Western University
Scholarship@Western

Paediatrics Publications | Paediatrics Department

4-18-2016

Insulin-positive, Glut2-low cells present within mouse pancreas exhibit lineage plasticity and are enriched within extra-islet endocrine cell clusters

Christine A. Beamish
Western University

Brenda J. Strutt
Western University

Edith J. Arany
Western University, earany@uwo.ca

David J. Hill
Western University

Follow this and additional works at: https://ir.lib.uwo.ca/paedpub

Citation of this paper:
Beamish, Christine A.; Strutt, Brenda J.; Arany, Edith J.; and Hill, David J., "Insulin-positive, Glut2-low cells present within mouse pancreas exhibit lineage plasticity and are enriched within extra-islet endocrine cell clusters" (2016). *Paediatrics Publications*. 671.
https://ir.lib.uwo.ca/paedpub/671

Islets

ISSN: 1938-2014 (Print) 1938-2022 (Online) Journal homepage: https://www.tandfonline.com/loi/kisl20

Insulin-positive, Glut2-low cells present within mouse pancreas exhibit lineage plasticity and are enriched within extra-islet endocrine cell clusters

Christine A. Beamish, Brenda J. Strutt, Edith J. Arany & David J. Hill

To cite this article: Christine A. Beamish, Brenda J. Strutt, Edith J. Arany & David J. Hill (2016) Insulin-positive, Glut2-low cells present within mouse pancreas exhibit lineage plasticity and are enriched within extra-islet endocrine cell clusters, Islets, 8:3, 65-82, DOI: 10.1080/19382014.2016.1162367

To link to this article: https://doi.org/10.1080/19382014.2016.1162367

View supplementary material

Published online: 20 Apr 2016.

Submit your article to this journal

Article views: 1214

View related articles

View Crossmark data

Citing articles: 13 View citing articles

Full Terms & Conditions of access and use can be found at
https://www.tandfonline.com/action/journalInformation?journalCode=kisl20

ISLETS
2016, VOL. 8, NO. 3, 65–82
http://dx.doi.org/10.1080/19382014.2016.1162367

Taylor & Francis
Taylor & Francis Group

RESEARCH PAPER

Insulin-positive, Glut2-low cells present within mouse pancreas exhibit lineage plasticity and are enriched within extra-islet endocrine cell clusters

Christine A. Beamish[a,d,e], Brenda J. Strutt[b,e], Edith J. Arany[b,c,d,e], and David J. Hill[a,b,d,e]

[a]Department of Physiology and Pharmacology, Western University, London, ON, Canada; [b]Department of Medicine, Western University, London, ON, Canada; [c]Department of Pathology, Western University, London, ON, Canada; [d]Children's Health Research Institute, London, ON, Canada; [e]Lawson Health Research Institute, St Joseph Health Care, London, ON, Canada

ABSTRACT

Regeneration of insulin-producing β-cells from resident pancreas progenitors requires an understanding of both progenitor identity and lineage plasticity. One model suggested that a rare β-cell sub-population within islets demonstrated multi-lineage plasticity. We hypothesized that β-cells from young mice (postnatal day 7, P7) exhibit such plasticity and used a model of islet dedifferentiation toward a ductal epithelial-cell phenotype to test this theory. RIPCre;Z/AP$^{+/+}$ mice were used to lineage trace the fate of β-cells during dedifferentiation culture by a human placental alkaline phosphatase (HPAP) reporter. There was a significant loss of HPAP-expressing β-cells in culture, but remaining HPAP^{+} cells lost insulin expression while gaining expression of the epithelial duct cell marker cytokeratin-19 (Ck19). Flow cytometry and recovery of β-cell subpopulations from whole pancreas *vs.* islets suggest that the HPAP^{+}Ck19^{+} cells had derived from insulin-positive, glucose-transporter-2-low (Ins^{+}Glut2^{LO}) cells, representing 3.5% of all insulin-expressing cells. The majority of these cells were found outside of islets within clusters of <5 β-cells. These insulin^{+}Glut2^{LO} cells demonstrated a greater proliferation rate *in vivo* and *in vitro* as compared to insulin^{+}Glut2^{+} cells at P7, were retained into adulthood, and a subset differentiated into endocrine, ductal, and neural lineages, illustrating substantial plasticity. Results were confirmed using RIPCre;ROSA- eYFP mice. Quantitative PCR data indicated these cells possess an immature β-cell phenotype. These Ins^{+}Glut2^{LO} cells may represent a resident population of cells capable of forming new, functional β-cells, and which may be potentially exploited for regenerative therapies in the future.

ARTICLE HISTORY
Received 2 February 2016
Revised 25 February 2016
Accepted 1 March 2016

KEYWORDS
β-cell; differentiation; duct; Glut2; islet; pancreas; plasticity; progenitor cell

Introduction

It has been proposed that all new β-cells in the postnatal pancreas arise from pre-existing β-cells, both across the lifespan and during adaptive responses to physiological metabolic demands.[1,2] However, a contribution from "facultative" progenitor cells, which may require a stimulus of injury or pathologic metabolic stress, dependent on the maturational status of cells, is also potentially important.[3] This is supported by the reported presence of progenitor β-cells with multi-lineage plasticity in both adult mouse and human islets.[4] These rare, pancreas-derived, multipotential progenitor (PMP) cells express insulin, albeit at reduced proportions as compared to proto-typical β-cells, and display β-progenitor characteristics, with increased expression of *Nkx6.1, Pdx1*, and *Ngn3*, and importantly, a lack of *Glut2*. [5] These PMPs were identified after culturing dispersed islets in clonal sphere-forming conditions, generating rare PMP sphere colonies which displayed features of both neural and pancreatic precursors.[4,5]

In the embryo, pancreatic β-cells develop from bipotent trunk cells in the ductal epithelium and therefore share a common precursor population with duct cells. [6] Islets have been demonstrated to undergo an apparent dedifferentiation *in vitro* to an epithelial, ductal phenotype as a model for β-cell plasticity.[7–9] Dedifferentiation is defined here as the loss of mature and functional characteristics from a partially or terminally differentiated cell type, and which, in some cases, may occur prior to trans-differentiation, or the change from one differentiated phenotype to

CONTACT David J. Hill david.hill@lhrionhealth.ca Lawson Health Research Institute, St. Joseph's Health Care, 268 Grosvenor Street, London, Ontario N6A 4V2, Canada

Supplemental material data for this article can be accessed on the publisher's website.

© 2016 Taylor & Francis Group, LLC

another.[10,11] A minority compartment of β-cells within islets which demonstrated the capacity for dedifferentiation *in vitro* has been reported previously, with 1.4%[12] and 0.5%[13] of β-cells dedifferentiating *in vitro* from mouse, and <5% of human β-cells dedifferentiating *in vitro* using similar culture conditions,[7] although none of these studies characterized the rare plastic cells. These low rates of dedifferentiation might indicate that only rare β-cells are capable of survival and phenotypic transition, and potentially suggesting β-cell heterogeneity. We have further examined the potential role of postnatal PMP-like cells in the plasticity of β-cells using an *in vitro* approach. We hypothesized that plasticity of β-cells would be greatest in early life and that postnatal day 7 (P7) would be an optimal age to identify and study resident PMP-like cells. Our strategy was to use RIPCre;Z/AP$^{+/+}$ [14,15] and RIPCre;ROSA-eYFP$^{+/+}$ transgenic mice where the majority of β-cells are genetically tagged with a human placental alkaline phosphatase (HPAP) and enhanced yellow fluorescent protein (eYFP) reporters, respectively, to investigate and characterize the identity, location, and fate of β-cells that demonstrate phenotypic plasticity.

Methods

Animals

All animal experimentation was approved by the Western University Animal Use Ethics Committee, in accordance with the Canadian Council on Animal Care. Rat insulin promoter (RIP) Cre$^{+/+}$ mice (*B6.Cg-Tg(Ins2-cre)25Mgn/J*)[14] (Jackson Laboratories, strain 3573) were crossed with a reporter mouse strain containing an ALPP/HPAP gene construct (*CAG-Bgeo/ALPP)1Lbe/J*) (LacZ/HPAP)[15] (Jackson Laboratories, strain 3919). Genotyping of RIPCre;Z/AP$^{+/+}$ mice was accomplished by PCR amplification using primers for the RIPCre (FW - gcggtctggcagtaaaaactatc and RV-gtgaaacagcattgctgtcactt, 100 bp) and Z/AP (FW-ccgcttcccatatgtggctctgtcc and RV – gcatgagctcagtgcggttccacac, 548bp) transgenes from P5 pups. LacZ^{-} littermates were used as controls. A second reporter strain containing a yellow fluorescent protein gene construct (*B6.129X1-Gt(ROSA)26Sortm1(EYFP)Cos/J*) (R26R- EYFP) were used to confirm findings (Jackson Laboratories, strain 6148).

Islet dedifferentiation

Reagents were obtained from Life Technologies unless otherwise stated. Islets were isolated from P7 mice as described,[16,17] and cultured in dedifferentiation medium containing DMEM/F-12 (Gibco,12400–024), 10% FBS (12483020), 1 μg/mL insulin (Sigma Chemical, I0516), 100 ng/mL cholera toxin (Cedarlane Labs, 101B(LB)), 1 μM dexamethasone (Sigma, D4902), 0.1 mg/mL soybean trypsin inhibitor (Gibco, 17075–029), and 10 ng/mL epidermal growth factor (EGF) (Sigma, E4127).[7–9] Whole islets were plated 25/well in a 6-well plate coated with 1 mL type I (rat-tail) collagen. All cell culture medium contained 100 mmol/L penicillin/streptomycin (15140–122) and 10 mmol/L fungizone (15290–018) and was changed every 2–3 d.

Redifferentiation of dedifferentiated cells

After 1 week in ductal dedifferentiation culture, collagen was digested using Collagenase XI (Sigma, C9263), and the cell monolayer dispersed into single cell suspension using Dispase (Corning, IWR-CACB354235) and Accutase (BD Biosciences, SCR005 (CH)). Cells were cultured for endocrine redifferentiation in medium previously shown to promote endocrine maturation as described by Russ *et al*, [18] and similar to Seaberg *et al*, [4] consisting of DMEM/F12, 25 mmol/L D-glucose, 1% bovine serum albumin (BSA) (Sigma, A7906), insulin/transferring/selenium (ITS) (Sigma, I1884), N2 (17502–048) and B27 supplements (17504–044), 10 mmol/L nicotinamide (Sigma, N0636), 8 nm exendin-4 (a kind gift from Dr. Savita Dhanvantari, Lawson Health Research Institute), and 8 nm activin-A (Cedarlane Labs, CL2950R-10), in 6-well tissue culture plates coated with Matrigel (1:25 dilution) (BD Biosciences, 354234).

Fluorescent immunohistochemistry

Pancreata were fixed in 4% (v/v) paraformaldehyde (PFA) (Electron Microscopy Sciences, 15710–5) for 24 h, washed in PBS for 24 h, and suffused in 30% sucrose overnight prior to embedding in OCT (Tissue Tek, 4583) and frozen at −80°C. Pancreata were sectioned at 8 nm. Cultured cells were dispersed from monolayers and affixed to glass-bottom dishes (MatTek Corp, P35-0-^{14}C) pre-adsorbed with diluted Cell-Tak (BD Biosciences, CACB354240), then fixed with 4% PFA for 15 min and stored in PBS. The rabbit HPAP antibody (Abcam) required antigen retrieval at 95°C in 10 mmol/L sodium

citrate for 20 min, and Ki67 required antigen retrieval using 100 mmol/L Tris-EDTA at 100°C for 30 min. The mouse HPAP antibody (Sigma) required a Mouse-on-Mouse immunostaining kit (Vector Laboratories, FMK-2201). Isolated cells and pancreas sections were permeabilized with 0.3% triton-X-100/PBS for 10 min before blocking with Background Sniper (Biocare Medical, BS96614) for 8 min and washed. Primary antibodies (Table 1) were incubated overnight at 4°C in Antibody Diluent Solution (Dako, 79149). Secondary antibodies were appropriately matched to the primary antibody and tissue sections/cells incubated at RT for 2 h in the dark. Mice were injected with 2 μl 10mmol/L EdU per gram body weight 24 h prior to dissection, or, for *in vitro* incubation, 0.05% (v/v) was added to culture medium for 6 h prior to fixation, and stained with the EdU Click-It Reaction kit (C10340). Apoptosis was determined using an *In Situ* Cell Death Detection kit (TUNEL) (Roche, 12156792910). DAPI (4, 6-diamidino-2 phenylindole, dihydrochloride) (1/500, D1306) was used as a counterstain for cell identification. MatTek dishes and slides were imaged on a Zeiss LSM 510 Duo Vario confocal microscope (Carl Zeiss Ltd, Oberkochen, Germany) located at The Biotron (Western University), and counted manually using LSM 5 software.

Quantification of cells

HPAP$^+$ cells were quantified by co-staining with insulin in P7 pancreas sections (>250 insulin$^+$ cells/section). Isolated islets from each mouse were cultured separately. At least 5 replicate cultures were performed, with >2000 cells counted per time point/replicate. Due to low reporter protein presence after culturing, all HPAP$^+$ cells were counted per MatTek dish. At least 20 fields of view (FOV) were captured and total cell numbers counted (>50 cells/FOV). The proportion of HPAP$^+$/total cells was calculated by extrapolating the average number of cells/FOV, then projecting this number to total FOV/dish.

Table 1. Antibodies used for immunofluorescent histochemistry.

Protein	Species	Company	Cat. No.	Dilution
Insulin	mouse	Sigma Chemical	I2018	1:2000
Insulin	rabbit	Santa Cruz	sc-9168	1:200
Insulin	guinea pig	Abcam Inc.	ab7842	1:300
Glut2	goat	Santa Cruz	sc-7580(C-19)	1:100
Glut2	rabbit	Millipore EMD	07–1402	1:500
Glucagon	rabbit	Santa Cruz	sc-13091	1:200
Somatostatin	mouse	Santa Cruz	sc-25262	1:200
Ck19	mouse	Dako	m0888	1:100
Ck19	rabbit	Abcam	ab15463	1:100
Ki67	mouse	BD Biosciences	550609	1:50
HPAP	mouse	Sigma	A2951	1:500
HPAP	rabbit	Abcam	ab133602–3	1:200
GPM6a	mouse	MBL International	D055–3	1:100
GFAP	rabbit	Millipore	AB5804	1:200
β-III tubulin	mouse	Sigma	T5076	1:500
Amylase	mouse	Abcam	sc12821	1:50
GFP	rabbit	Abcam	ab290	1:300

Glut2: glucose transporter 2
CK19: cytokeratin19
HPAP: human placental alkaline phosphatase
GPM6a: glycoprotein m6a
GFAP: glial fibrillary acidic protein
GFP: green fluorescent protein.

Fluorescent activated cell sorting

Pancreata from P7 mice were perfused with 2 ml digestion buffer (1.0 mg/ml collagenase V (C9263), 0.2 mg/ml BSA, 0.1 mg/ml soybean trypsin inhibitor, in HBSS), pooled, and incubated at 37°C for 30 min in a shaking water bath. Cells were washed and further dispersed with 0.25% trypsin (25300054), shaken in a 37°C water bath for 5 min, then drawn twice through 14 G, 18 G, and 22 G needles. Enzymes were inactivated by the addition of 5 ml media, containing HBSS (Sigma, H1387) + 10% FCS + 0.1 mg/ml DNase I (18068–015). After washing, the resulting cells were re-suspended in 1 ml Red Blood Cell Lysis Buffer (Sigma, R7757) for 1 min, then inactivated by adding 5ml HBSS + 10% FCS + 0.1 mg/ml DNase I. Dissociated cells were filtered through a 40 μm nylon mesh (BD Biosciences, CA21008). Primary (GPm6a and Glut2, Table 2) and secondary antibodies were incubated sequentially for 60 min each to label β-cells. Seven-Aminoactinomycin D (7-AAD) (BD Pharminogen, 51-68981-E) was added as a viability marker. Cells were sorted on a Becton Dickinson FACSAria III cell sorter running FACSDiVa software (v 6.1.2) at the

Table 2. Primer sequences designed for the amplification of mouse cDNA by qPCR.

Gene	Sense primer (5′-3′)	Antisense Primer (5′-3′)	Band Size (bp)
Insulin	agccccggggaccttcagac	gcgggtcgaggtgggcctta	134
Pdx1	gttggatagccggagagatg	agtttggagcccaggttg	151
Glut2	acaccggaatgttcttagcc	gtgagagaagccgaggaaag	109
Ngn3	ggcgcctcatcccttggatg	cagtcacccacttctgcttcg	161
Nkx6.1	tctggctgtgggatgttagc	tcatctcggccatactgtgc	106
MafB	ggtataaacgcgtccagcag	cgagtttctcgcacttgacc	138
CycloA	atggtcaaccccaccgtgt	ttctgctgtctttggaactttgtc	102

Pdx1: pancreatic duodenal homeodomain box 1
Glut2: glucose transporter 2
Ngn3: neurogenin3
MafA: V-maf musculoaponeurotic fibrosarcoma oncogene homolog A
Nkx6.1: homeobox protein Nkx_6.1
Cyclo A: Cyclophilin A.

London Regional Flow Cytometry Facility at Robarts Research Institute at Western University (London, Ontario). Gating strategy is shown in supplemental Figure S1.

Glucose-stimulated insulin secretion (GSIS)

Cells recovered from flow cytometric sorting were cultured overnight in DMEM/F-12 + 10% FBS, or dedifferentiated and redifferentiated *in vitro*, and stimulated as described for static GSIS assays using 2.8 and 16.7 mmol/L D-glucose.[19] Insulin release was determined using an Ultrasensitive Insulin RIA (range 0.02–1 ng/ml) (St Joseph Health Care, London Canada).

Neural-lineage differentiation

Ins^{+}(GPm6a^{+} or YFP^{+})$Glut2^{HI/LO}$ cells recovered after sorting were cultured in Neurocult Neural Stem Cell (NSC) Basal Media (StemCell Technologies, Inc. Inc., 5700) with Proliferation Supplement (StemCell Technologies, Inc., 5702) plus EGF and basic fibroblast growth factor (bFGF) (PHG0266) as per manufacturer's instructions on low attachment plates (Corning, 29446-3-030) for 7–14 d. Intact neurospheres were transferred to Matrigel-coated (1/25) MatTek dishes, and cultured with NSC Basal Media with Differentiation Supplement (StemCell Technologies, Inc. Inc., 5703) for 7–14 d.

Quantitative PCR

Total RNA ($\leq$1 μg) from isolated P7 islets (n = 5) or neurospheres produced by GFP^{+}$Glut2^{LO}$ cells recovered from flow cytometry (n = 4) was extracted using RNeasy Plus Micro kits (Qiagen, 74034) and Qiashredders (Qiagen, 79564). RNA was reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, 170–8840). Quantitative PCR experiments were performed using the $2^{-\Delta\Delta C}{}_{T}$ method after confirmation of parallel PCR amplification efficiencies on a Real-Time PCR ABI Prism 7500. IQ SYBR Green (Bio-Rad, 170–8882) was employed for detection of PCR products, using primer sequences provided in Table 2. PCR reactions were run in duplicate using an initial denaturation at 95°C for 5 min, followed by cycles of denaturation (95°C for 15 sec), primer annealing (60°C for 1 min) and transcript extension (50°C for 2 min) for 40 cycles.

Statistical analysis

Data are expressed as mean ± SEM, and comparisons between groups analyzed using a Student's t-test or one-way ANOVA with a minimum acceptable level of significance of $p < 0.05$. Statistical analysis was performed using GraphPad Prism software (v. 5.01, La Jolla, CA).

Results

Loss of islet phenotype after culture

Freshly isolated Islets from 7-day old mice lost their 3-dimensional architecture within 1 week of culturing in epithelial-cell promoting/dedifferentiation conditions, forming a rapidly expanding monolayer which could be maintained for up to 4 weeks (Figs. 1A-C).

Intact islets did not demonstrate immunostaining for the ductal marker cytokeratin-19 (Ck19) (Fig. 1D, white bar). After 1 week in dedifferentiation medium, 74.7 ± 3.8% of cells present expressed Ck19 (hatched bar, $p < 0.001$), and this phenotype was maintained throughout the remaining culture period (4 weeks, black bar). In freshly isolated islets, 5.3 ± 0.8% of cells were shown to be undergoing proliferation by EdU localization (total EdU^{+}/total DAPI^{+}) (Fig. 1E, white bar). After islet dedifferentiation culture for 1 week, this increased to 33.1 ± 8.2% (hatched bar, $p < 0.001$); but dropped thereafter to 10.8 ± 3.7% (black bar, $p < 0.01$), revealing the generation of a new proliferative, epithelial cell monolayer which expressed a ductal marker. Immunostaining for the acinar cell marker amylase (Fig. S2A, green) was seen in pancreas sections outside of the islet (Fig. S2A, insulin, red), and was absent after 1 week in the dedifferentiated cell monolayer (Fig. S2B), as was insulin.

Lineage tracing illustrates rare β-cell dedifferentiation

We sought to determine the extent to which specifically β-cells contributed to islet cell dedifferentiation *in vitro* using the HPAP lineage tracer localized by immunohistochemistry of P7 RIPCre;Z/AP$^{+/+}$ mice. Insulin-expressing β-cells (Fig. 2A, green) were identified by the HPAP reporter protein (Fig. 2A, red) within islets *in situ* with an efficiency of 85.3 ± 1.2% (Fig. 2A, merged, and C, white bar). The staining frequency of HPAP in extra-islet β-cell clusters containing <5 β-cells was significantly lower at 39.0 ± 6.9% ($p <$

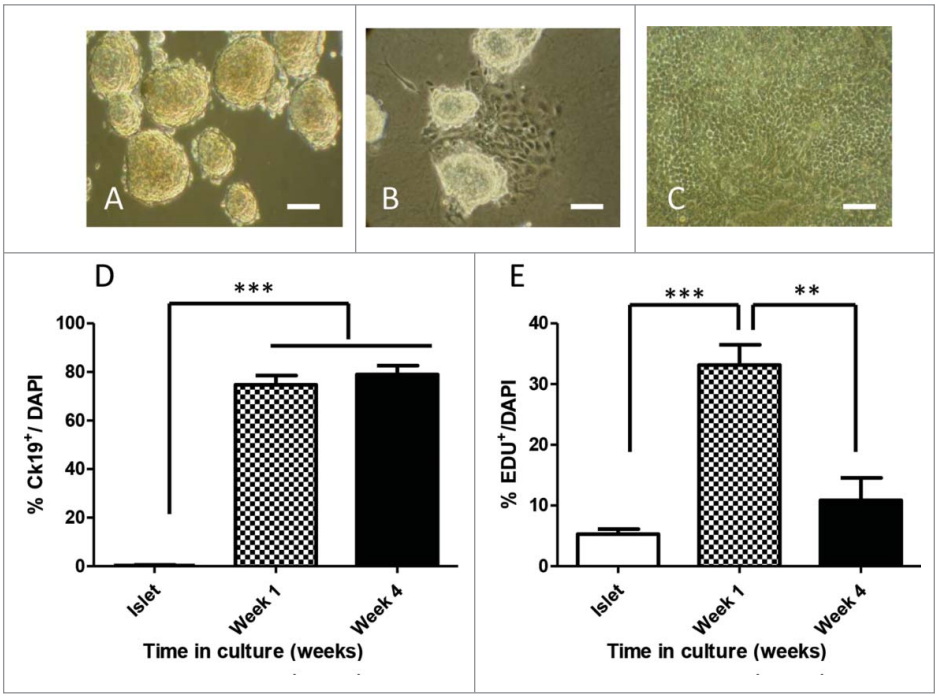

Figure 1. *In vitro* dedifferentiation of neonatal mouse islets. Photomicrographs depicting neonatal (P7) mouse islets immediately following isolation (A), 4 d after plating on collagen under dedifferentiation culture conditions (B), and after 1–4 weeks *in vitro* (C). The total proportion of cytokeratin-19 ($Ck19^+$)-expressing cells significantly increased after islets (D, white bar) were cultured in ductal epithelial promoting conditions (D, hatched bar = 1 week; black bar = 4 weeks) and which was maintained. The cell proliferation index (total $EdU^+/DAPI^+$ cells, E) increased after islets (E, white bars) were cultured for ductal dedifferentiation for 1 week (E, hatched bar), and decreased thereafter (E, black bar, 4 weeks). Size bars denote 50 μm, $n > 10$ experiments, data are represented as % mean ± SEM, $^{**}p < 0.01$, $^{***}p < 0.001$.

0.01) (Fig. 2B, arrows, and C, black bar). Within tissue sections, β-cells constituted 78.9 ± 4.4% of total islet cells; thus, given the labeling efficiency, 67.3 ± 0.9% of total islet cells expressed HPAP before isolation and culture. Pancreas sections from P7 RIPCre;$Z/AP^{-/-}$ control littermates did not exhibit HPAP immunostaining (Fig. S3A, insulin, green; HPAP, red). Ck19-expressing cells were identified around ducts (Fig. S3B, red) but not within $HPAP^+$ islets (Fig. S3B, green) in P7 RIPCre;$Z/AP^{+/+}$ pancreas sections. The extra-islet endocrine cell clusters were not included in the following whole islet analysis due to the physical limitations of handpicking these structures.

After islet isolation and *in vitro* culture, there was a progressive loss of insulin-immunoreactive cells over 1 week (Fig. 2D, red), and the frequency of HPAP expression dropped significantly to <0.2% of cells present in the ductal epithelial monolayer (Fig. 2D, green, 67.3 ± 0.9% *vs* 0.14 ± 0.03% $HPAP^+$/total cells, $p < 0.0001$, islet *vs* 1 week). This represented 14–95 immunostained $HPAP^+$ cells retained per MatTek dish. Of the surviving $HPAP^+$ cells that no longer exhibited immunoreactive insulin (Fig. 2E, HPAP, green), 87.5 ± 3.9% demonstrated expression of Ck19 (Fig. 2E, red) (islet *vs* 1 week, $p < 0.0001$) which was maintained during longer cultures. Analysis of the proliferation rate of these remaining HPAP-tagged cells by dual staining with EdU showed a significant increase after 1 week of culture compared to freshly isolated islets (Fig. 2F, 3.5 ± 0.2% isolated islets (white bar) *vs*. 25.6 ± 1.8% 1 week *in vitro* (hatched bar), $p < 0.001$), but decreased thereafter (Fig. 2F, black bar, 4 weeks). Visualization of apoptosis showed that a major loss of insulin-immunoreactive cells occurred after 5 d of culture (Fig. 2G, insulin, green; TUNEL, red). Due to the nature of the reporter protein, which tracks the β-cell directly and not insulin production, these results indicate that a minority of β-cells survived *in vitro* with a higher proliferation rate than islet β-cells *in situ*, but demonstrated a phenotypic change with a loss of insulin and the expression of the ductal-epithelial cell marker Ck19, suggesting a dedifferentiation process. Subsequent re-culture of these remaining HPAP-expressing cells in media previously shown to

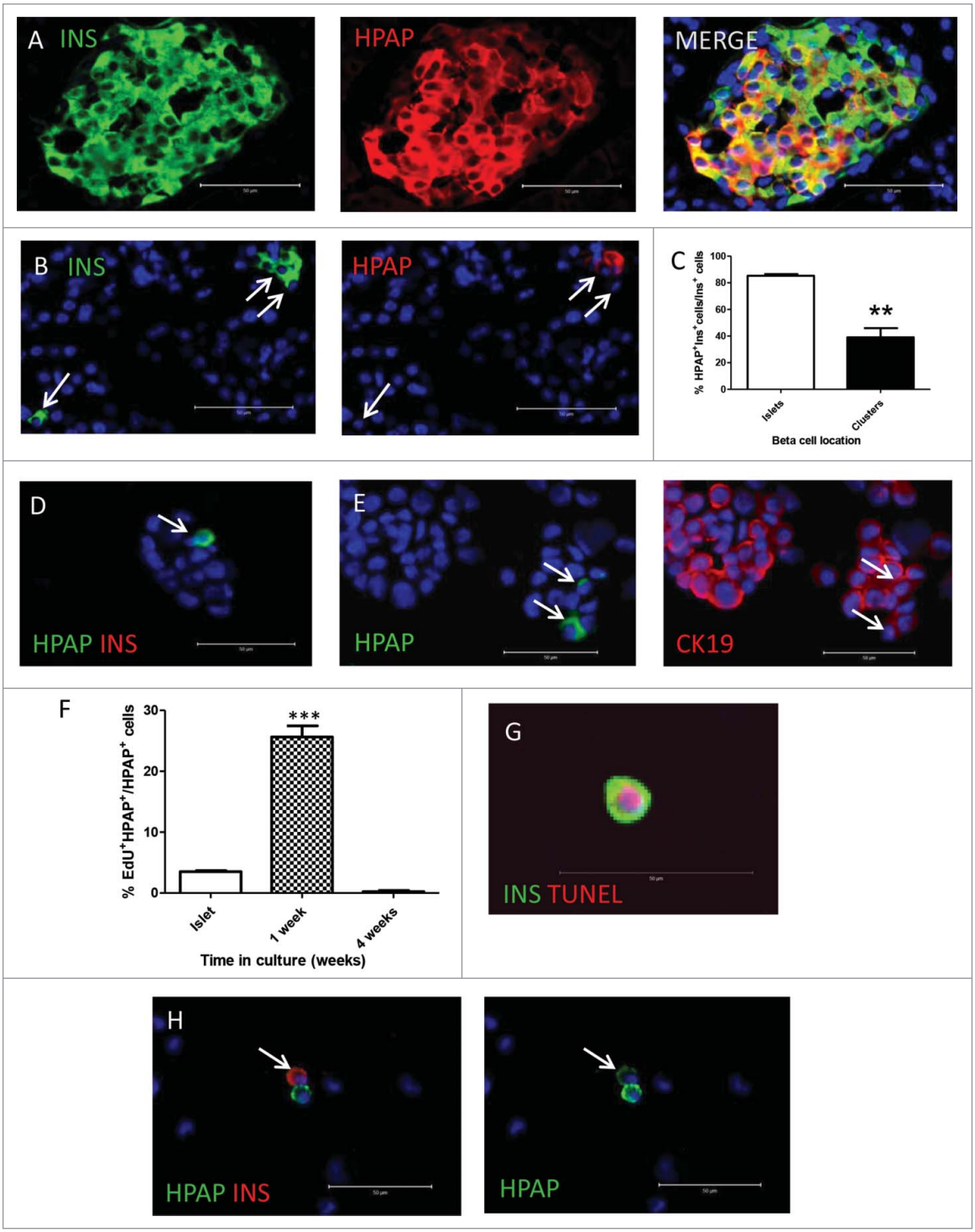

Figure 2. Specificity of HPAP to the β-cell using RIPCre;Z/AP$^{+/+}$ mice and lineage tracing of the β-cell during *in vitro* islet dedifferentiation. RIPCre;Z/AP$^{+/+}$ transgenic mouse model showing insulin^{+} β-cells (green, A) tracked by the reporter protein HPAP (red, A), and quantified in (C). The proportion of insulin-expressing β-cells (B, green) co-labeled by HPAP (B, red) in β-cell clusters containing fewer than 5 β-cells was significantly lower than in islets (B, C, black bar). After islets were cultured in dedifferentiation media for 1 week, all insulin expression was lost (D, red), and only a minority of cells retained HPAP expression (D, green). The majority of cells present in culture after 1 week immunostained for Ck19 (E, red), as did the rare remaining HPAP^{+} cells (green). These HPAP^{+}Ck19^{+} were proliferative at 1 week in culture (F, hatched bar) but proliferation decreased thereafter (F, 4 weeks, black bar). There was a significant increase in β-cell apoptosis after 5 d in dedifferentiation conditions (G, insulin, green; TUNEL, red). After replating the resultant epithelial-like monolayer of cells in redifferentiation medium, the majority of the rare, remaining HPAP^{+} cells (H, green) re-expressed insulin (H, red). Scale bar denotes 50 μm, $n > 6$, data are represented as % mean $\pm$ SEM, $^{**}p < 0.01$, $^{***}p < 0.001$.

promote endocrine redifferentiation[18] revealed that greater than 90% of these cells expressed insulin over 4 weeks of culture (Fig. 2H, insulin, red; HPAP, green) demonstrating substantial phenotypic plasticity. We next examined the defining characteristics of this subpopulation of β-cells.

Characterization of β-cell subpopulations by Glut2 and GPM6a presence

We examined whether the subpopulation of HPAP$^+$ tagged β-cells that survived tissue culture and had an apparent phenotypic plasticity were comparable to the previously reported rare, pancreas-derived multipotential progenitor (PMP) cells in mouse pancreas.[5] Distinguishing characteristics of these PMP cells were their reduced, but detectable expression of insulin while having extremely low or absent expression of the glucose transporter-2 (Glut2), and the expression of transcription factors normally associated with endocrine pancreas development during organogenesis.[5] As the proportion of these progenitor cells in islets was reported to be only 1–5/10,000,[5] flow cytometric sorting of β-cells from RIPCre;Z/AP$^{+/+}$ dispersed whole mouse pancreata was initially used to optimize yield. Cells were immunostained using antibodies for GPM6a,[20] a cell surface marker shown to specifically identify the β-cell, and differentially sorted for the presence of Glut2. GPM6a (red) immunostained, insulin-expressing (green) β-cells are shown in Figure 3A. Flow cytometry demonstrated that GPM6a-expressing β-cells were sorted into 3 fractions: GPM6a^{HI}Glut2^{HI} cells (Fig. 3B, red, representing typical β-cells) and GPM6a$^+$Glut2$^{MID/LO}$ cells (Fig. 3B, green); further split arbitrarily into GPM6a^{+-} Glut2^{MID} and GPM6a$^+$Glut2^{LO} fractions and collected separately. At P7, GPM6a$^+$Glut2^{LO} cells represented 0.9 ± 0.2% of all live cells sorted from whole pancreas, compared with 0.4 ± 0.2% of cells identified as GPM6a$^+$Glut2^{MID} and 1.0 ± 0.2% possessing a GPM6a$^+$Glut2^{HI} phenotype (Fig. 3B, n=12 sorts). Insulin immunostaining was more prominent in the GPM6a$^+$Glut2^{HI} cells than in the other populations (Fig. 3C, red). GPM6a$^+$Glut2^{HI} β-cells were immunopositive for Glut2 (Fig. 3C, green, top panel), but this was greatly diminished in the GPM6a$^+$Glut2^{MID} cells (middle panel), and absent in the GPM6a$^+$Glut2^{LO} cells (lower panel). These data indicate that neonatal mouse β-cells exist within a spectrum of Glut2 presence.

These cell populations were recovered after cell sorting and cultured in the presence of 16.7 mmol/L D-glucose for 75 min prior to measurement of secreted insulin. As expected, GPM6a$^+$Glut2^{HI} cells readily secreted insulin (18.6 ± 1.8 ng), whereas insulin secretion from the same number of GPM6a^{+-} Glut2^{LO} cells was not detected (<0.02 ng), demonstrating that the GPM6a$^+$Glut2^{LO} cells were functionally unresponsive to glucose (n=3, 6000 sorted cells/experiment).

When cultured in dedifferentiation media conditions, the majority of GPM6a^{HI}Glut2^{HI} β-cells did not survive (not shown), similar to unsorted, islet-derived β-cells. Conversely, GPM6a$^+$Glut2$^{MID/LO}$ cells survived *in vitro*, retained the HPAP β-cell tag, lost insulin immunoreactivity, and immunostained for Ck19 (Fig. 3D, arrows, Ck19 (red), HPAP (green)). These GPM6a$^+$Glut2^{LO} cells exhibiteda ~2-fold higher proportion of Ck19$^+$ cells after 4 weeks dedifferentiation than did the GPM6a$^+$Glut2^{MID} cells (72.4 ± 7.7% of Glut2^{LO} *vs* 42.7 ± 8.9% of Glut2^{MID} cells, $p < 0.01$, $n = 5$), possibly suggesting that the GPM6a$^+$Glut2^{MID} cells represent a transition state between Glut2^{LO} and Glut2^{HI}. When these dedifferentiated cells were subsequently cultured in redifferentiation medium, HPAP$^+$ cells (Fig. 3E, green) derived from GPM6a$^+$Glut2^{LO} cells displayed insulin expression (red). However, under these conditions the resulting cells released considerably less insulin than did freshly separated GPM6a$^+$Glut2^{HI} β-cells (above) in a low or high glucose environment (2.8 mmol/L, 14.1 ± 0.4 pg, and 16.7 mmol/L glucose, 18.9 ± 0.4 pg insulin/5×10^4 cells, $n = 3$). These results are consistent with the magnitude of insulin release observed from *de novo* β-cells generated from human stem cells[21] that demonstrated an immature phenotype *in vitro*. Moreover, glucose responsiveness may be only obtained after *in vivo* transplantation and maturation.[4,22]

To confirm the above findings using RIPCre;Z/AP$^{+/+}$ mice sorted by GPM6a, we utilized a separate ROSA-eYFP reporter strain to label β-cell progeny (RIPCre;ROSA-eYFP$^{+/+}$ mice). Figure 4A demonstrates YFP (GFP, green) labeling of β-cells (insulin, red) within P7 pancreas sections. Flow-cytometric sorting of RIPCre;ROSA-eYFP$^{+/+}$ P7 mouse pancreas by

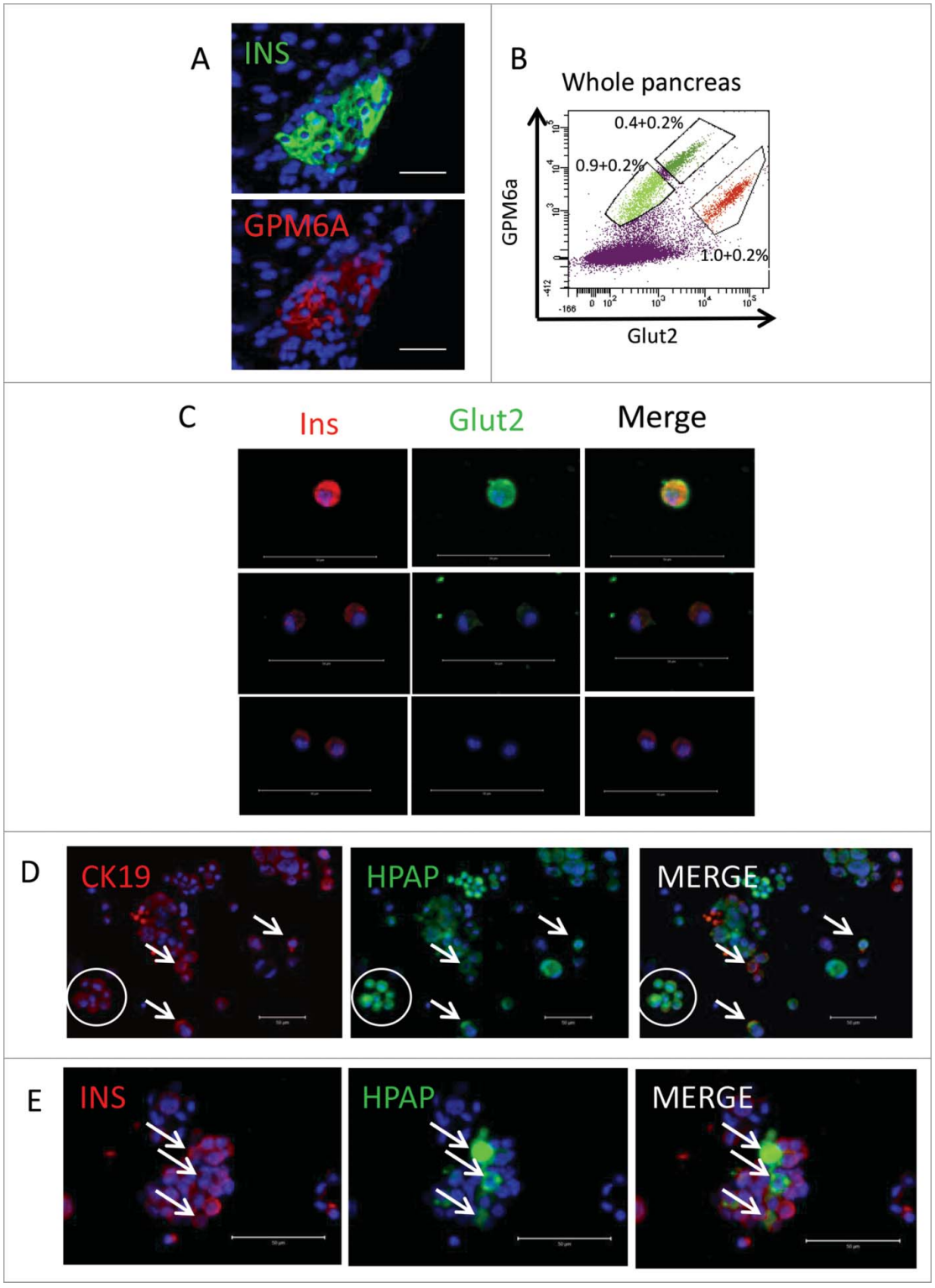

Figure 3. Pancreatic β-cells sorted by flow-cytometry using Glut2 and GPM6a presence. Insulin-expressing β-cells (A, green) could be identified by GPM6a (A, red) using immunofluorescence. Whole mouse P7 pancreas (B, left) was dispersed and sorted into distinct populations of insulin^{+} (GPM6a^{+}) cells according to differential Glut2 expression: GPM6a^{+}Glut2^{+} (B, red) and GPM6a^{+} Glut2$^{MID/LO}$(green), further split into GPM6a^{+-} Glut2^{LO} (B, green) and GPM6a^{+}Glut2^{MID} (B, left, darker green, upper). Cells recovered after sorting (C) were immunostained for Glut2 (green) and insulin (red). Insulin staining (C, red) was more prominent in proto-typical β-cells (GPM6a^{+}Glut2^{HI}, top panel) as compared to GPM6a^{+}Glut2$^{MID/LO}$ cells. Glut2 immunostaining (C, green) was diminished in the GPM6a^{+}Glut2^{MID}cells (C, middle), and was absent in the GPM6a^{+}Glut2^{LO} cells (C, green, bottom). After flow cytometry, cell recovery, and 1 week in dedifferentiation culture, the majority of Ins^{+}Glut2^{LO} cells co-expressed Ck19 (red) and HPAP (green) (D, arrows and circle). Redifferentiation of dedifferentiated ductal-like cells after 4 weeks in culture demonstrated the β-cell lineage reporter HPAP (E, green) co-localizing with insulin (E, red, arrow). Scale bar denotes 50 μm, $n = 5$.

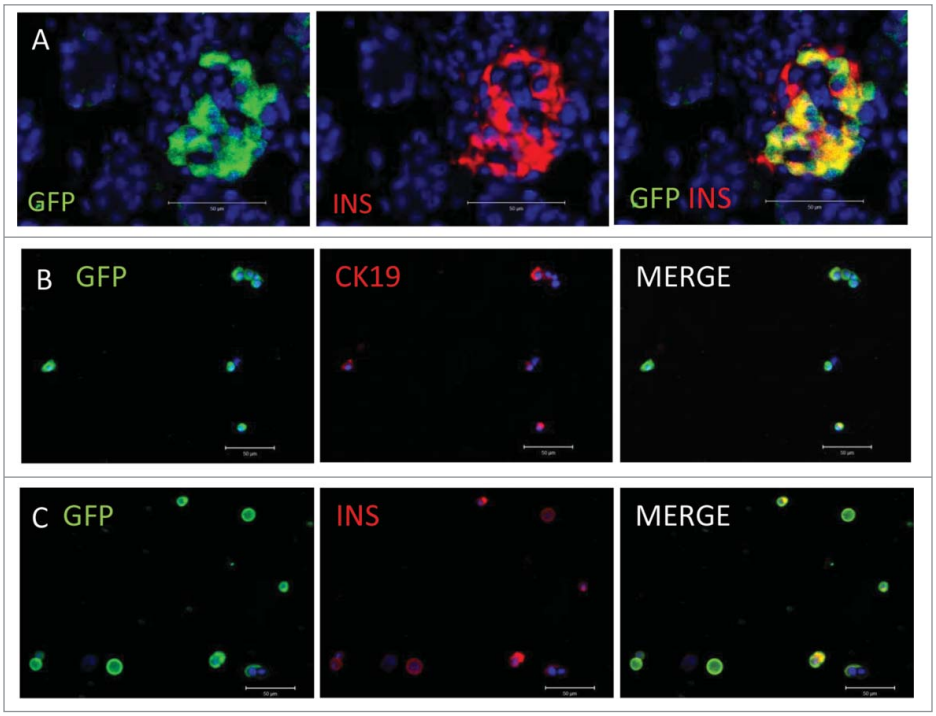

Figure 4. Confirmation of the β-cell origin of GPM6a^{+}Glut2^{LO} cells using the reporter strain ROSA-eYFP. (A) insulin (red) and GFP (green) immunostained β-cells are shown within RIPCre;ROSA-eYFP$^{+/+}$ P7 mouse pancreas sections ($n = 5$). After sorting live cells by flow cytometry from whole pancreas using GFP and Glut2, the GFP^{+}Glut2^{LO} cells dedifferentiated *in vitro* to ductal-lineage cells (B, Ck19, red and GFP, green, $n = 4$). These cells were able to redifferentiate back into insulin-expressing cells *in vitro* (C, insulin, red; GFP, green, $n = 4$).

Glut2 and GFP expression yielded similar proportions of cells as previously found using RIPCre;Z/AP mice, with GFP^{+}Glut2^{LO} cells representing 0.8±0.3% of total live cells from whole pancreas, compared with 2.6± 0.7% of cells being GFP^{+}Glut2^{HI}. Once placed in ductal dedifferentiation media, GFP^{+}Glut2^{LO} cells similarly lost insulin immunoreactivity and dedifferentiated into GFP^{+}Ck19^{+} cells (Fig. 4B, GFP (green) and Ck19 (red)), whereas GFP^{+}Glut2^{HI} cells did not dedifferentiate (not shown). Dedifferentiated GFP^{+}Ck19^{+} cells were then able to redifferentiate back into insulin-expressing cells (Fig. 4C, GFP (green) and insulin (red)). Therefore results were consistent using 2 different β-cell lineage reporters.

Ins^{+}Glut2^{LO} cells generate neurospheres and neural-lineage cells in vitro

To assess their cellular plasticity, GPM6a^{+}Glut2^{LO} cells from RIPCre;Z/AP$^{+/+}$ mice were recovered after FACS and cultured in neural proliferation conditions. A subset (1/200) of these GPM6a^{+}Glut2^{LO} cells proliferated and formed neurospheres (Fig. 5A), while GPM6a^{+}Glut2^{HI} cells did not proliferate (Fig. 5B). After subsequent neural differentiation culture, the GPM6a^{+}Glut2^{LO}-derived neurospheres formed cells with neuronal-like processes (Figs. 5C, D), and demonstrated immunostaining for the neuronal marker β-III tubulin (Fig. 5E, green) and the astrocyte marker glial fibrillary acidic protein (GFAP) (Fig. 5F, red). Both of these resulting cell phenotypes expressed the β-cell marker HPAP (Fig. 5E, red, and Fig. 5F, green), indicating a lineage plasticity of GPM6a^{+}Glut2^{LO} cells such that all neurospheres generated both neuronal and astrocytic cell types.

Neurospheres were similarly generated after *in vitro* culture of GFP^{+}Glut2^{LO} cells isolated from RIPCre; ROSA-eYFP$^{+/+}$ mice. These neurospheres expressed YFP (GFP, green) and insulin (red) as shown in Figure 5G, indicating their β-cell origin. Neurospheres demonstrated a gene expression profile consistent with immature β-cells, showing decreased expression of *Ins* and *Glut2*, and an increased expression of *MafB* and *Ngn3*, when compared to isolated P7 islets by qPCR (Fig. 5H, $p < 0.05$). While there was a trend toward increased expression of *Pdx1* and *Nkx6.1* in

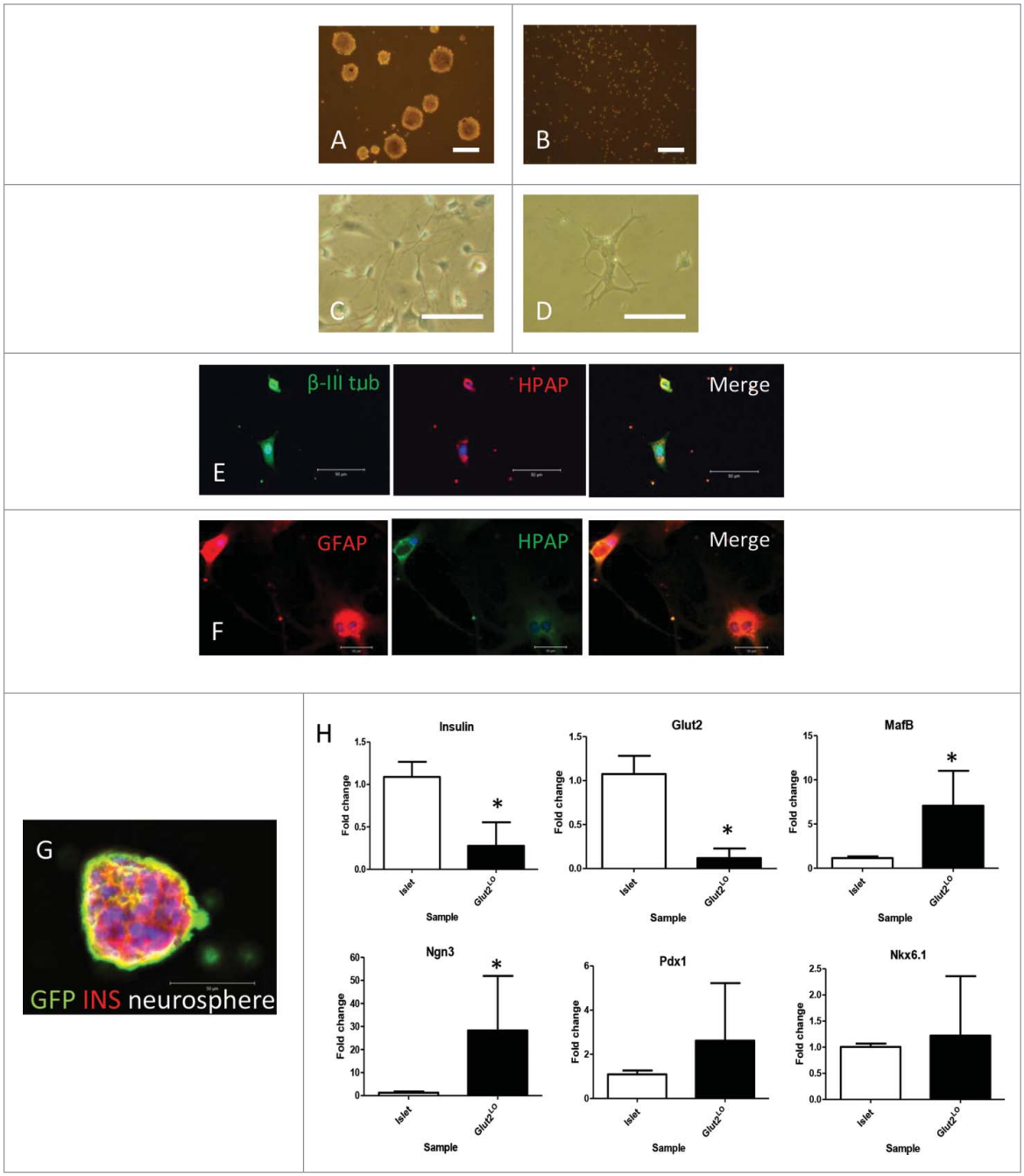

Figure 5. Lineage plasticity of pancreatic Glut2^{LO} β-cells. GPM6a^{+} cells recovered from whole mouse RIPCre;Z/AP$^{+/+}$ P7 pancreas after flow cytometry and cell sorting based on Glut2 presence were placed in neural proliferation conditions. Neurospheres (A) were generated from GPM6a^{+}Glut2^{LO} cells, compared to non-proliferative GPM6a^{+}Glut2^{HI} cells (B). After culture in neural differentiation conditions, the neurospheres generated multiple morphologies, including neuron-like processes (C, D). When subjected to immunofluorescent staining, cells with a neuronal appearance were visualized by β-III tubulin (E, green), and astrocytes by GFAP (F, red). These neural-lineage cells co-stained with HPAP (red, E; green, F; $n = 5$). Neurospheres (G, GFP, green, and insulin, red) were similarly generated from sorted GFP^{+}Glut2^{LO} β-cells derived from RIPCre;ROSA-eYFP$^{+/+}$ P7 pancreas, when cultured under neural proliferation conditions, and demonstrated an immature β-cell phenotype by quantitative PCR with decreased expression of *Ins* and *Glut2*, and increased expression of *Ngn3* and *MafB* (H, $^{*}p < 0.05$, neurospheres *vs* P7 islets, $n = 4$). Scale bar denotes 100 μm (A, B), 50 μm (C-G).

the neurospheres, high variability between samples resulted in no significant change when compared to neonatal islets.

Ins^{+}Glut2^{LO} cells are primarily found outside of islets

Flow cytometric sorting was performed on β-cells recovered from isolated RIPCre;Z/AP$^{+/+}$ mouse islets as opposed to whole pancreas to separate GPM6a^{+}-Glut2^{HI}, GPM6a^{+}Glut2^{MID}, and GPM6a^{+}Glut2^{LO} cells. In contrast to whole pancreas, GPM6a^{+}Glut2^{MID} cells could not be recovered from isolated islets, and the population of GPM6a^{+}Glut2^{LO} cells represented only 0.3 ± 0.1% of total live cells (Fig. 6A, green) compared with 54.0 ± 4.8% of cells being GPM6a^{+}Glut2^{HI} (red). Thus, the abundance of GPM6a^{+}Glut2^{LO} cells was 3 times lower in islets than in whole pancreas, suggesting a predominantly extra-islet localization.

To identify the anatomical location of the insulin-immunoreactive, but Glut2-low/negative cells, mouse pancreas sections were examined. While some Ins^{+}Glut2^{LO} cells were located in islets (Fig. S4, insulin, red; Glut2, green, arrow indicates an Ins^{+}Glut2^{LO} cell), the majority of these cells were located outside of islets in small β-cell clusters (1–5 β-cells), with many of these cells also undergoing proliferation. A representative Ins^{+}Glut2^{LO}EdU^{+} cell is shown in Figure 6B. When quantified, Ins^{+}Glut2^{LO} cells represented 3.5 ± 0.2% of all insulin-expressing β-cells in the P7 mouse pancreas by fluorescent immunocytochemistry (Fig. 6C, 1935 cells, n=5 pancreata). When stratified by β-cell location, Ins^{+}Glut2^{LO} cells represented 1.2 ± 0.3% of insulin^{+} cells within islets (>6 β-cells), but 20.5 ± 3.6% of insulin^{+} cells within extra-islet β-cell clusters (<5 β-cells, clusters) (Fig. 6C, 23/1703 cells and 45/232 cells, respectively). Furthermore, the proliferation rate of insulin^{+} cells was higher in β-cell clusters than in islets at P7 by EdU presence (Fig. 6D, 12.6 ± 0.7% *vs* 2.8 ± 0.2% respectively). When further sub-analyzed by phenotype, 12.8 ± 4.1% of Ins^{+}EdU^{+} cells lacked Glut2 within islets, and 20.1 ± 5.6% within clusters (Fig. 6E), illustrating that at P7 Ins^{+}Glut2^{LO} cells are proliferative both within and outside of the islet as compared to Ins^{+}Glut2^{HI} β-cells.

It is possible that the sub-population of Ins^{+}Glut2^{LO} β-cells in P7 mouse pancreas represents an immature population only present in early postnatal life. We therefore quantified their presence in adult mouse pancreas (3 months age) using immunostaining for insulin, Glut2, and Ki67. In adults, the proportion of insulin-expressing β-cells found in extra-islet clusters was significantly lower than at P7 (Fig. 6F, 15.5 ± 1.8% *vs* 5.3 ± 1.7%, 7 d *vs* 3 mo, ** $p < 0.01$), demonstrating that most β-cells are located within proto-typical islets during adult life. The β-cell sub-population of interest, Ins^{+}Glut2^{LO} cells were still present in adult pancreas, although their abundance was also lower than in the neonate (Fig. 6G, 3.5 ± 0.2% *vs* 0.8 ± 0.2%, 7 d *vs* 3 mo, ** $p < 0.01$), coinciding with a decrease in cluster proportion and illustrating endocrine pancreas maturation to a functional phenotype. A similar age-related change was found in total β-cell proliferation following immunostaining for insulin and Ki67 (Fig. 6H, 6.1 ± 0.8% *vs* 0.8 ± 0.1%, 7 d *vs* 3 mo, *** $p < 0.001$). These data provide strong evidence for a substantial proportion of Ins^{+}Glut2^{LO} cells predominantly present within extra-islet endocrine cell clusters in the young mouse and decreasing with age.

HPAP^{+} cells tag non-insulin-expressing cells with advancing age

In the neonatal RIPCre/Z/AP$^{+/+}$ mouse (<14 d), HPAP^{+} cells that immunostained for glucagon (Gcg) or somatostatin (Sst) were extremely rare (Fig. 7A and B, white bars), whether quantified by proportion of marker/HPAP (A) or HPAP/marker (B). However, when older (>1 y) RIPCre;Z/AP$^{+/+}$ mice were examined, the proportion of HPAP-labeled cells (green) that also immunostained for glucagon (Fig. 7A and B, black bars, and C, red) or somatostatin (Fig. 7A and B, black bars, and D, red) increased. These results indicate that some of the HPAP-tagged cells demonstrate a trans-lineage endocrine fate as mice age.

Discussion

In agreement with previous reports, we show here that rare β-cells, tracked by HPAP or GFP reporter protein expression, demonstrate the capacity for dedifferentiation to a hormone-negative, ductal-epithelial-like phenotype expressing Ck19.[8,9] We hypothesized that these cultured, reporter-labeled, Ck19-expressing and insulin-negative cells represented the progeny of Ins^{+}Glut2^{LO} cells, reported to denote a pancreatic multipotent progenitor (PMP) cell pool.[5] We have shown that these PMP-like cells can be isolated from

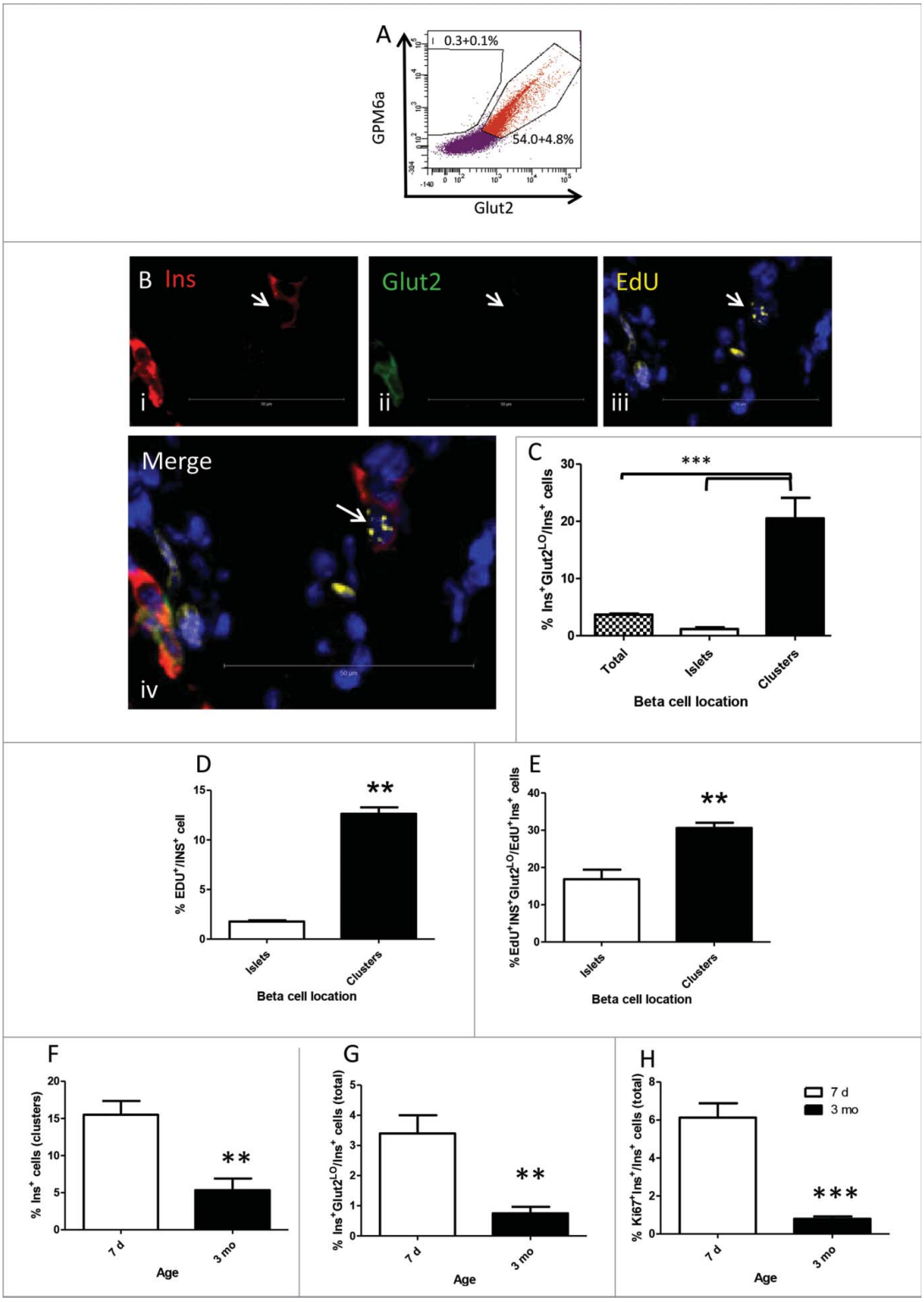

Figure 6. $Ins^{+}Glut2^{LO}$ β-cells are primarily found outside of islets. FAC-sorted $GPM6a^{+}Glut2^{LO}$ cells isolated from P7 islets represented 0.3 $\pm$ 0.1% of live cells (A), a lower proportion relative to that found in whole pancreas (see Fig. 3B). (B) Mouse P7 pancreas section immunostained for insulin (i, red), Glut2 (ii, green), EdU (iii, yellow), and merged images (iv), demonstrating an $Ins^{+}Glut2^{LO}EdU^{+}$ cell (arrow) located in an extra-islet β-cell cluster. Quantification of $Ins^{+}Glut2^{LO}$ cells by β-cell grouping indicated their primary location in clusters as compared to islets (C, $^{***}p < 0.001$ cluster *vs* total and islet). The proliferation rate of Ins^{+} cells at P7 was higher in extra-islet clusters than in islets (D, $^{**}p < 0.01$), and there was a high occurrence of $Ins^{+}Glut2^{LO}$ cell proliferation within islets and clusters at P7 (E, $^{**}p < 0.01$). The proportions of total extra-islet, insulin-expressing clusters (F, $^{**}p < 0.01$), total $Ins^{+}Glut2^{LO}$ cells (G, $^{**}p < 0.01$), and total β-cell proliferation by Ki67 (H, $^{***}p < 0.001$) all decreased from 7 d to 3 months of age in the mouse. Scale bar denotes 50 μm, $n > 5$. Data are represented as % mean $\pm$ SEM.

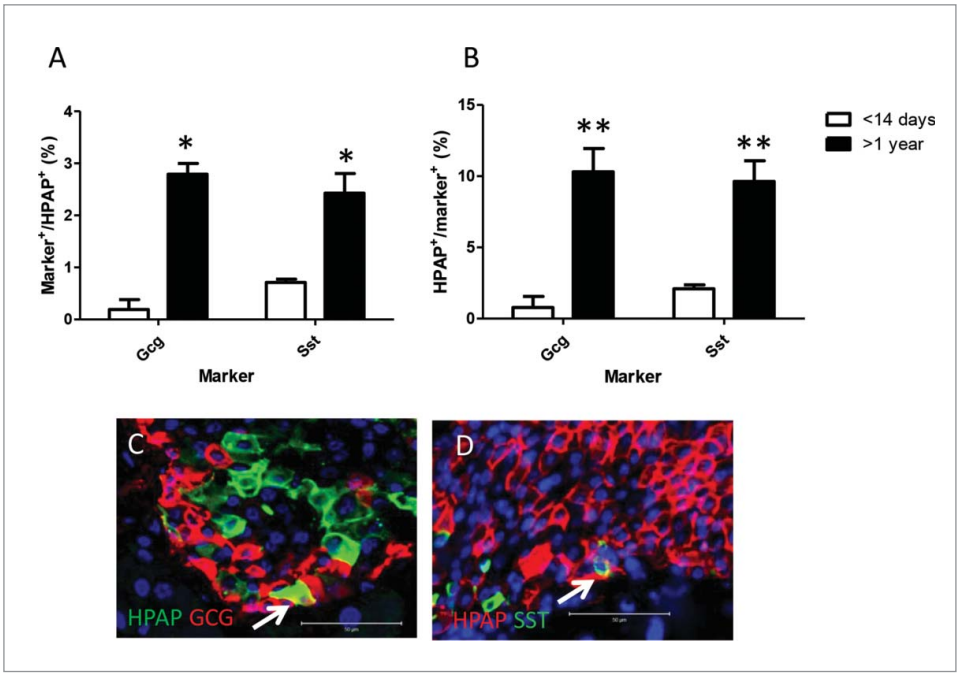

Figure 7. Alternate cell fates of HPAP^{+} cells *in vivo*. HPAP^{+} cells from RIPCre/Z/AP$^{+/+}$ neonatal (<14 d, $n = 5$) mouse pancreas sections rarely immunostained for non-insulin markers, including glucagon (Gcg) or somatostatin (Sst) (Fig. 7A and B, white bars) whether quantified by proportion of marker/HPAP (A) or HPAP/marker (B). In mice older than 1 y ($n = 4$), the proportion of HPAP^{+} (green) labeled non-insulin-expressing cells increased, including those containing glucagon (Fig. 7A and B, black bars, and C, red) or somatostatin (Fig. 7A and B, black bars, and D, red) as compared to young mice. Arrows indicate dual stained cells. Scale bar denotes 50 μm. Data are represented as % mean ± SEM, C and D, >1 y *vs* <14 d, t-test, * $p < 0.05$, ** $p < 0.01$.

neonatal mouse pancreas, and are capable of differentiation to a variety of cell types *in vitro*. Importantly, while Ins^{+}Glut2^{LO} cells can be found in intact islets as reported,[5] the majority are located in small, extra-islet endocrine cell clusters, where they have a significantly greater proliferative capacity at P7 than do the Ins^{+}Glut2^{HI} cells. These findings add potential physiological relevance to the initial observations of van der Kooy *et al*,[4,5] indicating that there are multipotent cells within the pancreas which express insulin and persist into adulthood.

The proportion of total lineage-plastic cells shown here in the neonate is higher than the reported rate in adult mouse as found by Smukler and colleagues;[5] however our approaches were different. Smukler *et al.*[5] used a clonal sphere assay to identify single multi-potential β-cells within dispersed islets. From this, the authors were able to ascertain that these cells demonstrated low or absent Glut2 expression. We have used this reduced/absent Glut2 expression as a tool to identify and segregate subsets of β-cells, and found that there is a relatively higher population of such cells in the young mouse pancreas. We also found that only a subset (1/200) of these Ins^{+}Glut2^{LO} cells are indeed multipotent under the neural culture conditions used. Since 0.3% of live cells in islets represent GPm6a-immunostained Glut2^{LO} β-cells, the number of resident multipotent cells would be approximately 1 in 1500 β-cells within islets. However, we have shown that the richest anatomical location of GPM6a^{+}Glut2^{LO} cells capable of proliferating and forming neurospheres is not islets but the abundant small extra-islet endocrine cell clusters, which were not examined in the former analysis. The presence of Ins^{+}Glut2^{LO} cells persists into adult life, although with a reduced abundance relative to neonates. This is due, in part, to a reduction in the number of extra-islet endocrine cell clusters with age.

Pancreatic endocrine cell and duct cell dedifferentiation or trans-differentiation have emerged as verified modes of cell plasticity *in vivo*, at times acting in an attempt to maintain β-cell mass, but also perhaps an erroneous fate decision made by the cell during stress. Expansive reviews showing the depth of evidence were recently published by Weir *et al.*[23] and Puri *et al.*[10] As an Illustration, rare β-cells were

shown to trans-differentiate to α-cells during type 2 diabetes progression,[24] further confounding the loss of β-cell mass, and representing β-cell failure.[25] In another study, β-cells lost insulin expression after partial pancreatectomy, becoming proliferative, Smad-7^{+} cells, and adopting a pancreatic polypeptide (PP) identity, similarly representing a dedifferentiation step during injury.[26] Endocrine cell trans-differentiation was shown in a type 1 diabetes model, with autoimmunity furthering the loss of β-cell mass in a trans- lineage progression from α- to β- to δ- cell phenotypes, ultimately resulting in an excess of δ- cells.[27] It was recently shown that β-cell dedifferentiation state may be assessed using the presence of Urocortin 3 (Ucn3) as a marker of β-cell maturity,[19,28] and this dedifferentiated (non-functional) phenotype could be rescued by the addition of Alk5, which acted through the inhibition of TGFβ receptor 1 signaling.[29] This finding supports the concept that endocrine cell dedifferentiation can be reversible.

The notion that progenitor cells may paradoxically express mature markers, such as insulin, could explain why such cells in the pancreas have remained elusive. Interestingly, it does satisfy the evidence that postnatal β-cells arise from pre-existing β-cells,[1] but, as shown here, not all insulin-expressing cells are of equivalent phenotype. Other precedents exist for mature marker presence in stem cell populations, such as neural stem cells expressing the astrocyte marker GFAP.[30] We have shown that Ins^{+}Glut2^{LO} cells represent 1.2% of β-cells in the neonatal islet by immunostaining, but only a subset of these cells are capable of differentiation to pancreatic islet and ductal phenotypes; the proportion of plastic cells still being higher than that found by Smukler *et al.*[5] However, a striking 3.5% of insulin-expressing cells within whole pancreas at P7 lacked Glut2, and up to 21% of these cells are found within clusters. This suggests that the highest source of β-cells demonstrating lineage plasticity is found outside of the islet, and these extra-islet clusters are most abundant in the neonatal period and thereafter decline with advancing age.[31] The absence of Glut2 expression is likely not the sole identifier of a progenitor for a β-cell however, since the co-expression of other markers such as Ngn3 and Nkx6.1 has been proposed.[5,32] Reports have demonstrated the presence of putative β-cell progenitors during adulthood, with or without diabetes, and showed that these cells exhibited higher expression levels of Pdx1 and MafB, and low/absent expression levels of genes indicative of mature gene function, including Nkx6.1 and Glut2.[33,34] Furthermore, these β-cells could be subdivided into those with the ability to transition to mature β-cells in culture, and those that maintained a progenitor phenotype throughout, illustrating β-cell heterogeneity and differing states of functional maturity.[34]

Beta cell heterogeneity is a burgeoning concept, with our data contributing multiple ways to stratify the β-cell: by age (neonatal vs adult); by location (islet vs cluster); and by expression of transporters or transcription factors (Glut2, MafB, Ngn3), all of which may confer degrees of function. These data support evidence from others indicating β-cell heterogeneity in relation to proximity to other cell types, vasculature, and innervation patterns.[35] Neonatal β-cells have been shown to require a glucose threshold for maturation, and β-cells from extra-islet clusters in young mice are functionally altered than those within islets, including differential expression of the peptide hormone Ucn3.[19] It is well established that mouse β-cells in the immediate postnatal period are immature, with poor GSIS and lower expression of Glut2 compared to adult β-cells.[36–38] Furthermore, human infant β-cells demonstrate some plasticity in insulin secretory machinery.[39] Indeed, one recent study showed tripotent progenitor cells present in P7 mouse pancreas, generating endocrine, exocrine, and ductal lineages dependent on *in vitro* conditions,[40] but did not define the cells capable of cellular plasticity. Our data suggests that a lack of Glut2 expression may reflect not only β-cells with decreased function, but also a subset of potential progenitor cells. Interestingly, Stolovich-Rain *et al.* showed that after selective β-cell ablation using doxycycline-mediated diphtheria toxin, surviving β-cells in the mouse pancreas displayed significantly decreased levels of Glut2, purportedly due to glucose toxicity.[41] While injury models were not examined in the present study, it must be assessed whether a decrease in Glut2 expression is a necessary mechanism during regeneration, a direct result of environmental toxicity, or whether the surviving cells exhibited Glut2 expression at all. As we have shown a higher proliferation rate *in situ* in the Ins^{+}Glut2^{LO} cells, it is conceivable that these could clonally expand in an attempt to replace β-cells after injury, with subsequent maturation to functional β-cells exhibiting high Glut2 expression. Indeed, this was recently

shown in a follow-up report from Razavi *et al.*, showing a selective survival, proliferation, and differentiation potential of insulin^{+}Glut2^{low} cells toward a β-cell phenotype after diabetes presence in both human and mouse. [42]

Russ *et al.* suggested that human β-cells were able to dedifferentiate much more readily than mouse β-cells *in vitro*.[12] Despite distinctions between human and rodent islets with respect to architecture[43] and higher prevalence of GLUT1 utilization over GLUT2,[44–48] Smukler *et al.* demonstrated that Ins^{+}Glut2^{LO} cells from human islets had a similar phenotype, occurrence, and lineage plasticity to those found in mice.[5] Moreover, GLUT2 has been recently implicated in human neonatal diabetes[49] and its importance for glucose transport into human β-cells has been demonstrated.[50] If Ins^{+}GLUT2^{LO} cells are as abundant in the small endocrine clusters of human pancreas from young donors as we have shown here in mice, the inclusion of these cells may enhance the effectiveness and longevity of human islet transplants.

Dor *et al.* showed that β-cells within extra-islet clusters had a similar labeling index with a genetic lineage tag as did those in larger islets, and concluded that these represented small islets, or islets with β-cells undergoing apoptosis, rather than reflecting a new source of β-cells derived from a population with progenitor capabilities.[1] Our experiments differ in that; 1) we used a non-inducible transgenic model which results in the labeling of a much higher percentage of β-cells allowing for greater discrimination in sub-population changes, and 2) we studied neonatal as opposed to adult mice. Within this paradigm, we have shown that there is significantly lower lineage labeling with HPAP in β-cells located within β-cell clusters than in anatomically mature islets in P7 mice (39% versus 85%), which is likely to reflect the generation of new β-cells from progenitors within the clusters during early postnatal life. While the number of extra-islet clusters decreases with age in rodents, they are still found in adulthood.[31] Chintinne *et al.* found that the majority of β-cells in young rats were present in small aggregates ($<$50 μm diameter, including single cells) which formed postnatally, and concluded that the neogenesis and clustering of these aggregates is a key process to attaining β-cell mass in adulthood.[51] Significantly, it was recently reported that after pancreatic duct ligation of 8 week old mice, the highest proliferation of insulin-expressing cells had occurred in small islets of $<$20 β-cells, and which had derived from Ngn3^{+} cells within these small β-cell clusters.[52] Our data would suggest that such β-cells in mice represent, in part, a population that has recently differentiated from insulin^{-} progenitors, which consequently did not tag with HPAP.

As indicated by Murtaugh, [53] the study by Smukler *et al.*[5] used the inducible RIPCreER; Z/EG model to tag β-cells and follow them *in vivo*: a model in which only a subset of β-cells were tagged, and which, it has been suggested, may inappropriately label a fraction of acinar cells over an extended chase, raising concern over RIPCreER transgene stringency. [54] The present study used the non-inducible strain RIPCre to tag a high proportion of β-cells with HPAP before islet isolation and *in vitro* culture. All lineage tracing experiments contain inherent limitations, including the assumption of reporter indelibility without dilution, and the theoretical possibility that Cre driven by a minimal promoter may be subject to promiscuous expression in non-native conditions, such as tissue culture. As only ~0.2% of islet-derived cells present in dedifferentiation culture after 1 week immunostained for the β-cell reporter HPAP, we propose that this number does not over-represent the surviving β-cell population. The stoichiometric analysis is further complicated by the relative rarity of the Ins^{+}Glut2^{LO} cell population, and the observation that in their primary location, the extra-islet endocrine clusters, fewer neonatal β-cells immunostained for HPAP than did those within islets. However, the cytoplasmic HPAP reporter in our model required us to employ a proxy β-cell marker, GPM6a [20] for cell sorting, thus we did not sort β-cells based on a putatively random event. Furthermore, while lineage tracing analyses were reliant on Cre-based reporter expression, Ins^{+}Glut2^{LO} cells themselves were identified *in vivo* and showed differential expression based on age, providing physiological relevance and a future avenue for study. Also, as demonstrated, not all Ins^{+}Glut2^{LO} cells were multipotent. Thus we have identified Ins^{+}Glut2^{LO} cells using different techniques (FACS and immunofluorescence), multiple markers for β-cells (insulin, GPM6a, HPAP, and eYFP), and 2 separate antibodies for Glut2, indicating that these findings are collectively unlikely to represent artifact.

This work builds on evidence that the mouse and human pancreas contain insulin-expressing multipotential cells, which can be identified by the absence

of Glut2.[4,5] Determining the fate of these progenitors during maturation, and the age at which their numbers cease to be a feasible source of renewable insulin-producing cells, may elucidate the extent of postnatal β-cell plasticity.

Abbreviations

Ck19	cytokeratin 19
Cyclo A	Cyclophilin A
Glut2	glucose transporter 2
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GPM6a	glycoprotein M6a
GSIS	glucose stimulated insulin secretion
HPAP	human placental alkaline phosphatas
MafB	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B
Ngn3	neurogenin3
Nkx6.1	homeobox protein Nkx_6.1
Pdx1	pancreatic duodenal homeodomain box 1
RIA	radioimmunoassay
YFP	yellow fluorescent protein

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We wish to thank K. Chadwick for cell sorting, K. Nygard for confocal microscopy assistance, and A.R. Cox, M.D Seferovic, and D.A. Hess for experimental discussion and reading of the manuscript.

Funding

This work was supported by grants from the Canadian Institute of Health Research (MOP-15263), the Alan Thicke Center for Juvenile Diabetes Research, and the Program of Experimental Medicine (R0362A06) through the Department of Medicine at the Schulich School of Medicine, Western University, London ON, Canada.

References

[1] Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 2004; 429:41-6; PMID:15129273; http://dx.doi.org/10.1038/nature02520

[2] Teta M, Rankin MM, Long SY, Stein GM, Kushner JA. Growth and regeneration of adult beta cells does not involve specialized progenitors. Dev Cell 2007; 12:817-26; PMID:17488631; http://dx.doi.org/10.1016/j.devcel.2007.04.011

[3] Nir T, Melton DA, Dor Y. Recovery from diabetes in mice by beta cell regeneration. J Clin Invest 2007; 117:2553-61; PMID:17786244; http://dx.doi.org/10.1172/JCI32959

[4] Seaberg RM, Smukler SR, Kieffer TJ, Enikolopov G, Asghar Z, Wheeler MB, Korbutt G, van der Kooy D. Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. Nat Biotech 2004; 22:1115-24; http://dx.doi.org/10.1038/nbt1004

[5] Smukler SR, Arntfield ME, Razavi R, Bikopoulos G, Karpowicz P, Seaberg R, Dai F, Lee S, Ahrens R, Fraser PE, et al. The adult mouse and human pancreas contain rare multipotent stem cells that express insulin. Cell Stem Cell 2011; 8:281-93; PMID:21362568; http://dx.doi.org/10.1016/j.stem.2011.01.015

[6] Zhou Q, Law AC, Rajagopal J, Anderson WJ, Gray PA, Melton DA. A multipotent progenitor domain guides pancreatic organogenesis. Dev Cell 2007; 13:103-14; PMID:17609113; http://dx.doi.org/10.1016/j.devcel.2007.06.001

[7] Hanley SC, Pilotte A, Massie B, Rosenberg L. Cellular origins of adult human islet in vitro dedifferentiation. Lab Investig 2008; 88:761-72; PMID:18490899; http://dx.doi.org/10.1038/labinvest.2008.41

[8] Yuan S, Rosenberg L, Paraskevas S, Agapitos D, Duguid WP. Transdifferentiation of human islets to pancreatic ductal cells in collagen matrix culture. Differentiation 1996; 61:67-75; PMID:8921586; http://dx.doi.org/10.1046/j.1432-0436.1996.6110067.x

[9] Jamal A-M, Lipsett M, Sladek R, Laganière S, Hanley S, Rosenberg L. Morphogenetic plasticity of adult human pancreatic islets of Langerhans. Cell Death Differ 2005; 12:702-12; PMID:15818398; http://dx.doi.org/10.1038/sj.cdd.4401617

[10] Puri S, Folias AE, Hebrok M. Plasticity and Dedifferentiation within the Pancreas: Development, Homeostasis, and Disease. Cell Stem Cell 2015; 16:18-31; PMID:25465113.

[11] Okada T. Transdifferentiation in animal models: fact or artifact? Dev Growth Differ 1986; 28:213-21; http://dx.doi.org/10.1111/j.1440-169X.1986.00213.x

[12] Russ HA, Bar Y, Ravassard P, Efrat S. In vitro proliferation of cells derived from adult human beta-cells revealed by cell-lineage tracing. Diabetes 2008; 57:1575-83; PMID:18316362; http://dx.doi.org/10.2337/db07-1283

[13] Weinberg N, Ouziel-Yahalom L, Knoller S, Efrat S, Dor Y. Lineage tracing evidence for in vitro dedifferentiation but rare proliferation of mouse pancreatic beta-cells. Diabetes 2007; 56:1299-304; PMID:17303800; http://dx.doi.org/10.2337/db06-1654

[14] Gannon M, Shiota C, Postic C, Wright CV, Magnuson M. Analysis of the Cre-mediated recombination driven by rat insulin promoter in embryonic and adult mouse pancreas. Genesis 2000; 26:139-42; PMID:10686610; http://dx.doi.org/10.1002/(SICI)1526-968X(200002)26:2%3c139::AID-GENE12%3e3.0.CO;2-7

[15] Lobe CG, Koop KE, Kreppner W, Lomeli H, Gertsenstein M, Nagy A. Z/AP, a double reporter for cre-mediated recombination. Dev Biol 1999; 208:281-92; PMID:10191045; http://dx.doi.org/10.1006/dbio.1999.9209

[16] Hehmke B, Kohnert KD, Odselius R. The use of a new dextran gradient medium for rapid isolation of functionally intact neonatal rat pancreatic islets. Diabetes Res 1986; 3:13-6; PMID:2420503

[17] Cox AR, Beamish CA, Carter DE, Arany EJ, Hill DJ. Cellular mechanisms underlying failed beta cell regeneration in offspring of protein-restricted pregnant mice. Exp Biol Med 2013; 238:1147-59.

[18] Russ HA, Sintov E, Anker-Kitai L, Friedman O, Lenz A, Toren G, Farhy C, Pasmanik-Chor M, Oron-Karni V, Ravassard P, et al. Insulin-producing cells generated from dedifferentiated human pancreatic beta cells expanded in vitro. PLoS One 2011; 6:e25566; PMID:21984932; http://dx.doi.org/10.1371/journal.pone.0025566

[19] Blum B, Hrvatin SSŠ, Schuetz C, Bonal C, Rezania A, Melton DA. Functional beta-cell maturation is marked by an increased glucose threshold and by expression of urocortin 3. Nat Biotech 2012; 30:261-4; http://dx.doi.org/10.1038/nbt.2141

[20] Dorrell C, Grompe MT, Pan FC, Zhong Y, Canaday PS, Shultz LD, Greiner DL, Wright CV, Streeter PR, Grompe M. Isolation of mouse pancreatic alpha, beta, duct and acinar populations with cell surface markers. Mol Cell Endocrinol 2011; 339:144-50; PMID:21539888; http://dx.doi.org/10.1016/j.mce.2011.04.008

[21] Hrvatin S, O'Donnell CW, Deng F, Millman JR, Pagliuca FW, DiIorio P, Rezania A, Gifford DK, Melton DA. Differentiated human stem cells resemble fetal, not adult, β cells. PNAS 2014; 111:3038-43; PMID:24516164; http://dx.doi.org/10.1073/pnas.1400709111

[22] Kroon E, Martinson L a, Kadoya K, Bang AG, Kelly OG, Eliazer S, Young H, Richardson M, Smart NG, Cunningham J, et al. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotech 2008; 26:443-52; http://dx.doi.org/10.1038/nbt1393

[23] Weir GC, Aguayo-Mazzucato C, Bonner-Weir S. B-Cell Dedifferentiation in Diabetes Is Important, But What Is It? Islets 2013; 5:233-7; PMID:24356710; http://dx.doi.org/10.4161/isl.27494

[24] White MG, Marshall HL, Rigby R, Huang GC, Amer A, Booth T, White S, Shaw JAM. Expression of mesenchymal and α-cell phenotypic markers in islet β-cells in recently diagnosed diabetes. Diabetes Care 2013; 36:3818-20; PMID:24062329; http://dx.doi.org/10.2337/dc13-0705

[25] Talchai C, Xuan S, Lin HV, Sussel L, Accili D. Pancreatic β cell dedifferentiation as a mechanism of diabetic β cell failure. Cell 2012; 150:1223-34; PMID:22980982; http://dx.doi.org/10.1016/j.cell.2012.07.029

[26] El-Gohary Y, Tulachan S, Wiersch J, Guo P, Welsh C, Prasadan K, Paredes J, Shiota C, Xiao X, Wada Y, et al. A smad signaling network regulates islet cell proliferation. Diabetes 2014; 63:224-36; PMID:24089514; http://dx.doi.org/10.2337/db13-0432

[27] Piran R, Lee S-H, Li C-R, Charbono A, Bradley LM, Levine F. Pharmacological induction of pancreatic islet cell transdifferentiation: relevance to type I diabetes. Cell Death Dis 2014; 5:e1357; http://dx.doi.org/10.1038/cddis.2014.311

[28] Van der Meulen T, Xie R, Kelly OG, Vale WW, Sander M, Huising MO. Urocortin 3 marks mature human primary and embryonic stem cell-derived pancreatic alpha and beta cells. PLoS One 2012; 7:e52181

[29] Blum B, Roose AN, Barrandon O, Maehr R, Arvanites AC, Davidow LS, Davis JC, Peterson QP, Rubin LL, Melton DA. Reversal of β cell de-differentiation by a small molecule inhibitor of the TGFβ pathway. Elife 2014; 3:e02809; PMID:25233132

[30] Morshead CM, Garcia AD, Sofroniew MV, van Der Kooy D. The ablation of glial fibrillary acidic protein-positive cells from the adult central nervous system results in the loss of forebrain neural stem cells but not retinal stem cells. Eur J Neurosci 2003; 18:76-84; PMID:12859339; http://dx.doi.org/10.1046/j.1460-9568.2003.02727.x

[31] Jo J, Kilimnik G, Kim A, Guo C, Periwal V, Hara M. Formation of pancreatic islets involves coordinated expansion of small islets and fission of large interconnected islet-like structures. Biophys J 2011; 101:565-74; PMID:21806924; http://dx.doi.org/10.1016/j.bpj.2011.06.042

[32] Gu G, Dubauskaite J, Melton DA. Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 2002; 129:2447-57; PMID:11973276

[33] Liu H, Guz Y, Kedees MH, Winkler J, Teitelman G. Precursor cells in mouse islets generate new beta-cells in vivo during aging and after islet injury. Endocrinology 2010; 151:520-8; PMID:20056825; http://dx.doi.org/10.1210/en.2009-0992

[34] Szabat M, Luciani DS, Piret JM, Johnson JD. Maturation of adult beta-cells revealed using a Pdx1/insulin dual-reporter lentivirus. Endocrinology 2009; 150:1627-35; PMID:19095744; http://dx.doi.org/10.1210/en.2008-1224

[35] Merkwitz C, Blaschuk OW, Schulz A, Lochhead P, Meister J, Ehrlich A, Ricken AM. The ductal origin of structural and functional heterogeneity between pancreatic islets. Prog Histochem Cytochem 2013; 48:103-40; PMID:24100070; http://dx.doi.org/10.1016/j.proghi.2013.09.001

[36] Jermendy A, Toschi E, Aye T, Koh A, Aguayo-Mazzucato C, Sharma A, Weir GC, Sgroi D, Bonner-Weir S. Rat neonatal beta cells lack the specialised metabolic phenotype of mature beta cells. Diabetologia 2011; 54:594-604; PMID:21240476; http://dx.doi.org/10.1007/s00125-010-2036-x

[37] Aye T, Toschi E, Sharma A, Sgroi D, Bonner-Weir S. Identification of markers for newly formed beta-cells in the perinatal period: a time of recognized beta-cell immaturity. J Histochem Cytochem 2010; 58:369-76; PMID:20051380; http://dx.doi.org/10.1369/jhc.2009.954909

[38] Richardson CC, Hussain K, Jones PM, Persaud S, Löbner K, Boehm A, Clark A, Christie MR. Low levels of glucose transporters and K+ATP channels in human pancreatic

beta cells early in development. Diabetologia 2007; 50:1000-5; PMID:17380317; http://dx.doi.org/10.1007/s00125-007-0644-x

[39] Manning Fox JE, Seeberger K, Dai XQ, Lyon J, Spigelman AF, Kolic J, Hajmrle C, Joseph JW, Kin T, Shapiro AMJ, et al. Functional Plasticity of the Human Infant β-Cell Exocytotic Phenotype. Endocrinology 2013; 154:1392-9; PMID:23449893; http://dx.doi.org/10.1210/en.2012-1934

[40] Ghazalli N, Mahdavi A, Feng T, Jin L, Kozlowski MT, Hsu J, Riggs AD, Tirrell D a, Ku HT. Postnatal Pancreas of Mice Contains Tripotent Progenitors Capable of Giving Rise to Duct, Acinar, and Endocrine Cells In Vitro. Stem Cells Dev 2015; 0:1-14

[41] Stolovich-Rain M, Hija A, Grimsby J, Glaser B, Dor Y. Pancreatic beta cells in very old mice retain capacity for compensatory proliferation. J Biol Chem 2012; 287:27407-14; PMID:22740691; http://dx.doi.org/10.1074/jbc.M112.350736

[42] Razavi R, Najafabadi HS, Abdullah S, Smukler S, Arntfield M, van der Kooy D. Diabetes enhances the proliferation of adult pancreatic multipotent progenitor cells and biases their differentiation to more beta-cell production. Diabetes 2015; 64:1311-1323; PMID:25392245.

[43] Brissova M, Fowler MJ, Nicholson WE, Chu A, Hirshberg B, Harlan DM, Powers AC. Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. J Histochem Cytochem 2005; 53:1087-97; PMID:15923354; http://dx.doi.org/10.1369/jhc.5C6684.2005

[44] De Vos A, Heimberg H, Quartier E, Huypens P, Bouwens L, Pipeleers D, Schuit F. Human and rat beta cells differ in glucose transporter but not in glucokinase gene expression. J Clin Invest 1995; 96:2489-95; PMID:7593639; http://dx.doi.org/10.1172/JCI118308

[45] Ferrer J, Benito C, Gomis R. Pancreatic islet GLUT2 glucose transporter mRNA and protein expression in humans with and without NIDDM. Diabetes 1995; 44:1369-74; PMID:7589840; http://dx.doi.org/10.2337/diab.44.12.1369

[46] Heimberg H, De Vos A, Pipeleers D, Thorens B, Schuit F. Differences in glucose transporter gene expression between rat pancreatic alpha- and beta-cells are correlated to differences in glucose transport but not in glucose utilization. J Biol Chem 1995; 270:8971-5; PMID:7721807; http://dx.doi.org/10.1074/jbc.270.15.8971

[47] McCulloch LJ, van de Bunt M, Braun M, Frayn KN, Clark A, Gloyn AL. GLUT2 (SLC2A2) is not the principal glucose transporter in human pancreatic beta cells: implications for understanding genetic association signals at this locus. Mol Gen Metab 2011; 104:648-53; http://dx.doi.org/10.1016/j.ymgme.2011.08.026

[48] Coppieters KT, Wiberg A, Amirian N, Kay TW, Von Herrath MG. Persistent glucose transporter expression on pancreatic beta cells from longstanding type 1 diabetic individuals. Diabetes Metab Rev 2011; 27:746-54; http://dx.doi.org/10.1002/dmrr.1246

[49] Sansbury FH, Flanagan SE, Houghton JA, Shuixian Shen FL, Al-Senani AM, Habeb AM, Abdullah M, Kariminejad A, Ellard S, Hattersley AT. SLC2A2 mutations can cause neonatal diabetes, suggesting GLUT2 may have a role in human insulin secretion. Diabetologia 2012; 55:2381-5; PMID:22660720; http://dx.doi.org/10.1007/s00125-012-2595-0

[50] Van de Bunt M, Gloyn a L. A tale of two glucose transporters: how GLUT2 re-emerged as a contender for glucose transport into the human beta cell. Diabetologia 2012; 55:2312-5; PMID:22696037; http://dx.doi.org/10.1007/s00125-012-2612-3

[51] Chintinne M, Stange G, Denys B, In 't Veld P, Hellemans K, Pipeleers-Marichal M, Ling Z, Pipeleers D. Contribution of postnatally formed small beta cell aggregates to functional beta cell mass in adult rat pancreas. Diabetologia 2010; 53:2380-8; PMID:20645074; http://dx.doi.org/10.1007/s00125-010-1851-4

[52] Van de Casteele M, Leuckx G, Baeyens L, Cai Y, Yuchi Y, Coppens V, De Groef S, Eriksson M, Svensson C, Ahlgren U, et al. Neurogenin 3(+) cells contribute to β-cell neogenesis and proliferation in injured adult mouse pancreas. Cell Death Dis 2013; 4:e523; PMID:23470530; http://dx.doi.org/10.1038/cddis.2013.52

[53] Murtaugh LC. Stem cells and β cells: the same, but different? Cell Stem Cell 2011; 8:244-5; PMID:21362562; http://dx.doi.org/10.1016/j.stem.2011.02.010

[54] Blaine S, Ray KC, Anunobi R, Gannon M, Washington MK, Means AL. Adult pancreatic acinar cells give rise to ducts but not endocrine cells in response to growth factor signaling. Development 2010; 137:2289-96; PMID:20534672; http://dx.doi.org/10.1242/dev.048421