be selected from transplant-quality cadaveric tissues taken from donors with no known metabolic disease; body weight within a BMI range of 18–25 kg/m<sup>2</sup>. Tissues utilized were representative of diverse ethnic backgrounds, and inclusive of both sexes (48% female, 13/27 samples). Paraffin blocks were chosen from mid-pancreas whenever possible, and human pancreas tissues were fixed within 6–12h of cold ischemia to control for changes post-mortem.

All animal procedures were approved by the Western University Animal Use Ethics Committee, in accordance with the Canadian Council on Animal Care guidelines. Forty-one samples of mouse pancreas from both sexes (42% female, 17/41) were collected from our existing colony with a C57Bl/6 genetic background. All mice were maintained at the Lawson Health Research Institute, St Joseph Health Care, London, Ontario, Canada with food and water provided *ad libitum*. Mice were killed by exposure to CO<sub>2</sub> and pancreata were fixed and prepared as previously described (Beamish *et al.* 2016).

Mouse and human tissues of various ages were chosen to illustrate major developmental stages, including early

**Table 1** Human pancreas samples.

Case no.	Sex	Age	Developmental stage
1	F	22 weeks G	Fetal
2	F	30 weeks G	Fetal
3	F	30 weeks G	Fetal
4	M	2 days	Infant
5	F	11 days	Infant
6	M	14 days	Infant
7	F	5 years	Child
8	M	6 years	Child
9	M	6 years	Child
10	F	6 years	Child
11	F	9 years	Child
12	M	10 years	Adolescent
13	M	11 years	Adolescent
14	F	14 years	Adolescent
15	M	19 years	Young adult
16	F	20 years	Young adult
17	F	28 years	Young adult
18	M	36 years	Young adult
19	F	41 years	Adult
20	M	50 years	Adult
21	F	52 years	Adult
22	M	54 years	Adult
23	M	56 years	Adult
24	M	65 years	Late adult
25	F	72 years	Late adult
26	M	72 years	Late adult
27	M	79 years	Late adult

**Figure 1**

Lifespan of the mouse and human. The entire lifespan was examined in human (A) and mouse (B), with the ages of pancreatic tissue donor selected to illustrate major developmental stages, including early development, infancy, childhood/weaning, puberty/sexual maturation, and adulthood, as represented by each red star. When possible, developmental points were matched. A significant proportion of mouse islet development occurs postnatally, such that 20-week gestation in humans is analogous to postnatal day 8 in mice (Pan & Wright 2011);  $n \geq 4$  mice, and  $\geq 3$  humans/star.

endocrine pancreas development, infancy, childhood, adolescence/sexual maturation, and early, mid, and late adulthood; the characteristics of human sample are shown in Table 1, and a schematic of life stages for both species is shown in Fig. 1.

### Induction of hyperglycemia by STZ

To assess the changes in the population of Ins<sup>+</sup>Glut2<sup>LO</sup> cells following the induction of diabetes, streptozotocin (STZ, Sigma) was injected on day 7 of age (100  $\mu$ g/g body weight, in 0.1M citrate buffer). Control littermates were injected with 0.1M citrate buffer (Sham). Before killing the mice, blood samples from the tail vein were taken and glucose was measured after a 4-h fast using a One Touch Ultra glucometer (Lifescan INC, Milpitas, CA, USA). Mice were killed on days 9, 14, 21, or 28 of age and pancreata were dissected and processed for histological assessment.

### Beta cell morphometry

Two pancreas sections per mouse were immunostained with insulin as described previously (Cox *et al.* 2010), and imaged and analyzed using the Northern Eclipse software (v7.0; Empix Imaging, Mississauga, ON, Canada). Total pancreas area was traced at 2.5 $\times$  magnification, and  $\beta$ -cell area was traced at 40 $\times$  magnification by insulin immunoreactivity. Beta-cell mass was calculated by multiplying the fractional  $\beta$ -cell area by the weight of the pancreas (mg).

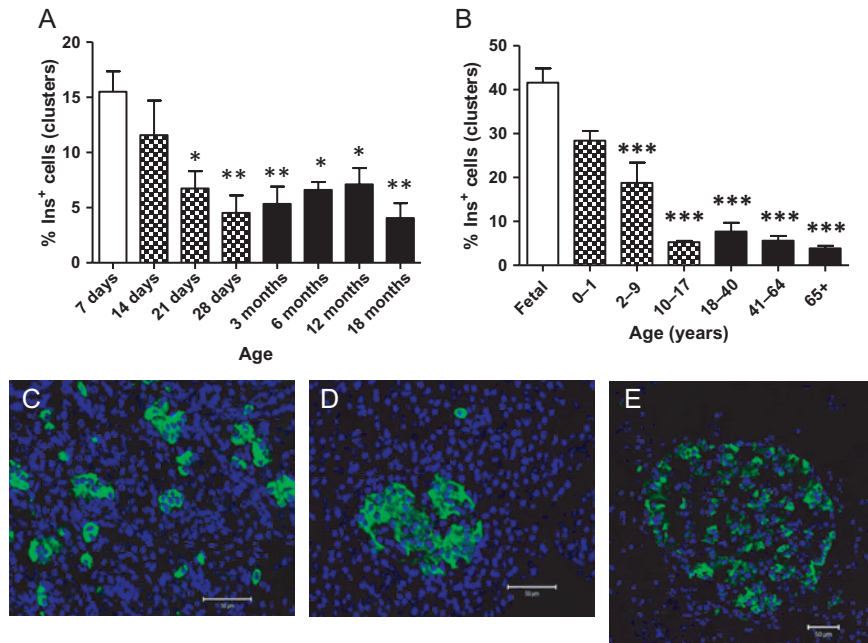
### Fluorescent immunohistochemistry

Fluorescent immunohistochemistry was performed using antibodies for insulin, GLUT2 and Ki67 as described previously (Beamish *et al.* 2016). GLUT2 immunostaining in human pancreas samples required tyramide signal amplification (TSA) (Perkin Elmer) (McCulloch *et al.* 2011). Ki67 antigen visualization required antigen retrieval at 95 $^{\circ}$ C in Tris-EDTA buffer for 30 min.

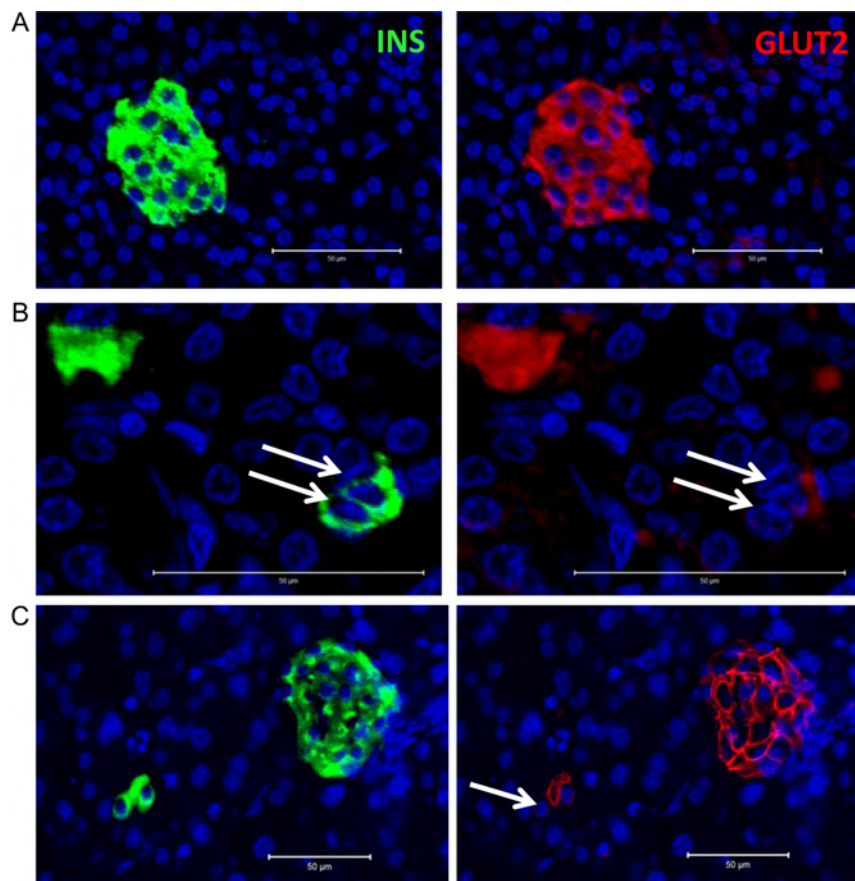
Tissue slides were imaged using a Zeiss LSM 510 Duo Vario confocal microscope (Carl Zeiss) at the Biotron Facility, Western University, and counted manually using the Zen software. Every  $\beta$  cell was counted per section, with minimum inclusion criteria being  $>300$  insulin<sup>+</sup>  $\beta$  cells/section for mice ( $\geq 2$  sections per pancreas, average 714  $\beta$  cells from at least 10 islets, average 15.6 islets per section), and  $>1200$  insulin<sup>+</sup>  $\beta$  cells/section of human pancreas (1–2 sections per pancreas from 1 block, average 1933  $\beta$  cells from at least 20 islets counted, average 48.9 islets). Controls were determined by cutting replicate sections from the same block ( $>100\mu$ m apart), and variance around the mean was  $<9\%$ .

### Statistical analysis

At least quadruplicate pancreata were analyzed for mouse and triplicate pancreata were analyzed for human samples per cohort per life stage (Table 1). Data are presented as percentage (%) mean  $\pm$  S.E.M. of insulin<sup>+</sup>  $\beta$ -cells, with statistics performed using the ANOVA-R and Tukey's post-test, or student's *t*-test, and with an acceptable level of significance of  $P < 0.05$ . Beta-cell proliferation index

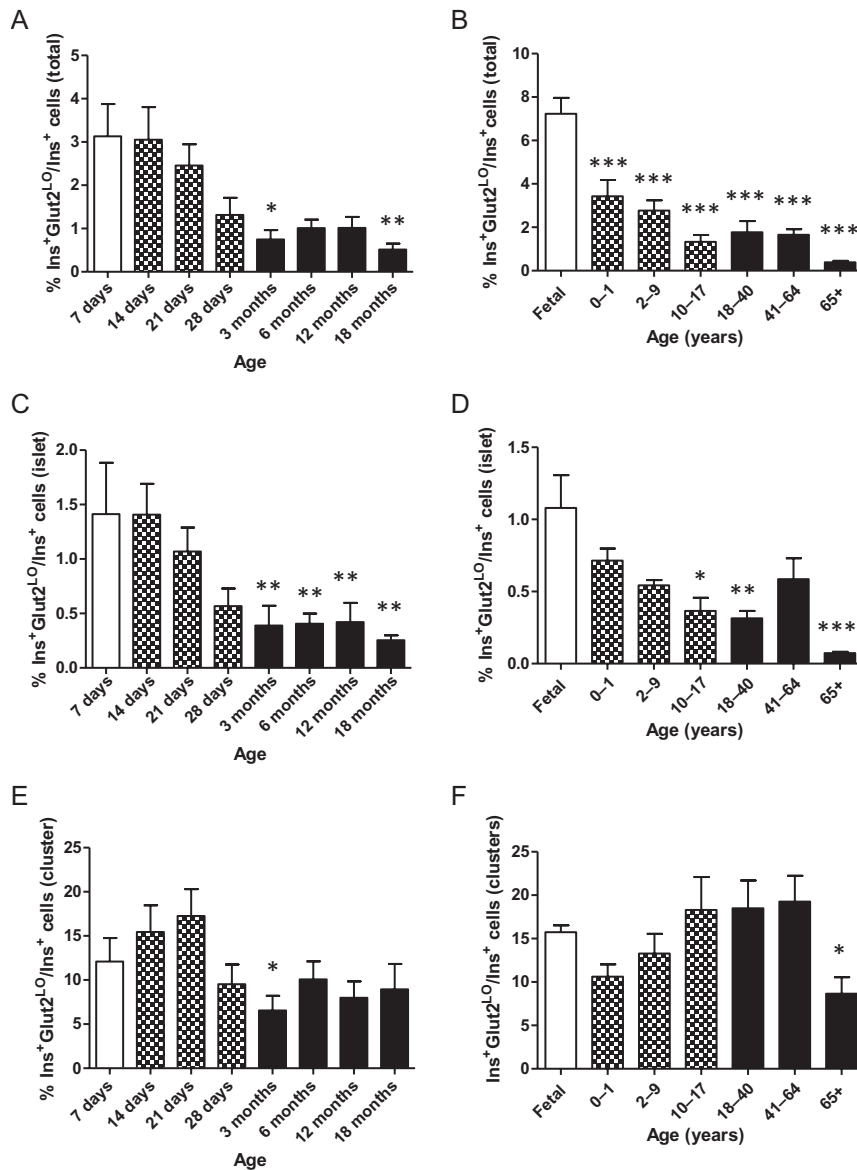
**Figure 2**

Beta cell cluster proportion decreases with age in mouse (A) and human (B) pancreas. Similar developmental points were grouped (day 7 (mouse) and fetal (human) samples, open bars; pre-weaning and sexual maturation (14–28 days) (mouse) and infancy/childhood/adolescence (human) samples, hatched bars; all adult time points, black bars). Beta cells were counted and allocated to the cluster (<5  $\beta$  cells together) or islet (>5  $\beta$  cells) compartments. (C, D, E) Representative images from human pancreas immunostained with insulin (green) illustrate changes during the maturation of islet structures, from fetal life (C, 30-week gestation female), puberty (D, 14-year-old female), and late adulthood (E, 72-year-old female). Data represent mean  $\pm$  s.e.m. proportion (%) of total  $\beta$ -cells counted; \* $P$  < 0.05, \*\* $P$  < 0.01, \*\*\* $P$  < 0.001 vs day 7 in mouse, and fetal in human;  $n$  > 3. Size bar denotes 50  $\mu$ m.

**Figure 3**

Insulin and GLUT2 immunostaining in human and mouse pancreas. Representative images of a human islet (A) and  $\beta$ -cell cluster (B) stained for insulin (green) and GLUT2 (red). Human Ins<sup>+</sup>Glut2<sup>LO</sup> cells are shown in B (arrows). Proto-typical membrane immunostaining for GLUT2 (red) and cytoplasmic insulin (green) in a mouse islet (C), as well as an Ins<sup>+</sup>Glut2<sup>LO</sup> cell in a mouse cluster (C, arrow). The non-membrane immunostaining of human GLUT2 was previously demonstrated by others (McCulloch *et al.* 2011), an effect of the TSA kit protocol. Size bar represents 50  $\mu$ m.



**Figure 4**

The Ins<sup>+</sup>Glut2<sup>LO</sup> cell proportion decreases with age. Percent Ins<sup>+</sup>Glut2<sup>LO</sup> cells relative to proportion of total  $\beta$  cells (A, B), islets (C, D), and clusters (E, F) as found in mouse (A, C, E) and human (B, D, F) pancreas. Data represent mean  $\pm$  s.e.m.; \* $P$  < 0.05, \*\* $P$  < 0.01; \*\*\* $P$  < 0.001 vs day 7 (A, C) or vs fetal (B, D, F);  $t$ -test 3 months vs 21 days (E);  $n$  > 5.

was assessed by non-linear regression. Statistical analysis was performed using the GraphPad Prism software (v. 5.01).

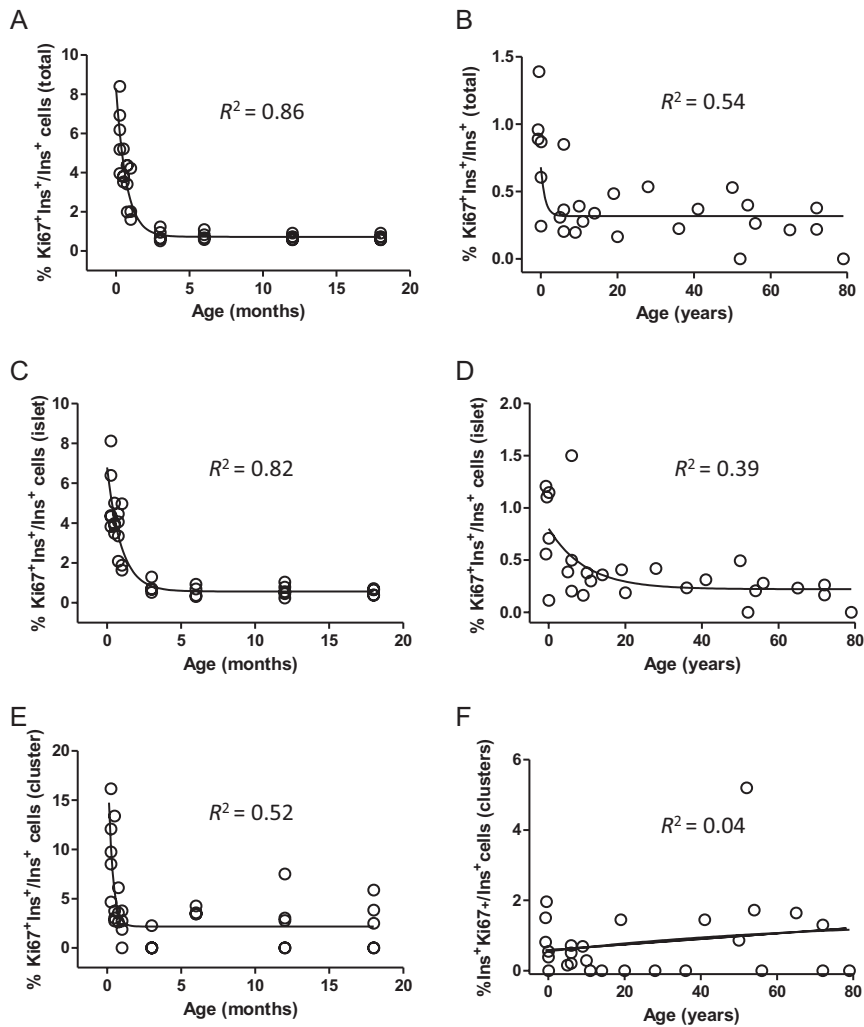
## Results

### $\beta$ -cell cluster proportion decreases with age in mouse and human pancreas

Pan and Wright showed that mice display significant postnatal development of the endocrine pancreas, such that P8 mouse pancreas is analogous to a 22-week gestation human fetal pancreas (Pan & Wright 2011). Thus, these time points were chosen to begin the analysis. At day 7,  $16.1 \pm 0.8\%$  of mouse  $\beta$  cells were

located in extra-islet clusters (Fig. 2A, white bar). This proportion decreased with advancing age, being significantly reduced by 18 months to only  $2.1 \pm 0.7\%$ ; the majority of  $\beta$  cells in the older animals being present in proto-typical islets (Fig. 2A, black bar, 18 months vs 7 day).

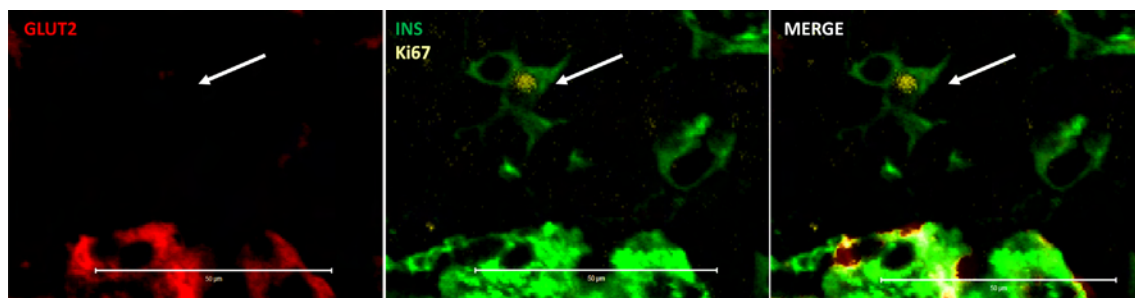
In contrast to mice,  $41.6 \pm 3.3\%$  of human  $\beta$  cells were located in clusters during the fetal life (Fig. 2B, white bar, and Fig. 2C, immunostaining positive for insulin (green)). This proportion was significantly larger than the equivalent population in mice (fetal vs 7 day,  $P$  < 0.0001,  $t$ -test). The relative proportion of  $\beta$  cells found in clusters within human fetal pancreas at childhood dropped significantly to  $18.8 \pm 4.6\%$  respectively (Fig. 2B, hatched bars), decreasing further to  $5.5 \pm 1.1\%$  at puberty

**Figure 5**

Frequency of β-cell proliferation decreases with age. Proliferation of insulin-expressing β cells by Ki67 presence is shown in total β cells (A, B), islets (C, D), and clusters (E, F) within mouse (A, C, E) and human (B, D, F) pancreas samples. Scatter graphs show the spread of data points between individual pancreata, and  $R^2$  values were calculated using non-linear regression analysis.

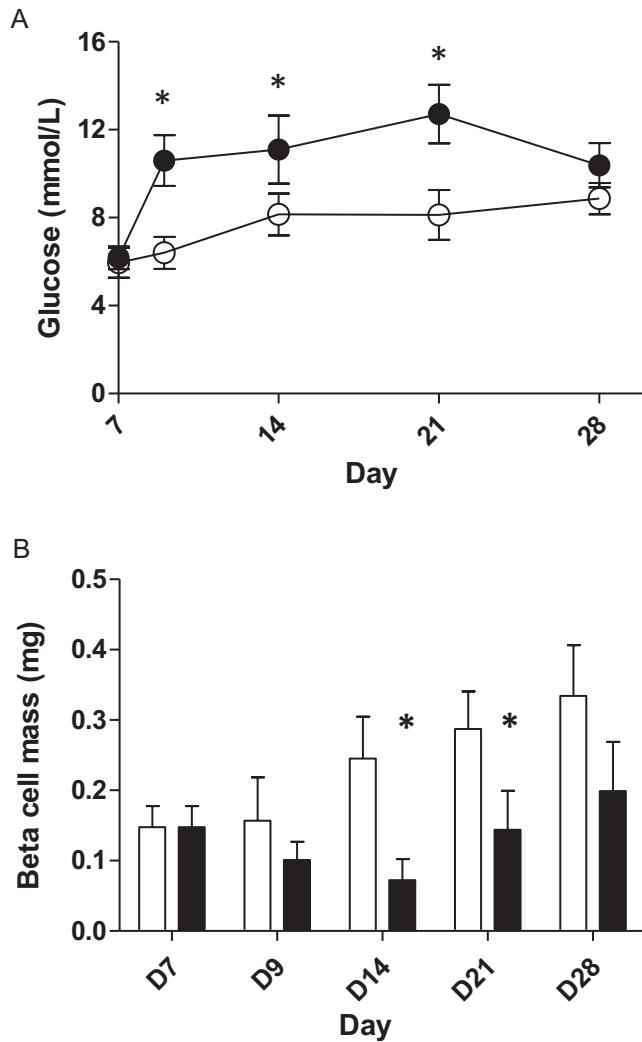
(Fig. 2B, hatched bar, fetal vs 10–17,  $P < 0.0001$ , and 2D). Thereafter, the majority of human β cells were maintained throughout the adulthood within islets (Fig. 2B, black bars, and Fig. 2D). In early adulthood, there was a significantly higher proportion of β cells present within

clusters in samples of human females than in males ( $11.0 \pm 0.3\%$  vs  $4.3 \pm 0.7\%$  respectively, 18–41 years,  $P < 0.05$ ,  $t$ -test, not shown). By late adulthood, there were very few β-cell clusters present within sections of human pancreas (Fig. 2E).

**Figure 6**

Ins<sup>+</sup>Glut2<sup>LO</sup>Ki67<sup>+</sup> cells are enriched within β-cell clusters. A β-cell cluster within a human fetal pancreas section demonstrates an Ins<sup>+</sup>Glut2<sup>LO</sup>Ki67<sup>+</sup> cell (arrow) immunostained for insulin (green, B), GLUT2 (red, A), and Ki67 (yellow, B). Size bar denotes 50 μm.



**Figure 7**

Partial regeneration of the endocrine pancreas in the young mouse following STZ administration. Blood glucose (A) and  $\beta$ -cell mass (B) following treatment with STZ on d 7 (closed circles/bars) or citrate buffer (open circles/bars) for up to 28 days. Data represent mean  $\pm$  S.E.M.; \* $P < 0.05$  vs day 7, \* $P < 0.05$  vs control for same day;  $n > 5$ .

### Ins<sup>+</sup>Glut2<sup>LO</sup> cell proportion decreases with age

We examined the population specifically of Ins<sup>+</sup>Glut2<sup>LO</sup> cells over the lifespan of both human and mouse. Sections of human pancreas demonstrated immunostaining for insulin (green) and GLUT2 (red) in an islet as shown in Fig. 3A. A representative image of human Ins<sup>+</sup>Glut2<sup>LO</sup> cells is shown in Fig. 3B (arrows) within the  $\beta$ -cell cluster compartment. The non-membrane immunostaining of human GLUT2 was also shown by others (McCulloch *et al.* 2011), an effect of the TSA protocol. For comparison, Fig. 3C demonstrates proto-typical membrane immunostaining for GLUT2 (red) and cytoplasmic insulin

(green) in a mouse islet, as well as an Ins<sup>+</sup>Glut2<sup>LO</sup> cell in an endocrine cluster (Fig. 3C, arrow).

In mice, the proportional presence of total Ins<sup>+</sup>Glut2<sup>LO</sup> cells decreased significantly from  $3.8 \pm 0.8\%$  at day 7 to  $0.6 \pm 0.2\%$  at month 3 (Fig. 4A, 7 day vs 3 month,  $P < 0.05$ ), which decreased further by 18 months (Fig. 4A,  $P < 0.001$ ). In humans, this decrease was exaggerated, with total Ins<sup>+</sup>Glut2<sup>LO</sup> cells proportionally declining after the fetal life (Fig. 4B, fetal vs all others,  $P < 0.0001$ ). There were proportionally twice as many Ins<sup>+</sup>Glut2<sup>LO</sup> cells in pancreas of human than that of mouse at the initial time point studied ( $7.8 \pm 0.9\%$  fetal vs  $3.8 \pm 0.8\%$  7 days,  $P < 0.05$ ).

The anatomical location of Ins<sup>+</sup>Glut2<sup>LO</sup> cells was then examined. Figure 4C shows that  $1.4 \pm 0.5\%$  of mouse  $\beta$  cells within islets lacked GLUT2 at day 7 (white bar), consistent with previous findings (Beamish *et al.* 2016). This proportion significantly decreased by early adulthood ( $0.4 \pm 0.2\%$ , 7 day vs 3 months,  $P < 0.001$ ), where it was maintained.

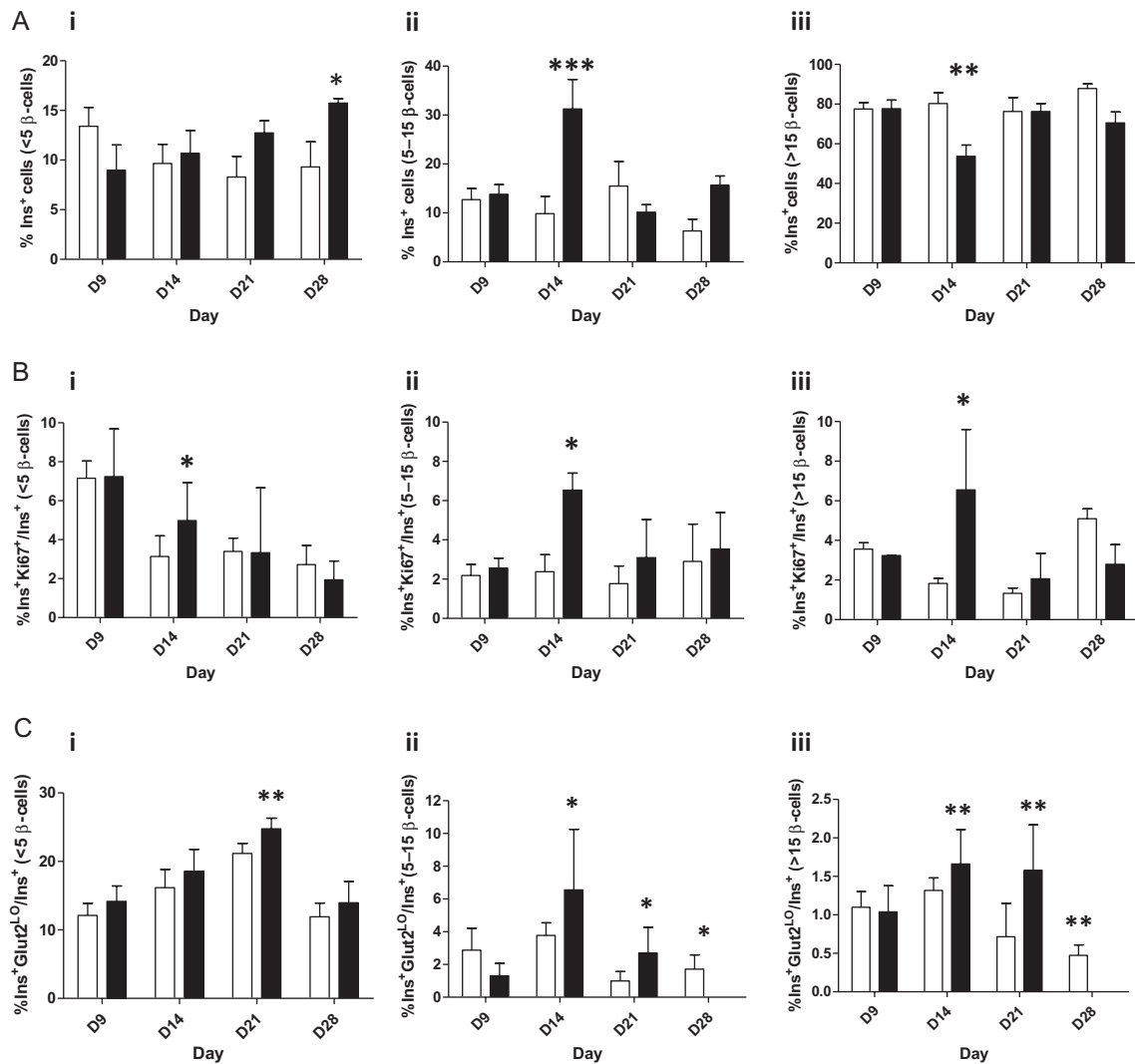
In humans,  $1.1 \pm 0.2\%$  of the  $\beta$  cells within islets demonstrated a lack of GLUT2 immunostaining during the fetal life, which decreased significantly by childhood, and further thereafter (Fig. 4D, fetal vs 10–17,  $P < 0.001$ ). There were few Ins<sup>+</sup>Glut2<sup>LO</sup> cells present in islets by late adulthood in the human pancreas (Fig. 4D,  $1.1 \pm 0.2$  vs  $0.07 \pm 0.01\%$ , fetal vs 65+,  $P < 0.0001$ ).

The proportion of Ins<sup>+</sup>Glut2<sup>LO</sup> cells within the cluster compartment was markedly different than that within the islet, with  $16.1 \pm 2.6\%$  of all mouse insulin-expressing cells lacking GLUT2 within clusters at day 7 (Fig. 4E), a peak at day 21, but a subsequent decline thereafter (Fig. 4E, *t*-test day 21 vs month 3).

In the human fetal pancreas, a similar proportion of Ins<sup>+</sup>Glut2<sup>LO</sup> cells was found in  $\beta$ -cell clusters as in the day 7 mouse ( $17.0 \pm 1.7\%$  vs  $16.1 \pm 2.6\%$ , fetal vs day 7). However, the Ins<sup>+</sup>Glut2<sup>LO</sup> cell proportional presence in clusters was maintained throughout most of life, only decreasing in late adulthood (Fig. 4F).

### $\beta$ -cell proliferation decreases with age

The proliferation index of total insulin<sup>+</sup>  $\beta$  cells was examined in mouse and human pancreas sections by Ki67 immunostaining. Beta-cell proliferation was highest at day 7 in the mouse and decreased thereafter, being maintained after weaning at  $\sim 0.6\%$  (Fig. 5A,  $R^2 = 0.86$ ). In the human pancreas, a similar trend was noted, with the highest total  $\beta$ -cell proliferation found in the fetal life, which dropped thereafter (Fig. 5B,  $R^2 = 0.54$ ). There was

**Figure 8**

The distribution of total  $\beta$ -cells, proliferating  $\beta$ -cells and Ins<sup>+</sup>Glut2<sup>LO</sup> cell abundance demonstrate ontological and anatomical differences during partial regeneration of  $\beta$ -cell mass in the young mouse. Animals were treated with STZ on d 7 (closed bars) or vehicle alone (open bars) and subsequently examined on days 9, 14, 21 or 28. The changes in the percentage of Ins<sup>+</sup> cells in clusters (<5  $\beta$ -cells, i), small islets (5–15  $\beta$ -cells, ii) or larger islets (>15  $\beta$ -cells, iii) are shown in A, the percentage of Ki67<sup>+</sup>-labeled Ins<sup>+</sup> cells in B, and the percentage of Ins<sup>+</sup>Glut2<sup>LO</sup> cells relative to all Ins<sup>+</sup> cells in C. Within  $\beta$ -cell clusters the relative number of Ins<sup>+</sup> cells decreased with age in control, but not STZ-treated mice. In clusters from control mice the percent Ins<sup>+</sup>Ki67<sup>+</sup> cells significantly decreased with age, but not so after STZ treatment, whilst a transient increase in Ins<sup>+</sup>Ki67<sup>+</sup> cells occurred in small and large islets at day 14 after STZ. The proportional presence of Ins<sup>+</sup>Glut2<sup>LO</sup> cells in clusters did not differ between control or STZ-treated mice, but were increased after STZ at day 14 in both small and large islets. Data represent mean  $\pm$  s.e.m.; \* $P$ <0.05, \*\* $P$ <0.01, \*\*\* $P$ <0.001 vs day 9;  $n$ >5.

a significant variability in the prenatal and perinatal samples, ranging from 0.9% to 2.3% and 0.2% to 0.9%, respectively (Fig. 5B).

Proliferation of  $\beta$  cells within islets followed similar patterns as was found for total cells, with rates of replicating  $\beta$ -cells highest in mice at day 7, and which decreased significantly by early adulthood (Fig. 5C,  $R^2=0.86$ ). In the human pancreas, a comparable trend was found, with proliferation of  $\beta$  cells being highest during the fetal life, and decreasing thereafter (Fig. 5D,  $R^2=0.39$ ). There was

considerable variability in the  $\beta$ -cell proliferation rate within the islet in the fetal and infant samples, ranging from 0.6% to 2.2% and 0.1% to 1.2%, respectively. After adolescence the presence of  $\beta$ -cell proliferation within islets was low but detectable at less than 0.5% of  $\beta$ -cells (Fig. 5D).

Within clusters,  $\beta$ -cell proliferation dropped significantly in mice from day 7 to day 28 (Fig. 5E,  $R^2=0.52$ ). Interestingly, this trend was not demonstrated in human  $\beta$ -cell clusters, which showed variable proliferation throughout life (Fig. 5F,  $R^2=0.04$ ).

We have previously shown that Ins<sup>+</sup>Glut2<sup>LO</sup> cells have a higher rate of proliferation than do GLUT2-expressing insulin-positive cells in the neonatal life, particularly in extra-islet clusters (Beamish *et al.* 2016). We extended this analysis throughout life for both mouse and human pancreas using Ki67 as a marker of DNA synthesis. Consistent with our previous findings,  $8.4 \pm 4.2\%$  of proliferating  $\beta$  cells lacked GLUT2 in the islet, and  $18.8 \pm 4.9\%$  in the clusters at day 7 in the mouse. At the equivalent age in humans (fetal), 2/3 of pancreas samples exhibited cells with an Ins<sup>+</sup>Glut2<sup>LO</sup>Ki67<sup>+</sup> phenotype ( $3.3 \pm 5.7\%$  within islets vs  $16.67 \pm 15.74\%$  within clusters,  $7.9 \pm 6.9\%$  total). This distribution was maintained during infancy (0–1 year,  $14.1 \pm 17.2\%$  total, 2/3 samples), and childhood (2–9 years,  $6.9 \pm 10.9\%$  total, 2/4 samples). Interestingly, 83.3% of these cells were found in clusters in the human pancreas, and a similar 78.8% in the mouse pancreas. After day 7 in the mouse and childhood in the human, the proportion of Ins<sup>+</sup>Glut2<sup>LO</sup>Ki67<sup>+</sup> cells diminished greatly, occurring rarely at all subsequent ages, and predominantly in clusters. A representative Ins<sup>+</sup>Glut2<sup>LO</sup>Ki67<sup>+</sup> cell within a fetal human pancreas section is shown in Fig. 6 (arrow) immunostained for insulin (green), GLUT2 (red) and Ki67 (yellow).

### Changes in Ins<sup>+</sup>Glut2<sup>LO</sup> cell present during the $\beta$ -cell mass regeneration after neonatal STZ exposure

To assess the possible contribution of Ins<sup>+</sup>Glut2<sup>LO</sup> cells to  $\beta$ -cell regeneration after the induction of diabetes, neonatal (day 7) mice were treated with STZ. Analysis was further sub-divided into three  $\beta$ -cell grouping-size categories (clusters, <5  $\beta$  cells; small islets, 5–15  $\beta$  cells; and large islets, >15  $\beta$  cells), so as to identify dynamic changes in the distribution during the  $\beta$ -cell regeneration in the early post-natal mouse pancreas. Relative hyperglycemia was noted 2 days after the STZ treatment and persisted until day 21, but glucose levels were similar in STZ-treated and control mice by day 28 (Fig. 7A). The presence of hyperglycemia correlated with a 70% reduction of  $\beta$ -cell mass after the STZ treatment compared to that of control animals by day 14 (Fig. 7B). However,  $\beta$ -cell mass was partially recovered and was not significantly different between STZ-treated and control mice by day 28.

The proportion of Ins<sup>+</sup> cells was determined within clusters, small islets, or larger islets for STZ-treated or control mice (Fig. 8A). Within clusters, the relative number of Ins<sup>+</sup> cells in control mouse pancreas significantly decreased with age (Fig. 8A-i). This was not seen for clusters

from STZ-treated mice, and at day 28 the percentage of Ins<sup>+</sup> cells was significantly higher than that in controls. No age-related changes in the percentage presence of Ins<sup>+</sup> cells within small or larger islets were observed in control mice (Fig. 8A-ii and iii). At day 14 in STZ-treated animals there was a significant decrease in the percentage of Ins<sup>+</sup> cells present in larger islets, as would be expected and in agreement with a reduced  $\beta$ -cell mass, but a significant increase in percentage of Ins<sup>+</sup> cell present in small islets in the same tissues was observed.

We subsequently calculated the proportion of Ins<sup>+</sup> cells undergoing DNA synthesis (Ki67<sup>+</sup>) from the same pancreata (Fig. 8B). In clusters from control mice, the percentage of Ins<sup>+</sup>Ki67<sup>+</sup> cells significantly decreased with age, but not so after the STZ treatment (Fig. 8B-i). Within small and larger islets there was a significant but transient increase in percentage of Ins<sup>+</sup>Ki67<sup>+</sup> cells at day 14 after the STZ treatment compared to control mice involving both Ins<sup>+</sup>Glut2<sup>LO</sup> and Ins<sup>+</sup>Glut2<sup>HI</sup> cell populations (Fig. 8B-ii and iii).

The abundance of specifically Ins<sup>+</sup>Glut2<sup>LO</sup> cells was compared with all Ins<sup>+</sup> cells (Fig. 8C). In agreement with Fig. 4, the proportional presence of Ins<sup>+</sup>Glut2<sup>LO</sup> cells in control mice increased within clusters until day 21, before declining (Fig. 8C-i), and did not significantly differ after the STZ treatment. However, in both small and large islets there was a significant increase in the proportion of Ins<sup>+</sup>Glut2<sup>LO</sup> cells at day 14 that was absent in control mice (Fig. 8C-ii and iii). Thereafter, the population disappeared by day 28 in small and large islets (Fig. 8C-ii and iii) but was maintained in clusters (Fig. 8C-i).

These findings suggest that Ins<sup>+</sup>Glut2<sup>LO</sup> cells are not destroyed by STZ, as would be expected since STZ enters cells through GLUT2 transporters (Schnedl *et al.* 1994, Wang & Gleichmann 1998). Ins<sup>+</sup> cells were relatively more abundant in clusters and in smaller islets after the STZ treatment; and this was accounted for in part by a greater presence of Ins<sup>+</sup>Glut2<sup>LO</sup> cells in islets in advance of  $\beta$ -cell regeneration. This increase in Ins<sup>+</sup>Glut2<sup>LO</sup> cell abundance was associated with increased  $\beta$ -cell proliferation and suggests that they are dynamically linked to  $\beta$ -cell renewal. It also suggests that the Ins<sup>+</sup>Glut<sup>LO</sup> cells matured and coalesced from small  $\beta$ -cell clusters into islets.

## Discussion

Over the mammalian lifespan, the endocrine pancreas undergoes substantial changes in terms of cellular maturity, function, replication ability, and cell

macro-organization (Steiner *et al.* 2010). Previous studies have shown that a population of Ins<sup>+</sup>Glut2<sup>LO</sup>, lineage-plastic putative progenitor cells exist in adult human and mouse islets (Smukler *et al.* 2011), which are proportionally increased in the small, extra-islet clusters of  $\beta$  cells of young mice (Beamish *et al.* 2016). Hence, we sought to determine if this population was also present in similar extra-islet  $\beta$ -cell clusters in the human pancreas, as well as to assess how this cell population changes with age. We have found that the young human pancreas contains a significant proportion of Ins<sup>+</sup>Glut2<sup>LO</sup> cells both within and outside of the islet, and while this population decreases with advancing age in both species, such cells are still present in older adults.

Novel insights have emerged from this study regarding the pancreas maturation and remodelling at key developmental times such as birth and adolescence. A significant proportion of endocrine cell mass is present in the young human pancreas outside of proto-typical islet structures, with nearly 45% of  $\beta$  cells present in small clusters (<5  $\beta$  cells) during the fetal life, ~30% during infancy, and ~20% during childhood. This trend was noted previously (Meier *et al.* 2008), but not quantified *per se*. It could be questioned that if islets were sectioned at the periphery, then cell grouping size would be theoretically equivalent to that of the clusters; however, this would be a relatively rare event, the same bias would be applied consistently, and if that was true, then these data would not change with age as seen here. Although it may be similarly stated that the assignment of 5 or fewer  $\beta$  cells connoting a cluster is arbitrary, isolated human islets in transplant procedures are standardized to islet equivalent (IEQ) sizing, with a diameter of 150  $\mu$ m, or ~1500 cells in total (1560  $\pm$  20 cells) (Pisania *et al.* 2010). Including basic assumptions of spherical shape, mathematically calculated as  $V = 4/3\pi r^2$ , where  $r$  = the number of cells, and 1140/1560 of those cells being  $\beta$  cells, this volume calculation yields a standard islet diameter of 14.2 cells, or 13.0  $\beta$  cells, thus well above our 5  $\beta$ -cell restriction (which necessarily has a maximum radius of 2.5  $\beta$ -cells). Indeed, there is a lack of consistency in the nomenclature and size metrics in the literature when quantifying small  $\beta$ -cell aggregates or clusters. For instance, an 'islet' may be identified within pancreas sections as 4 (insulin-positive)  $\beta$ -cells (together) (Meier *et al.* 2008, Jo *et al.* 2011), in juxtaposition to ' $\beta$ -cell clusters' variably constituting <10  $\beta$  cells (Dor *et al.* 2004), or <20  $\beta$  cells (Van de Castele *et al.* 2013) when quantified by total cell number, or alternatively <50  $\mu$ m (Chintinne *et al.* 2010, 2012) or <60  $\mu$ m (Kilimnik *et al.*

2012) when determined by the  $\beta$ -cell area. Within the confines of our model, the proportion of  $\beta$  cells present as single cells/small clusters (i.e., <5  $\beta$ -cells) outside of the islet only declines substantially during puberty, and is thereafter maintained at ~5% in adults. This dramatic decrease was not seen in mouse, which showed a more gradual decline after weaning. However, in both species,  $\beta$ -cell proliferation in the cluster compartment was extremely low (mouse)/absent (human) by this stage of development, which may indicate a shift in cell plasticity. This low rate of  $\beta$ -cell proliferation during adolescence has been noted previously (Meier *et al.* 2008), although not specifically in reference to  $\beta$  cells outside of islets. It is of interest to note that the incidence of type 1 diabetes in humans peaks between 10 and 14 years (i.e., puberty/adolescence) (Gan *et al.* 2012), which coincides with the developmental shift to larger islets and fewer  $\beta$ -cell clusters, as demonstrated here. We propose that in addition to the demands of somatic growth necessary at this age (Meier *et al.* 2008) and the relative insulin resistance associated with puberty as measured by homeostatic model assessment (HOMA) (Jeffery *et al.* 2012), adolescence may be an especially vulnerable window for diabetes if  $\beta$ -cell expansion from small clusters is a declining option.

It has been shown by others that the young human pancreata contain a higher proportion of small islets relative to large islets than that is found in mice (Kilimnik *et al.* 2012). Intriguingly, there is evidence that small islets are functionally superior to large ones after transplant (Lehmann *et al.* 2007, Suszynski *et al.* 2014). Although the physiological relevance of extra-islet  $\beta$  cells in the human pancreas remains to be determined, the implications of this data are clear: efforts should be made to retain all  $\beta$  cells, especially within clusters, for use in human islet transplant preparations. It was recently shown that islet transplants from young donors generate superior results, although islet yields are poorer compared to those from adults (Meier *et al.* 2014). Data shown here provide further support for the utilization of islets specifically from young donors, which we contend to contain a higher proportion of Ins<sup>+</sup>Glut2<sup>LO</sup>  $\beta$ -cell progenitors present within small islets/ $\beta$ -cell clusters. In mice, the difference in the Ins<sup>+</sup>Glut2<sup>LO</sup> cell population within  $\beta$ -cell clusters during the immediate postnatal time period and up to day 21 is consistent with data indicating a shift in  $\beta$ -cell plasticity after a change in diet at weaning (Stolovich-Rain *et al.* 2015).

In the human pancreas the proportional presence of  $\beta$  cells undergoing replication was reported to be 3.2%

at the fetal life, 2.5% at birth, and reduced to 0.1% after 6 months of age (Kassem *et al.* 2000, Meier *et al.* 2008, 2010). Higher rates of proliferation were noted in small islets and  $\beta$  cells found scattered throughout the pancreas, and decreased with age, leading to the conclusion that larger islets were created both from proliferation as well as from the coalescence of small clusters (Jo *et al.* 2011), and that this cluster aggregation decreased with age (Jeon *et al.* 2009). By adulthood, there is consensus that  $\beta$ -cell proliferation is extremely low, at ~0.1–0.5% (Müller *et al.* 1990, Meier *et al.* 2008, Cnop *et al.* 2010, Perl *et al.* 2010). Rates of  $\beta$ -cell proliferation in rodents have been shown to be similar to those in humans, with 1–3%  $\beta$  cells replicating at any time in the young rat (Finegood *et al.* 1995), but only ~0.2% by adulthood (Müller *et al.* 1990, Kushner 2006). Recently this  $\beta$ -cell proliferation consensus was challenged, with evidence that the length of time post-mortem that the tissue was exposed to ischemia negatively affected the number of cells staining positively for Ki67, thus artificially minimizing the proliferation rate of adult human  $\beta$  cells (Sullivan *et al.* 2015). Our results are consistent with the observed variability between the young human pancreata; however, we have shown the rate of  $\beta$ -cell proliferation in mouse and human in adulthood to be slightly higher than that reported by others, which may be explained by the relatively high number of cells examined in this study, the stringent inclusion criteria of brief human cadaveric cold ischemia time prior to fixation, as well as the inclusion of small endocrine clusters. Although we acknowledge that the Ki67 labelling rate in humans may be underestimated, the inter-sample variability in adult tissues was not high, thus lending validity to these findings. Moreover, our human data was comparable to findings in mice, whose pancreata were fixed immediately after dissection.

An important question is whether Ins<sup>+</sup>Glut2<sup>LO</sup> cells have a physiological function at times of metabolic stress or pancreas damage. It is conceivable that  $\beta$  cells undergo a normal cycle of developmental transition in glucose-responsiveness, expressing Glut2 in high and low proportion. Similarly, these cells could be dismissed as simply senescent  $\beta$ -cells. Neither of those alternative hypotheses would support the evidence that the population of Ins<sup>+</sup>Glut2<sup>LO</sup> cells decreases with age, or transiently increases after STZ ablation, as demonstrated here. Other have shown that human Ins<sup>+</sup>Glut2<sup>LO</sup>  $\beta$  cells within islets obtained from pancreas donors with diabetes demonstrated a higher differentiation and proliferative capacity *in vitro* than that from equivalent cell populations of non-diabetic donors (Razavi *et al.* 2014). That study did

not examine the contribution of Ins<sup>+</sup>Glut2<sup>LO</sup>  $\beta$  cells within clusters, which may represent a larger source of such cells *in vivo*, nor examine how the population changes with age. These cells may be represented within the small clusters of  $\beta$  cells retained in the pancreata of older 'Medalist' donor tissues (Keenan *et al.* 2010). These data further substantiate mounting evidence of  $\beta$ -cell heterogeneity, with newly identified markers being the variable presence of the antigens ST8SIA and CD9 which stratify  $\beta$ -cells into 4 subsets (Dorrell *et al.* 2016), the vesicular monoamine transporter 2 (VMAT2) (Saisho *et al.* 2008), the Wnt/planar cell polarity (PCP) gene *Flattop* (*Fltp*) (Bader *et al.* 2016), among others reported (Roscioni *et al.* 2016). Our data strongly align with evidence provided by Bader *et al.*, showing that immature  $\beta$ -cells can be identified by the lack of *Fltp*, are more proliferative, and have a significant reduction in *Slc2a2* (GLUT2) gene expression.

We and others have shown that young rodents retain the capacity to partially regenerate  $\beta$ -cell mass after STZ ablation, but this regenerative potential is mitigated in adulthood (Bonner-Weir *et al.* 1981, Portha *et al.* 1989, Wang *et al.* 1996, Thyssen *et al.* 2006, Tschen *et al.* 2009, Chamson-Reig *et al.* 2010, Cox *et al.* 2010, Marchand *et al.* 2010, Nicholson *et al.* 2010). This loss of regenerative capacity coincides with the decline seen here in both Ins<sup>+</sup>Glut2<sup>LO</sup> cell abundance, and a change in their anatomical location. When we examined the dynamic changes to Ins<sup>+</sup>Glut2<sup>LO</sup> cells during the compensatory  $\beta$ -cell regeneration that occurs after STZ administration to young mice, we found a positive association between the generation of new  $\beta$  cells, particularly in clusters and smaller islets, and an increased proportional presence of Ins<sup>+</sup>Glut2<sup>LO</sup> cells during the initial stages of regeneration. This could be consistent with an expansion in number and subsequent maturation of Ins<sup>+</sup>Glut2<sup>LO</sup> cells into functional  $\beta$  cells as a contributing mechanism to an adaptive increase in  $\beta$ -cell mass, as proposed by Szabat and coworkers who showed that  $\beta$  cells can transition from Pdx1<sup>+</sup>Ins<sup>LO</sup> to Pdx1<sup>+</sup>Ins<sup>+</sup> cells (Szabat *et al.* 2009). This is further supported with evidence from human Ins<sup>+</sup>Glut2<sup>LO</sup> cells *in vitro*, which showed that diabetes biased the maturation of those cells into a functional phenotype (Razavi *et al.* 2014), as well as aspects of  $\beta$ -cell lineage tracing experiments of induced pancreatic injury and regeneration (Liu *et al.* 2010).

Determining the role of resident Ins<sup>+</sup>Glut2<sup>LO</sup> cells during other pathological circumstances or in times of increased metabolic demand will further elucidate a potential role for this cell population in human health and pancreas development.



### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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