11-28-2014 12:00 AM

Pancreatic Beta Cell Plasticity and Involvement of Insulin-Expressing Progenitor Cells

Christine A. Beamish
The University of Western Ontario

Supervisor
Dr. David Hill
The University of Western Ontario

Graduate Program in Physiology and Pharmacology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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Insulin^{+} progenitor β-cells

An integrated-article thesis

By

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Doctor of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

Abstract

Islet transplants have been successfully used as treatment for diabetes, but are limited by shortages of cadaveric insulin-producing β-cells. An alternate source may be the dedifferentiation, expansion, and subsequent redifferentiation of pancreatic islets or β-cells using *in vitro* techniques prior to transplant. Based on protocols which described the dedifferentiation of human islets to ductal-like cells, we hypothesized that neonatal mouse islets could be similarly dedifferentiated *in vitro*.

Dedifferentiation techniques produced significant duct-like cells, but redifferentiation to insulin-expressing cells was limited. RIPCre;Z/AP$^{+/+}$ mice were consequently utilized to lineage trace β-cell fate during culture by a human placental alkaline phosphatase (HPAP) reporter. The proportion of HPAP^{+} β-cells decreased significantly in culture, but the rare remaining cells expressed the ductal marker CK19. Flow cytometric sorting of β-cell subpopulations from whole pancreas showed that the HPAP^{+}CK19^{+} cells had derived from insulin-positive, glucose-transporter-2-negative (Ins^{+}Glut2^{-}) cells, previously purported as insulin-expressing progenitor cells. In P7 mouse pancreas, these Ins^{+}Glut2^{-} cells represent 3.5% of all insulin^{+} cells, the majority of which were found outside of islets within β-cell aggregates (BCA, <5 β-cells). Ins^{+}Glut2^{-} cells demonstrated greater proliferation rates *in vivo* and *in vitro* as compared to Ins^{+}Glut2^{+} cells, and a subset could differentiate into endocrine, ductal, and neural lineages. We sought to quantify the presence of these progenitor cells throughout life in both mouse and human pancreata, and how their abundance and location changes with age. The presence of Ins^{+}Glut2^{-} cells in human and mouse pancreas demonstrated similar distributions and

ontogenies, being more abundant in BCA than islets at all ages sampled, and decreasing with age. Finally, neonatal rodents can regenerate β-cell mass after streptozotocin (STZ) exposure, but this response is mitigated in adulthood. As STZ accesses the β-cell via Glut2, we hypothesized that the β-cell regenerative capacity in early life involves mobilization of $Ins^{+}Glut2^{-}$ cells, and used RIPCreER;$Z/AP^{+/+}$ mice to trace these during damage and regeneration. We found that $Ins^{+}Glut2^{-}$ cells indeed survived STZ exposure, and subsequently matured into $Ins^{+}Glut2^{+}$ cells.

These $Ins^{+}Glut2^{-}$ cells represent a transitional cell type, the majority of which contribute to pancreas maturation, and a subset of which retains multipotential lineage capability.

Keywords

β-cell, cellular plasticity, development, diabetes, differentiation, duct, endocrine, Glut2, insulin, islet, lineage tracing, progenitor cell, regeneration, stem cell, streptozotocin, tissue culture

Co-authorship Statement

The studies described in data chapters 2-5 were performed by Christine A. Beamish in the laboratory of Dr. David Hill, with the assistance of the co-authors listed below.

For all experimentation, the contribution from Dr. David Hill was of an intellectual nature with respect to the experimental design, data analysis & interpretation, and manuscript preparation. All thesis chapters were written by Christine A Beamish with modifications and review by Dr. David Hill.

Chapter 2.

Brenda Strutt performed and assisted with analysis of the radioimmunoassays. NOD/scid IL-2rγ$^{-/-}$ mice were provided by Dr. David Hess. Transplantation experiments were performed by Dr. Andrew Pepper with assistance from Delfina Siroen. Endothelial cell co-culture experiments were performed in the laboratory of Dr. Gedes Cepinskas.

Chapter 3.

Sofia Mehta, Brendan Cheuk, and Yuzhen Tong performed some immunohistochemical staining. Brenda Strutt performed and assisted with analysis of the radioimmunoassays and qPCR experiments, as well as assisted with some tissue culture experiments. Dr. Kristin Chadwick performed the flow cytometric cell sorting.

Chapter 4.

Dr. Subrata Chakrabarti and Dr. Manami Hara, in association with Drs Piotr Witkowski and Michael Mills, provided human pancreas samples as sections on slides. Sofia Mehta performed immunohistochemical staining, and assisted with confocal microscopy.

Chapter 5.

Sofia Mehta performed some immunohistochemical staining, confocal microscopy, and pancreas morphometry analysis. Brandon Cheuk performed some immnohistochemical staining and pancreas morphometry analysis. Lynda McCaig assisted with mouse injections.

Acknowledgements

I would like to acknowledge the support, patience, and understanding of my supervisor and mentor, **Dr. David Hill**. This project took many years, and many hands, to accomplish and would not have been possible without his vision and dedication. I recognize the freedom and trust awarded in order to explore the limits of this project. I also very much appreciate the many opportunities to attend conferences, which significantly aided in my training as a scientist.

I thank **Brenda Strutt** for being a very talented technician and teacher, project collaborator, office-mate, and friend. I thank **Dr. Edith Arany** for support both professionally and personally, as an advocate and sounding-board when the view was unclear. I thank **Sheila Fleming** for her tireless optimism and immense foresight, and for teaching us all to make our world more beautiful. I acknowledge contributions from past Hill and Arany lab members, including **Kelly Marchand**, **Dr**. **Michael Nicholson**, **Dr. Astrid Chamson-Reig**, and **Dr. Sandra Thyssen**. Their friendship, leadership, and coordination over the years is much appreciated. I thank **Brandon Cheuk** for his steady resolve, and **Sofia Mehta** for her quiet, yet passionate, inquisitiveness and perseverance. I also thank **Dr. Aaron Cox** for his friendship, advice, and perspective while undertaking a parallel goal.

I thank the members of my advisory committee for their support throughout this process; **Dr. David Hess** for assistance with the transplantation experiments, as well as for experimental suggestions and manuscript revision of Chapter 4; **Dr. Subrata Chakrabarti** for

his quick collaboration on the human pancreas ontogeny; and **Dr. David O'Gorman** for long standing guidance since our shared days in the Han laboratory, and for assistance with quantitative PCR experimental design and analysis.

I thank **Karen Nygard** and **Nicole Bechard** for their assistance with confocal microscopy. Murphy's Law was alive and well when I was present at the Biotron. Their problem solving abilities and good-natured attitudes are commendable.

I thank the incredible support from my family, who have been fierce advocates of research, and diabetes research specifically, since I was a child. I thank my parents, **Paul** and **Maureen**, for instilling in all of their children the spirit of curiosity, the desire to strive for answers, and the examples they have set to contribute back to the world. I also thank my siblings **Katie, Alex,** and **Daniel**, for their encouragement, and for bolstering me when I needed it most.

Finally, I thank **Max**, for his love and steadfast support. I could not have done this without him.

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List of Abbreviations, Symbols, and Nomenclature

7-AAD	7-aminoactinomycin
α	alpha
β	beta
β-gal	beta galactosidase
δ	delta
ε	epsilon
°C	degrees Celsius
pg	picogram
ng	nanogram
µg	microgram
mg	milligram
g	gram
kg	kilogram
µL	microlitre
mL	millilitre
L	litre
mmol	millimole
mol	mole
mM	millimolar
pM	picomolar
µm	micrometre
ADP	adenosine di-phosphate
ALDH	aldehyde dehydrogenase
ANOVA	analysis of variance
ATP	adenosine tri-phosphate
β-act	beta-actin
β-tub	beta-tubulin
BCA	β-cell aggregate
BMI	body mass index
CAII	carbonic anhydrase II

cAMP	cyclic adenosine monophosphate
CK19	cytokeratin 19
CMV	cytomegalovirus
Cre	cyclization recombinase enzyme
Ct	cycle threshold
DAPI	4’,6–diamidino–2–phenlindole
DNA	deoxyribonucleic acid
DMEM/F-12	Dulbecco's modified eagle medium plus Ham’s F-12
Dx	doxycycline
e	embryonic day
E2F	family of DNA-binding transcription factors
EBM2	endothelial cell basal media-2
EC	endothelial cell
ECM	extracellular matrix
E-cad	e-cadherin
EDTA	ethylenediaminetetraacetic acid
EdU	5-ethynyl-2´-deoxyuridine
EGF	epidermal growth factor
eGFP	enhanced green fluorescent protein
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
ES	embryonic stem (cell)
FACS	fluorescent activated cell sort
FBS	fetal bovine serum
FGF-2	fibroblast growth factor 2
FOV	field of view
G	gaps (cell cycle); or gestation
Gck	glucokinase
GFAP	glial fibrillary associated protein
Glut2	glucose transporter-2
GPm6a	glycoprotein m6a
GSIS	glucose-stimulated insulin secretion

HbA_{1C}	glycated haemoglobin A_{1C}
HBSS	Hank's Buffered Salt Solution
HCl	hydrochloric acid
HGF	hepatocyte growth factor
Hnf1β	hepatocyte nuclear factor 1-beta
Hnf6	hepatocyte nuclear factor 6
HPAP	human placental alkaline phosphatase
IGF-II	insulin-like growth factor-2
Ins	insulin
IPGTT	intraperitoneal glucose tolerance test
ITS	insulin/transferrin/selenium
KGF	keratinocyte growth factor
Ki67	antigen identified by monoclonal antibody Ki67
LacZ	lactose operon, encoding β-galactosidase
LIF	leukemia inhibitory factor
loxP	locus of crossing over, bacteriophage P1
LSCM	laser scanning confocal microscopy
M	mitosis
MafA	V-maf musculoaponeurotic fibrosarcoma oncogene homolog A
MafB	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B
mRNA	messenger ribonucleic acid
MSC	mesenchymal stem cell
Muc1	mucin 1
n	sample size
NaCl	sodium chloride
NBT/BCIP	Nitro-Blue Tetrazolium Chloride/ 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt
NeuroD	neurogenic differentiation-1
Ngn3	neurogenin 3
NK	natural killer (cell)
Nkx2.2	NK2 transcription factor related, locus 2
Nkx6.1	NK6 homeobox 1

NOD/SCID IL-2rγ$^{-/-}$	non-obese diabetic/severe compromised immune deficient interleukin-2 recepter gamma knockout
OCT	optimal cutting temperature medium
P	postnatal day
p	p-value, probability
Pax4	paired box gene 4
Pax6	paired box gene 6
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDA	pancreatic ductal adenocarcinoma
PDEC	pancreatic ductal epithelial cells
PDGF	platelet-derived growth factor
PDL	partial duct ligation
Pdx1	pancreatic duodenal homeobox-1
PECAM	platelet endothelial cell adhesion molecule
PFA	paraformaldehyde
pRb	retinoblastoma protein
Px	partial pancreatectomy
qPCR	quantitative (real-time) RT-PCR
R26R	Rosa26 reporter
RIA	radioimmunoassay
RIP	rat insulin promoter
RNA	ribonucleic acid
RT	reverse transcription
rtTA	reverse tetracycline-dependent transactivator
S	DNA replication
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
sh	short hairpin
Sox9	sex determining region Y-box 9
STZ	streptozotocin
T1D	type 1 diabetes

TET-DTA	tetracycline operator–diphtheria toxin A
TBS	Tris-buffered saline
TGFα	transforming growth factor-alpha
TM	tamoxifen
TUNEL	terminal deoxynycleotidyl transferase dUTP nick end labeling
UV	ultra-violet
VR	Vector Red
VEGF	vascular endothelial growth factor
Vim	vimentin
Wnt1	wingless-type MMTV integration site family member 1
X-gal	5-bromo-4-chloro-indolyl-β-D-galactopyranoside

CHAPTER 1

Introduction

1.1 Diabetes

Diabetes mellitus is a metabolic disease of the pancreas, characterized by increased levels of glucose in the blood (hyperglycemia). It is caused by an insufficient production of insulin, as found in Type I diabetes (T1D), or by cells that do not effectively or appropriately respond to the insulin produced, as found in Type II diabetes (T2D). The hormone insulin is secreted by pancreatic β-cells located in the islets of Langerhans, a spherical cluster of endocrine cells containing predominantly insulin-producing β-cells. Without insulin, the body cannot derive energy from carbohydrate metabolism for use in its target tissues, including liver, muscle, and adipose. Patients with undiagnosed or untreated diabetes typically present with fatigue, increased urination, increased thirst, and weight loss.

Diabetes was first described >3000 B.C. by the Egyptians, and later by Greek physicians who coined the term *diabetes,* meaning, “to siphon”, referring to the large quantities of urine produced. The disease had also been described in India 400 B.C. as “honeyed urine” (1,2). For most of history, a diagnosis of type I diabetes was tantamount to a death sentence; it was not until 1922 that Frederick Banting and colleagues Charles Best, James Collip, and John Macleod discovered the hormone insulin, which was life-saving for patients with the disease (3,4).

Today, diabetes affects more than 285 million people worldwide, 9 million of them living in Canada with diabetes or pre-diabetes (5). This number is expected to surpass 438 million people worldwide by 2030. The vast majority of new cases is due to T2D, and attributable to a variety of factors, including an aging population, a sedentary lifestyle, and the worldwide

obesity epidemic (5). The cost to the Canadian healthcare system is expected to reach $16.9 billion by 2020 (5).

While a diagnosis of diabetes today does not carry with it the imminent death sentence it did a century ago, it is still the fourth leading cause of death by disease globally, with a morbidity rate of 4.6 million people annually (6). Death is associated with diabetes, but is commonly ascribed to complications of the disease, and only rarely to acute metabolic circumstances such as severe hyperglycemia and accompanying ketoacidosis, or its opposite, hypoglycemia (7). The long term sequelae of the disease are broadly classified into micro- and macro-vascular complications, as a result of both endothelial cell damage to small vessels, and elevated blood lipids which are deleterious to large vessels (7). Sustained exposure to hyperglycemia cause severe complications such as retinopathy (8,9), neuropathy (10), cardiovascular disease (11), and nephropathy (12). The majority of people with diabetes die of heart disease or stroke (6).

While the complications experienced are similar between diabetes types, <10% of all patients with the disease have T1D. T1D differentiates from T2D in that it is an autoimmune disease which specifically destroys the β-cells in the islets of Langerhans, mediated by T-cell activation and islet-specific autoantibody presence (13). The conditions which allow this autoimmune storm to proceed are still unclear, although a combination of environmental triggers, such as viral exposure (14), and genetic susceptibility, especially by the presence of the HLA alleles DR3-DQ2/DR4-DQ8 (15), have been implicated. T1D was historically termed “juvenile diabetes” as it was thought to strike only those under 30 years of age; it is now known that persons of any age can develop the disease (16), although it is still most

commonly diagnosed in the young. While pharmacological and bioengineering advances have improved the purity and delivery of the hormone, the current and standard treatment for T1D is still limited to exogenous insulin therapy. Despite advances, persons with T1D live, on average, 15 years less than those without the disease (6).

In 2000, a breakthrough procedure coined the "Edmonton Protocol" was introduced, in which islets of Langerhans were transplanted into patients with diabetes (17,18). This was an improvement from the only other transplant option – an entire pancreas – available solely to patients undergoing concurrent kidney transplant (19). Despite the relative success of islet transplants, it is limited by the need for immune-modulating drugs, as well as the high number of islets needed for transplant: the endocrine portion equivalent from up to three pancreata per recipient, derived from cadaveric sources (17). Even considering these limitations however, the benefits gained to patients seem to outweigh the risks, such as increased circulating C-peptide levels, decreased HbA_{1C}, and improved micro-vascular health (20). Indeed, recent refinements in the procedure have allowed most patients insulin injection independence for >3 years post-transplant (21).

1.2 Pancreas anatomy and function

The head of the pancreas is attached at the initial curve of the duodenum and stretches across the abdomen where the tail attaches to the spleen. The pancreas has dual functions: the exocrine (acinar) cells produce digestive enzymes and bicarbonate, which access the intestine by the highly branched ductal system; and the regulation of blood

glucose by the endocrine component, the islets of Langerhans, which account for ≤2% of the organ (22). A schematic illustration of the pancreas anatomy is shown in **Figure 1.1** (23).

The islets of Langerhans contain 5 distinct endocrine cell types, each of which produces its own hormone. Pancreatic α (alpha) cells release glucagon, which raises glucose by releasing glycogen from the muscle and liver, whereas β (beta) cells release insulin, which lowers blood glucose concentrations by stimulating glucose uptake in the liver, muscle and adipose tissues; these two hormones act synergistically to maintain glucose homeostasis. δ- (delta) cells produce somatostatin (or growth hormone-inhibiting hormone, GHIH), a peptide hormone that regulates the endocrine system and affects neurotransmission, cellular proliferation, and gastric emptying (24,25). ε- (epsilon) cells produce ghrelin, found also in the stomach, which acts to stimulate appetite (26–28) and is the counter-regulatory hormone to leptin. γ-cells (also termed PP cells) produce pancreatic polypeptide, which acts to self-regulate both endocrine and exocrine pancreas secretions (29,30).

In the rodent pancreas, α-cells are located in the mantle of the islet, and account for 14-20% of islet mass, whereas β-cells are located in the islet core, representing 75-80% of islet mass (31–33). δ -cells account for 10% of islet cell proportion (31), with γ-cells and ε -cells composing the remaining minority compartments. In the human, the proportional representation and islet architecture is more heterogeneous: the β-cell proportion varies widely from 28-75% (32,34,35), whereas α-cells represent 10-65% of cells, and δ-cells account for 1.2-22% (32). Moreover, the cells are not organized in a core-mantle

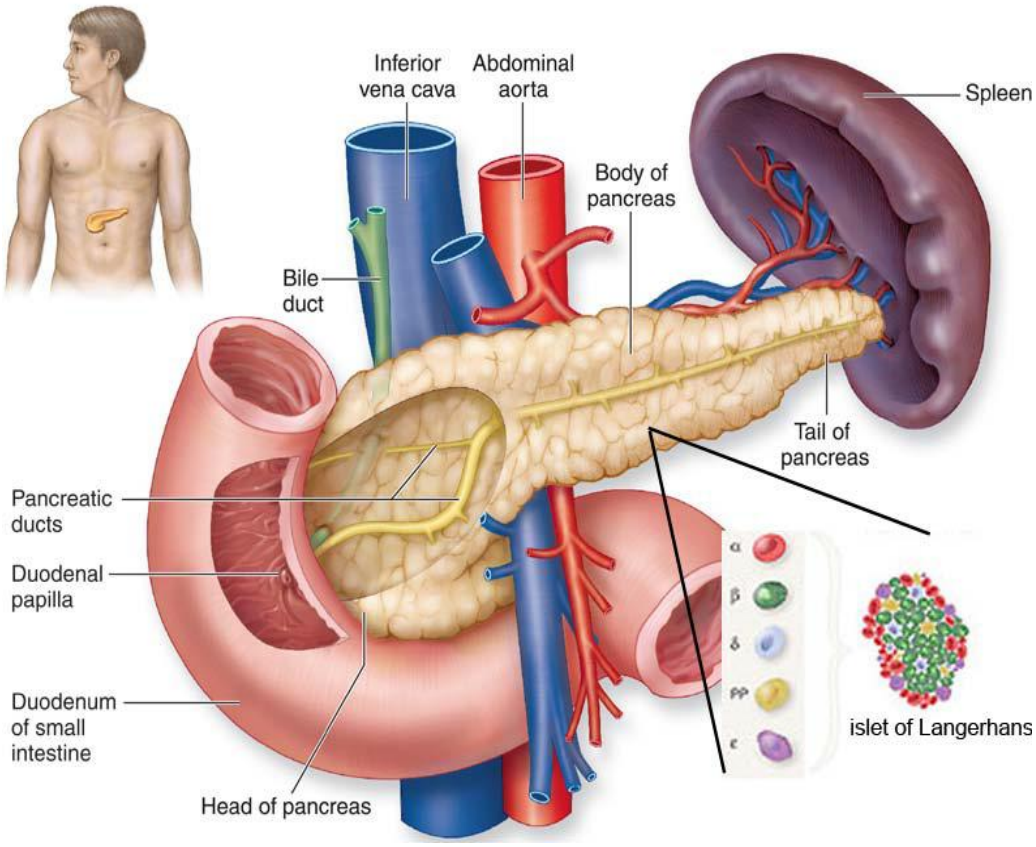

Figure 1.1. Anatomical location of the pancreas
The pancreas is located within the abdomen, and serves to accommodate dual functions: the production of digestive enzymes and bicarbonate by exocrine (acinar) cells, which access the intestine by the highly branched ductal system; and the regulation of blood glucose by the endocrine component, the islets of Langerhans, which account for only 1-2% of the organ. The islets contain 5 distinct cell types, each of which makes its own hormone: β (green, insulin), α (red, glucagon), δ (blue, somatostatin), γ (yellow, pancreatic polypeptide), and ε (purple, ghrelin).

Reproduced from McKinley, M. and O'Loughlin V.D. Human Anatomy, 2nd Edition, 2003.

arrangement as found in rodent islets (31,36), which may be due to alterations in their vasculature arrangement (36).

1.3 Pancreas development

Pancreas development begins in the mouse at embryonic day (e) 8.5 (**Figure 1.2** (37)), when the presence of the transcription factor and homeodomain protein Pancreatic duodenal homeobox-1 (Pdx1/Ipf1) delineates multiple structures, including the pancreas, stomach, duodenum, and bile duct (38,39), and which is expressed in all pancreatic cell progenitors (40). Pdx1 is known as the pancreatic master regulator, playing a critical role in early pancreas development, as well as β-cell differentiation and the maintenance of mature β-cell function later in development (41). $Pdx1^{-/-}$ mice present with complete pancreas agenesis and lethality shortly after birth (42), while inactivation of Pdx1 after bud formation prevents any further islet or acinar differentiation (43–45). By e9.0-9.5, two pancreatic lobes – dorsal and ventral – arise from the posterior foregut, in a region of pre-patterned epithelium (37,46,47), as shown in **Figure 1.2**.

This early epithelium begins an evagination process into the surrounding mesenchyme, as epithelial buds form at a bulge of primary pancreatic multipotent progenitor cells (47) (MPC, red, **Figure 1.3A** (48)). Each pancreatic bud develops into an extensively branched ductal tree between e10.5-e12.5, forming two primordial organs, in a phase termed the primary transition (**Figure 1.2**). Endocrine cells arise from progenitor cells present within the e9.5 gut endoderm in the ductal epithelium, while the acini and ducts

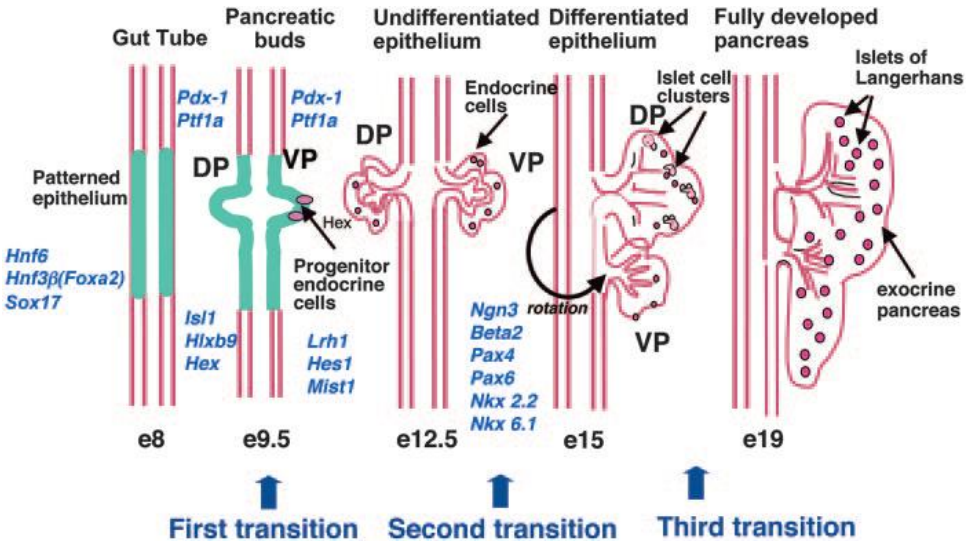

Figure 1.2. Mouse pancreatic development

On embryonic day 8 (e8), patterned epithelium of the primitive foregut forms dorsal pancreas (DP) and ventral (VP) buds by e9.5. These buds then develop into branching ducts and undifferentiated epithelium by e12.5, during the first (developmental) transition. The undifferentiated epithelium contains scattered single endocrine cells. The buds begin to differentiate into endocrine and exocrine cellular lineages by e14, with proliferation and expansion at the second transition. By e15, the two pancreata rotate, fuse, and form a nearly developed pancreas by e19, containing the endocrine cells organized into isolated clusters, termed the islets of Langerhans (third transition). The tertiary transition is characterized by the maturation of endocrine cells, continues for until weaning at postnatal day 21. The representative transcription factors expressed during the program of development are indicated in blue. The approximate embryonic age (in days) is designated for each stage of development.

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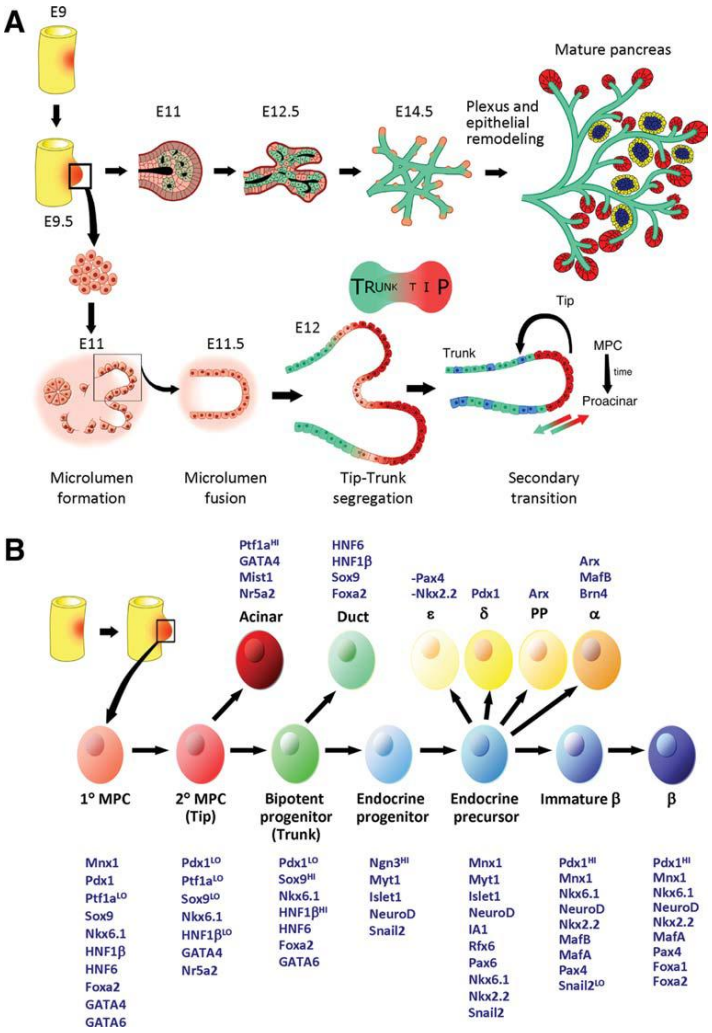

Figure 1.3. Mouse pancreas organogenesis

A: Upper diagram: A region of gut tube epithelium becomes pancreas-specified around E9, at a bulge of pancreatic multipotent progenitor cells (MPC, red), which expands to form a bud. At E11, cells within this bud initiate polarization and constriction, forming multiple microlumens. Coalescence and proliferation at e12.5 of the lumen produces epithelial tubes by e14.5. Ultimately, these tubes remodel, forming a ductal tree as part of the mature pancreas. **Lower diagram**: Epithelium specialization towards acinar (red), or endocrine/duct lineages (blue/green), which delineate from cells within the tip or trunk. Tip and trunk compartmentalization occurs around the time of tube formation (E12). Tip regions are distributed broadly throughout the epithelium. MPC that form tip regions (red) proliferate and undergo tip-splitting, causing outgrowth into the mesenchye, and generating additional trunk epithelium with bipotentiality for endocrine (blue) or duct (green) fates. 'Mature pancreas' shows that acinar cells (red) form the tips of branched ducts. Islets comprise β-cells (blue) surrounded by other endocrine cell types (yellow). **B:** Transcription factors expressed at each stage of pancreas development**.** Note that the same ductal epithelial transcription factors are expressed in early development as well as late.

Reproduced from Pan FC, Wright C. Pancreas organogenesis: from bud to plexus to gland. *Dev Dyn*. 2011;240(3):530–565. Permission granted from John Wiley and Sons.

develop by e14.5 (49). These early endocrine cells contain minimal amounts of glucagon; by e10 – e10.5, they co-express glucagon and insulin, then divide into distinct lineages (37). Early lineage tracing experiments by Herrera showed that adult α- and β-cells derive from independent lineages (50); however, recent experiments by the same group have suggested that α-cells retain the ability to transdifferentiate to β-cells under conditions of extreme β-cell loss (51), and by forced Pdx1 expression (52). Conversely, β-cells have been shown to trans-differentiate to α-cells during T2D diabetes progression (53), further confounding the loss of β-cell mass, and which was postulated to represent β-cell dedifferentiation, and failure as a result of stress (54).

By e12.5, these MPCs have proliferated, the epithelium has stratified, and epithelial tube formation has occurred (**Figure 1.3A**). Around e14, the pancreatic buds begin to differentiate into distinct endocrine and exocrine lineages, a phase termed the secondary transition. Zhou and colleagues found that this differentiation was due to "tip-trunk segregation" of the epithelium, where the tip cells become acinus, and the trunk cells remain as uncommitted trunk (duct-endocrine) cells (55) (**Figure 1.3A**). These bipotent trunk cells are identified by a signature of transcription factors including *Pdx1*, SRY (sex-determining region Y) box9 (*Sox9)*(56–58), hepatocyte nuclear factor 1 beta (*Hnf1β*/*Tcf2*) (59), and hepatocyte nuclear factor 6 *(Hnf6)* (60) (**Figure 1.3B**).

After the bifurcation of the exocrine compartment from duct-endocrine fates, the expression of the bHLH transcription factor neurogenin3 (*Ngn3*) represents a lineage switch, distinguishing cells that become endocrine pancreas from ductal epithelium (61–63) (**Figure 1.3B**). Ngn3 is expressed only in endocrine progenitors and is promptly down-regulated

during terminal differentiation (62). Ngn3$^{-/-}$ animals completely lack all pancreatic endocrine cell types (64).

Around e15, the dorsal and ventral pancreata rotate and fuse, forming a nearly complete pancreas by e19 as shown in **Figure 1.2**. Immature β-cells express multiple transcription factors after fate specification from Ngn3^{+} precursors, including v-Maf musculoaponeurotic fibrosarcoma onco- gene homologue A (*MafA)* and *MafB* (**Figure 1.4** (65)). Between e14-birth, MafA levels increase and MafB levels decrease in β-cells, and MafB expression becomes restricted to α-cells (65,66).

By e19, the endocrine cells have organized into clusters, the islets of Langerhans. Mature β-cells express multiple transcription factors, including Neurogenic Differentiation1 *(NeuroD/Beta2)* (67), Paired box gene4 (*Pax4)* (68), *Pax6* (49,69), Homeobox protein Nkx_2.2 (*Nkx2.2)* (70), and *Nkx6.1* (71). The transcriptional cascade during islet development is shown in **Figure 1.3B**. After e19-e21/P0 (birth), endocrine cells undergo further maturation during the third developmental transition, which continues for 2-3 weeks after birth (37). It is generally assumed, but not entirely proven, that islet cell regeneration in the postnatal period, or progenitor cell differentiation *in vitro*, will proceed by similar (canonical) pathways.

After bifurcating from the common bipotent trunk progenitor state shared with endocrine cells, duct cells maintain the expression of some transcription factors (*Sox9*, *Hnf1β*, and *Hnf6*) but notably lose expression of *Pdx1*, whilst gaining functional markers such as carbonic anhydrase II (*CAII*) (72) and cytokeratin 19 (*CK19*) (73).

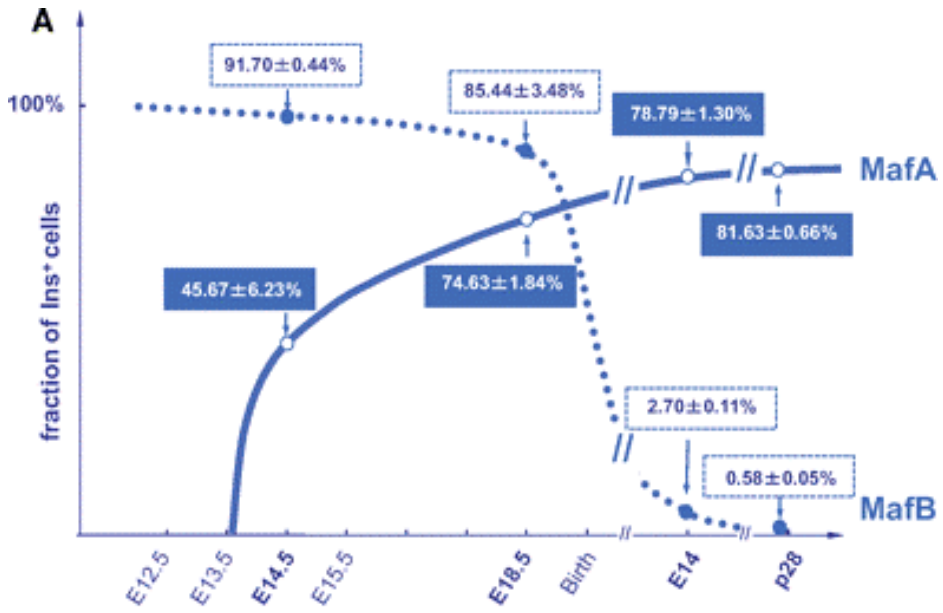

Figure 1.4. The transcription factors MafA and MafB undergo a developmental switch during β-cell maturation
In early development (e13.5), β-cells exhibit high expression of MafB and low levels of MafA; by the secondary transition at e14.5, MafA expression has increased significantly. By birth, these two transcription factors demonstrate a developmental switch, with MafA expression overtaking that of MafB. By postnatal day 28, MafA is expressed in high proportion in insulin-expressing β-cells, whereas MafA expression is generally restricted to the glucagon-producing α-cells.

In early postnatal mammalian life, further macro-organization of the endocrine pancreas occurs. Meier *et al* showed that neonatal human pancreas displayed significant small clusters of β-cells, and which organized into proto-typical islet structures during childhood (74). They, and others (75), found that these clusters of β-cells were more proliferative than those found within islets in the young human and rodent. The coordinated expansion of these small clusters, and fission of islet-like clusters contributes to the fully mature pancreas (75). Consistent with this, the proportion of β-cells present within clusters declines by adulthood, instead being found in islets (75,76).

1.4 β-cell function

In the β-cell, two other markers of mature function are glucokinase (Gck) and the glucose transporter-2 (Glut2). Gck is known as the "glucose sensor" (77,78), and is involved in the first step of glycolysis by phosphorylating glucose (79,80). Glut2 is a trans-membrane carrier protein which allows the facilitated movement of glucose across cell membranes in liver, kidney, and pancreatic β-cells, and is stimulated to initiate transport by high glucose concentrations outside of the cell. In humans, this protein is encoded by the *SLC2A2* gene (81). Glut2 is required for the initial step in glucose-induced insulin secretion by glucose uptake (82,83). In studying metabolic disease, is important to note that human β-cells use both GLUT2 and GLUT1 (preferentially GLUT1), whereas in the rodent pancreas, β-cells exclusively utilize Glut2 (84). Pdx1 is involved in trans-activating Glut2 in addition to its role in activating the insulin gene (41).

1.5 Sources of (new) β-cells, islet plasticity, and regeneration

The availability of a renewable pool of insulin-producing cells would alleviate the need for cadaveric-sourced islet transplants, and much work has focussed on identifying new or replicable sources of β-cells. The β-cell mass *in vivo* is maintained by a delicate balance of cellular growth and regression. In the neonatal period, rodents undergo significant remodelling of their pancreatic endocrine component, with increased rates of replication, neogenesis, and apoptosis (85). These processes lead to an increase in β-cell mass soon after weaning (86); but both replication and apoptosis drop significantly thereafter. Under normal circumstances, islet neogenesis (the generation of new pancreatic endocrine cells) in the rodent ceases after birth (87), although examples of endocrine regeneration after injury are well documented (88). Central to this debate are two expansive, yet related, questions: (i) under what circumstance(s) is β-cell regeneration possible? and, (ii) what is (are) the source(s) of the regenerating cells?

Two broad categories for the generation of any cell are the replication of pre-existing cells, and the differentiation from stem/progenitor cells. In the pancreas, these have been depicted by Bonner-Weir in **Figure 1.5** (89) as (i) replication of pre-existing β-cells, (ii) differentiation from ductal progenitor cells, (iii) differentiation from stem/progenitor cells outside of the ductal epithelium, and (iv) acinar transdifferentiation. The first 3 will be discussed in enhanced detail below.

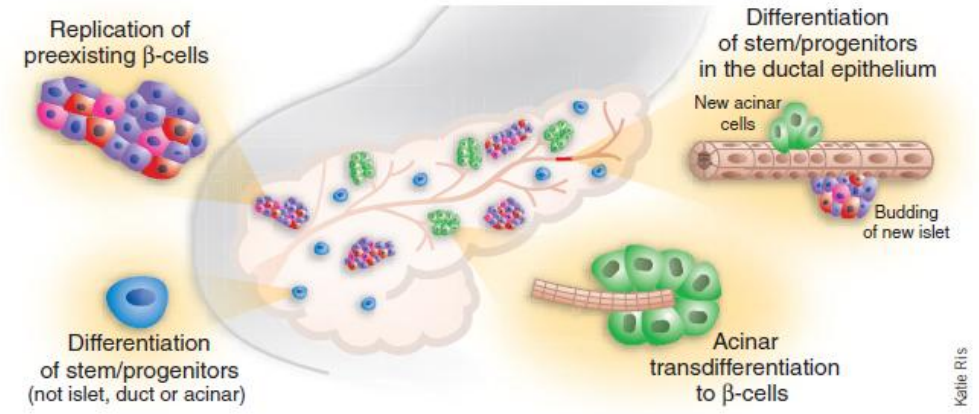

Figure1.5. Sources of new β-cells within the pancreas
Four broad categories of cell source within the pancreas are (i) replication of pre-existing β-cells, (ii) differentiation from progenitors present within the ductal epithelium, (iii) differentiation from stem/progenitor cells outside of the ductal compartment, and (iv) transdifferentiation from acinar cells.

Reproduced from Bonner-Weir S, Weir GC. New sources of pancreatic beta-cells. *Nat Biotech*. 2005;23(7):857–61. Permission granted from Nature Publishing Group.

1.5.1 Replication of pre-existing β-cells

1.5.1.1 β-cell turnover with age

In rodents, β-cell proliferation occurs postnatally at a rate of between 0.5% (90) to ~4% daily (85,91,92) between P30 – 100, and is countered by apoptosis rates of approximately 0.5% (91,92). This rate of proliferation then decreases to <1% per day after 1 year of age, with some estimates of β-cell replication at only ~0.2%/24 hours (93–95), indicating an increase in β-cell mass during early life (91). Multiple publications have addressed the issue of tissue turnover and β-cell mass expansion with age, and the consensus findings have suggested that the β-cell is capable of efficient compensatory proliferation in young animals, which diminishes with advancing age (88,95,96).

Human β-cell proliferation estimates have shown similarly low rates, with the few available publications indicating that the highest rate of β-cell replication occurs during the mid-fetal period (17-32 weeks gestation) at 3.2 ± 0.5% (97,98). By birth, the highest rate of proliferation was 2.6% in one case (74), and decreased thereafter, with β-cells replicating only 0.2%/day by early adulthood (74,98). Cnop *et al* showed that 97% of the human β-cell population was established by the age of 20 years (99). It was noted that in young humans, the proliferation was highest in small islets and scattered β-cells, there was wide variability between samples, and surprisingly, there was no wave of proliferation during adolescence as hypothesised (74). Furthermore, data from human pancreas studies suggest a mitigated ability of β-cell mass to proliferate based on physiologic demand when compared to young rodents (100), and that there was no adaptive response to increased insulin demands by replication in adult islets (99). **Figure 1.6** demonstrates the decline in human β-cell proliferation rate in the first 20 years of life (74).

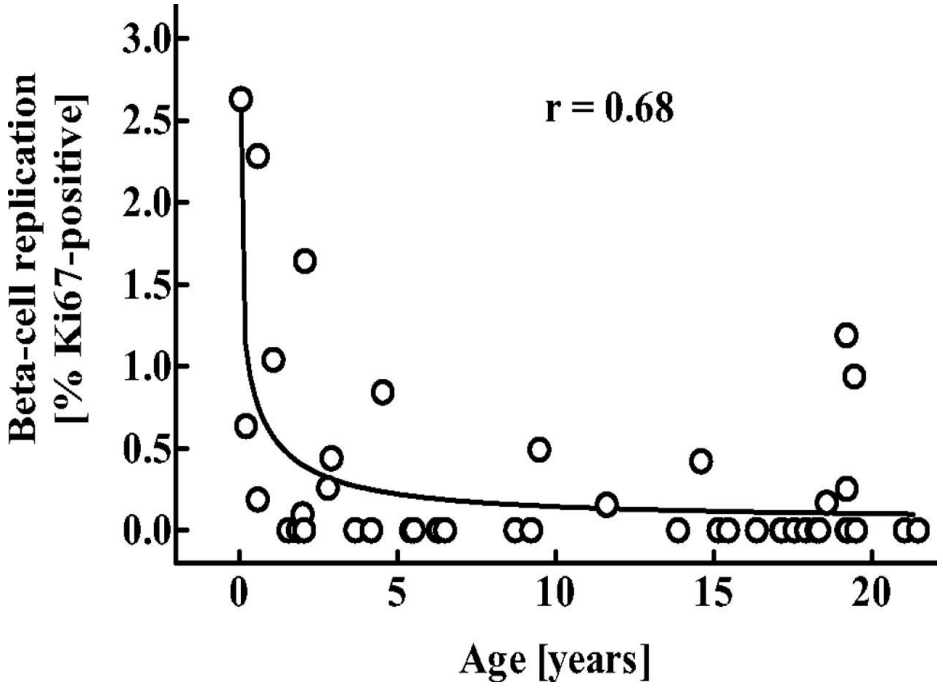

Figure 1.6. β-cell proliferation rate decreases with advancing age
While there is decreasing proliferation frequency with increasing age overall, intra-sample variability is high.

Reproduced from Meier JJ, Butler AE, Saisho Y, Monchamp T, Galasso R, Bhushan A, Rizza RA, Butler PC. Beta Cell Replication is the Primary Mechanism Subserving the Postnatal Expansion of beta-Cell Mass in Humans. *Diabetes*. 2008;57 (June):1584–1594.

The mechanisms which control the cell cycle in pancreatic β-cells are still not yet fully elucidated. Genetically, cell cycle progression requires DNA replication (S phase), and cell division or mitosis (M phase), which is halted between phases by a number of gaps (G). It has been shown that control of β-cell proliferation is greatly influenced by the cell cycle checkpoint at the G_1/S phase transition (101,102). Successful transition from the G_1/S checkpoint is accomplished via a family of transcriptional activators and repressors called the E2F proteins (102). The retinoblastoma protein (pRb), in addition to related pocket proteins p130 and p107, is regarded as the molecular brake on cell cycle progression, resulting in G_1/S arrest (102). pRb binds preferentially to E2F-1, -2, and -3, which serves to repress their transcriptional activity (102,103). This process is further regulated by a group of proteins such as cyclin D1/D2 (104) and their associated cyclin-dependent kinases (CDKs), specifically CDK4 (105), which act to promote β-cell proliferation. Conversely, proliferation can be inhibited by up-regulation of such proteins as the INK4 cell cycle inhibitors. One such negative regulator is $p16^{INK4a}$, which inhibits the CDK4-cyclinD2 complex in β-cells, and therefore inhibits cell cycle progression and cell proliferation. Moreover, expression of $p16^{INK4a}$ increases markedly with advancing age, further restricting the capacity for β-cell regeneration (96,106). This age-dependent decline in proliferation was recently shown to be a result of platelet-derived growth factor (PDGF) signalling, which controls the replication refractory period (107). Furthermore, it was also shown that nearly all G_1/S cell cycle control molecules are located in the cytoplasm and not the nucleus (108), but actively traffic to the nucleus for proliferation initiation (109). A detailed schematic illustrating the genetic control of β-cell replication is shown in **Figure 1.7** (109). Interestingly however, the age-dependent decline in β-cell proliferation was demonstrated to be reversible when adult quiescent β-

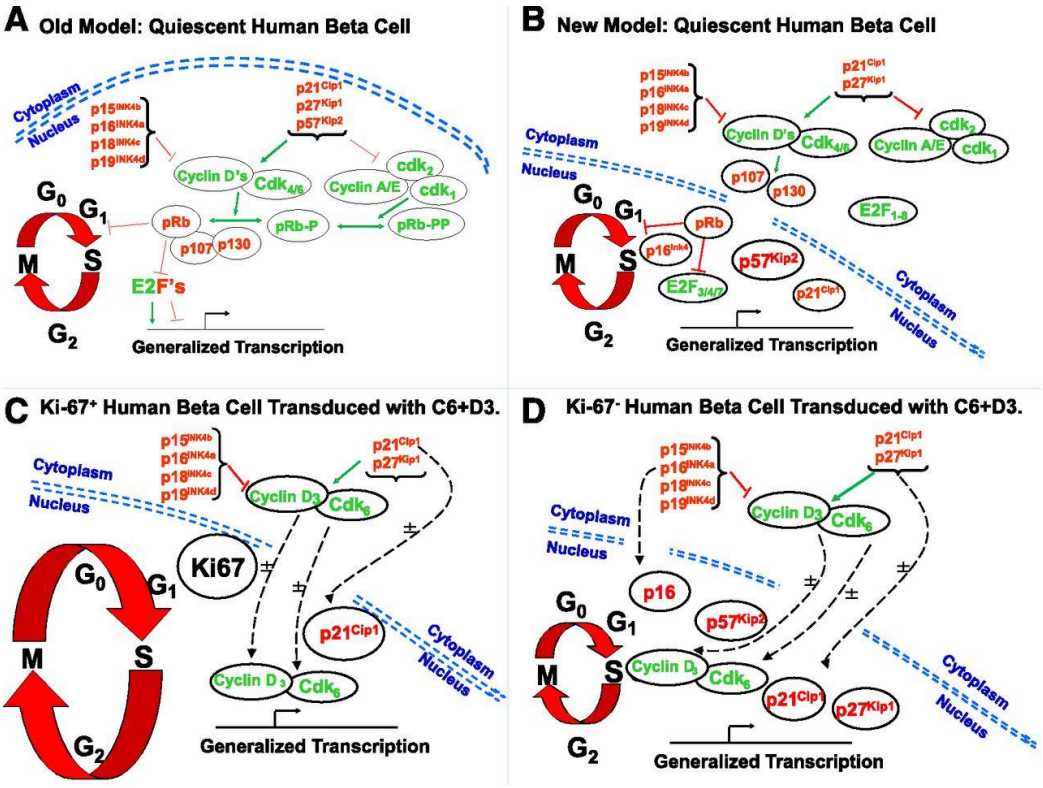

Figure 1.7. **Old and new models for β-cell proliferation and progression through the G_1/S checkpoint**

Previous (A) and recent (B) models of β-cells at quiescence, and new models of successful (C) and unsuccessful (D) transition through the G1/S checkpoint during β-cell proliferation. The new model indicates a trafficking of cell cycle inhibitors and associated proteins including Cdk6 and cyclin D3 from the cytoplasm into the nucleus.

Reproduced from Fiaschi-Taesch NM, Kleinberger JW, Salim FG, Troxell R, Wills R, Tanwir M, Casinelli G, Cox AE, Takane KK, Srinivas H, Scott DK, Stewart AF. Cytoplasmic-nuclear trafficking of G1/S cell cycle molecules and adult human β-cell replication: a revised model of human β-cell G1/S control. *Diabetes*. 2013;62(7):2460–70. Reprint available for teaching, copyright © 2013 American Diabetes Association, Inc.

cells were transplanted into young mice. This phenomena was postulated to be the result of a circulating β-cell trophic factor present in early life, and independent of *p16*^{INK4a} expression (110). This new data opens up the field to new therapeutic options for targeting β-cell replication and regeneration.

In a now-seminal publication, Dor and colleagues from the Melton group proposed that all (postnatally formed) adult β-cells arise from pre-existing β-cells, and not from stem cell differentiation (111). The group performed a pulse-chase experiment using the transgenic mouse model RIPCreER;Z/AP, which constitutively tags a subset of β-cells with a human placental alkaline phosphatase (HPAP) reporter protein after injection of the selective estrogen receptor modulater, tamoxifen (TM). The HPAP-tagged β-cells were then followed over the course of a year. They found that the ratio of tagged: untagged β-cells remained constant, indicating that no new β-cells arose from non- β-cell source, or that the contribution from stem cells was negligible. This was similarly concluded by the Kushner lab using sequential thymidine labelling of β-cells (112). However, there are caveats to this restrictive conclusion, including the lineage-tracing system utilized, which relies on interpretation of the frequency changes in labelling rates from a small percentage of tagged cells, with some inherent leakiness of the Cre transgene, as well as assessment on a small sample number (113).

The low rate of β-cell replication combined with the lack of definitive evidence for a β-cell stem/progenitor population would then suggest that the endocrine pancreas has a limited ability for growth in the postnatal setting; however, there are physiological instances documented where significant β-cell expansion is possible, such as during pregnancy (114–

116), or in response to obesity (34,117,118). Saisho and colleagues reported that in humans, a 50% increase in β-cell mass was found in (non-diabetic) obese patients (119). β-cell proliferation is also affected by various physiological stimuli, including glucose metabolism: sustained exposure to hyperglycemia has been shown to induce a modest increase in β-cell mass (120), and genetic defects in *Gck* show reduced β-cell proliferation, whereas activation of the enzyme increased proliferation (121). Related to these phenomena, insulin resistance in patients with T2D has been shown to promote an increase in β-cell proliferation (122–124). This has been suggested to be the result of a secreted protein termed "betatrophin" from liver and adipose cells, which selectively, and strikingly, increased β-cell proliferation in both mice and humans (125). The potential therapeutic opportunity provided by this new hormone is staggering in its scope for human disease modulation, predominantly for patients with T2D. However, recent evidence has somewhat tempered the supposition of this hormone to be the "magic bullet" for curing the disease alone, as the effects seen in mice were not replicated in human islet transplant experiments (126).

Combining both aging and disease, studies have examined islet/endocrine function from patients with long standing T1D. Recent reports have challenged the presumption that β-cell destruction is a one-time, static process, instead showing evidence for β-cell regeneration attempts in patients with long-term T1D (16,127). The Medalist Study examined histological sections from 9 pancreata donated post mortem of patients with >50-year duration of T1D (128). Of 4 pancreata selected based on positive antibody titres, two showed evidence of insulin$^+$ TUNEL$^+$ (apoptotic) cells, and one pancreas exhibited insulin$^+$ Ki67$^+$ (proliferative) cells, indicating active tissue turnover (128).

What is evident from these reports is that the rate of β-cell apoptosis outstrips the rate of β-cell regeneration/proliferation. However, reversal of T1D may be accomplished by mitigating the auto-immune destruction of the β-cell and allowing endogenous regeneration to occur. This could be provided by autologous hematopoietic stem cell transplant, which has shown to be effective in other autoimmune diseases including multiple sclerosis (129), or by immuno-modulatory therapy (130). However, the etiology and progression of T1D, with substantial loss of β-cell mass prior to overt symptoms, may limit full endogenous regeneration from occurring even if the autoimmune destruction can be controlled. Immuno-modulatory therapy may therefore be of use only for patients previously identified as at-risk for diabetes development, and not necessarily those with established disease present.

1.5.1.2 Models of β-cell injury and regeneration

Multiple injury models have been shown to induce endocrine regeneration, such as partial pancreatectomy (Px) (111,131,132), pancreatic duct ligation (PDL) (133–136), the injection of the β-cell toxin streptozotocin (STZ) or its related drug, alloxan, and recently, by genetically-based methods of specific β-cell ablation.

Partial pancreatectomy

Partial pancreatectomy involves the surgical resection of up to 90% of the pancreas in rodent models, and Bonner-Weir and colleagues have performed multiple studies demonstrating both endocrine and exocrine regeneration capacity in the weeks following surgery (131,132,137–139). Dor and colleagues also performed Px in parallel to the aging

model so as to ascertain if there was a difference in developmental origin of newly formed β-cells formed during regeneration. They concluded that after Px, similar to aging, β-cells from adult mice primarily arose from pre-existing β-cells, and not progenitor cell source (111).

Pancreas duct ligation

Pancreas duct ligation involves surgically obstructing the pancreatic duct, causing digestive enzymes to be shunted, and hence accumulate, in the ligated portion of the organ. This initiates severe inflammation and acinar apoptosis, similar to pancreatitis in humans (140). This model has been used extensively for islet regeneration (141–144), with new β-cells putatively arising from the ductal compartment (135). However, recent evidence has indicated that these results have been confounded by extensive (global) pancreatic damage and that endocrine regeneration does not occur (145).

STZ

STZ and alloxan are glucose mimetics and as such, access the β-cell via Glut2 (146,147); a structural comparison between the two compounds is shown in **Figure 1.8** (146). STZ (2-deoxymethyl-nitrosurea-glycopyranose) destroys the rodent β-cell by transferring a methyl group to DNA, leading to DNA fragmentation. Attempts to repair DNA by stimulation of poly (ADP-ribose) polymerase leads to cellular depletion of NAD^+ and ATP stores (148,149), and necrosis of the β-cell ultimately causes hyperglycemia within days of administration (147,150–152). However, due to its use of Glut2 as a mode of entry, other cell types which utilize this transporter are also damaged after drug administration (e.g.

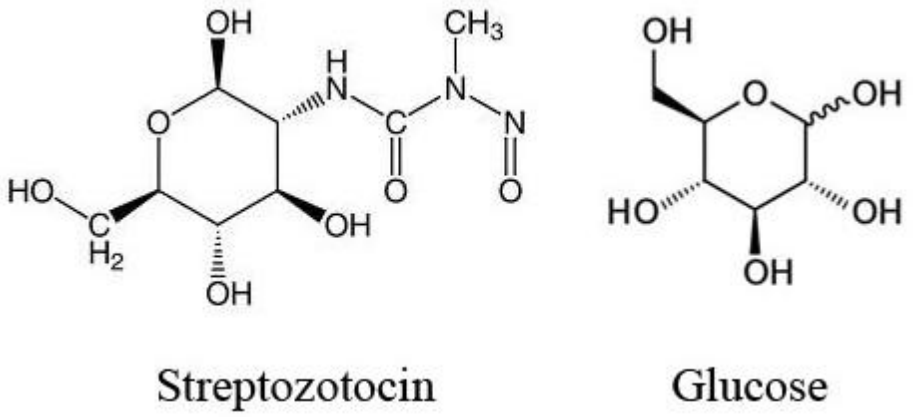

Figure 1.8. **Schematic illustration showing the structural similarities of the β-cell toxin streptozotocin (STZ) to glucose**

In rodents, both STZ and glucose enter the pancreatic β-cell via the glucose transporter Glut2.

liver, kidney), and care must be taken to avoid damage to distal organs which can confound interpretation of the diabetogenic effect of the drug (147). Moreover, human β-cells, which predominantly utilize GLUT1, are less susceptible to these effects than rodent β-cells (153). In rodents, STZ has also been shown to induce inflammation and peri-islet lymphocyte infiltration, suggesting that it not only causes β-cell apoptosis and hyperglycaemia, but also closely models TID disease development. However, this response is more classically seen after multiple low-dose STZ treatment, and not after a single bolus (154,155).

Interestingly, the regenerative capacity after administration of STZ is temporally defined: rodents given STZ during the neonatal time period can partially regenerate their endocrine pancreas (96,150,156–162), whereas the regenerative response is mitigated when the drug is administered in adulthood (96,146). The regenerative capacity also depends partly on the timing of STZ administration: a single bolus of 100 mg/kg of the toxin at P0 showed glucose normalization and β-cell mass re-establishment by 3 weeks of age (150), whereas STZ given after P5 showed a less-robust regenerative capacity (146), although it was still seen in mice up to 6 weeks of age as compared to adults (96). The mechanism(s) behind this temporal regeneration capacity have not been determined.

β-cell ablation by genetic manipulation

A new mode of β-cell damage is the Insulin-rtTA;TET-DTA model, combining a transgenic mouse strain that expresses the reverse tetracycline-dependent transactivator in pancreatic β-cells (Insulin-rtTA) with a mouse strain that expresses the diphtheria toxin A (DTA) subunit under a rtTA-responsive promoter (TET-DTA) (163). In the presence of doxycycline, rtTA induces the expression of DTA, causing β cell apoptosis (164). The benefit

of this model is that it specifically targets only the β-cell, and avoids confounding issues of inflammation, or effects on other organs that are observed in other injury models.

Nir and colleagues found that after giving doxycycline (Dx), 70-80% of β-cells were destroyed and overt hyperglycemia and diabetes was noted. Interestingly, upon withdrawal of Dx, insulin expression normalized, islets regained their architecture to 90%, and there was a remission of hyperglycemia, although full glucose tolerance was only complete after 8 months. These results were consistent regardless of the age at which the mouse β-cells were ablated. The authors then lineage traced the β-cells during this process, and found that the labelling indices of both regenerating and non-regenerating mice (injured and non-injured, respectively) were the same, and that proliferation rates were the same between the two groups, leading to the conclusion that β-cell regeneration is possible, but the cell source was exclusively pre-existing β-cells (164).

A separate publication from the same group then knocked out Pdx1-expressing pancreatic progenitor cells at multiple time points, using a similar diphtheria-toxin expressing strain (165). Here, the $Pdx1^{tTA}$ mouse was crossed with a $tetO^{DTA}$ mouse: addition of tetracycline maintained the normal developmental program, while cessation of tetracycline induced $Pdx1^{+}$-cell ablation. When tetracycline was administered throughout pregnancy or after e9.5, the progeny developed normally. However, if tetracycline was withdrawn entirely, or after e11.5, the resultant pups had near complete pancreatic agenesis. These results suggested that there are no stem cells in the embryonic or adult pancreas, and that final pancreas size (and by default, regenerative ability), is restricted by the number of progenitor cells present between e8.5-e12.5. Knocking out these progenitor cells before e12.5 completely abrogated the formation of $Pdx1^{+}$ pancreatic progenitor cells

with the ability to rescue normal pancreas development. The authors stated, "...our studies show that compensatory growth during pancreas development is either quite limited or does not occur at all. Thus, embryonic progenitor cells represent a critical and limiting determinant of pancreas size" (165).

Another study proposed that the restriction of β-cell proliferation in aged mice was a result of the injury model used (such as STZ, high fat diet, or partial pancreatectomy), and instead subjected their mice to β-cell damage by DTA using the transgenic approach as described previously (166). Addition of Dx to very old (25 month old) insulin-rtTA;TET-DTA mice caused a disruption in both normoglycemia and islet architecture; removal of the toxin resulted in a 3-fold increase of replication from basal rates. Addition of a glucokinase inhibitor, which enhanced glycolysis rates, had a similar effect (166). These results indicate that under multiple circumstances, β-cells are generated postnatally from pre-existing β-cells.

1.5.1.3 Generation of insulin-expressing cells *in vitro*

As has been shown, the majority of β-cells are formed by replication of pre-existing β-cells *in vivo*, which normally exhibit a low rate of cellular proliferation after birth. Since islets are in such high demand for use in transplant, then alternative sources of insulin-expressing cells are required, and expansion of (pre-existing) β-cells *in vitro* is an attractive option. Efforts to alter β-cell proliferation rate directly have generally failed, but differentiation techniques have been relatively more successful *in vitro* (167,168). Cellular identity is broadly determined by the interplay of genetic (and epigenetic) and

environmental factors which control gene expression in a cellular system (169). Therefore, instructive changes to either/both the environment and/or the genes present may effect a change in cell fate. Since all pancreatic cell types arise from a common progenitor cell type, *in vitro* differentiation is a conceivable approach. Dedifferentiation can be defined as the loss of mature, defining and functional characteristics from a partially or terminally differentiated cell type (169), whereas transdifferentiation is broadly defined as a change from one differentiated phenotype to another, involving morphological and functional phenotypic markers (170). These operational terminologies are utilized to describe the differentiation, dedifferentiation, or transdifferentiation of pancreatic cells towards or away from the genetic, morphological, and functional properties of mature β-cell. Importantly, cellular transdifferentiation can be induced by a multitude of factors, such as direct reprogramming induced by transcription factor over-expression, or alternatively by modified progression through dedifferentiation to a multipotent progenitor-like stage, and subsequent redifferentiation. These cellular fate (re)specifications are depicted by Puri *et al* in **Figure 1.9** (169), and show that terminal cellular differentiation in the endocrine pancreas is not as unidirectional as previously supposed.

One group published multiple reports over a number of years on the culture of human fetal pancreas cells, and demonstrated that these cells could be induced to expand and express insulin (171–177). They and others induced islet expansion and/or differentiation by the use of *in vitro* techniques, such as extracellular matrix selection (175,178,179), or specific combinations of growth factors such as hepatocyte growth factor (HGF) (180), fibroblast growth factor-2 (FGF-2), keratinocyte growth factor (KGF/FGF-7) and insulin-like growth factor – II (IGF-II) (177,181), activin, betacellulin, exendin-4, and HGF

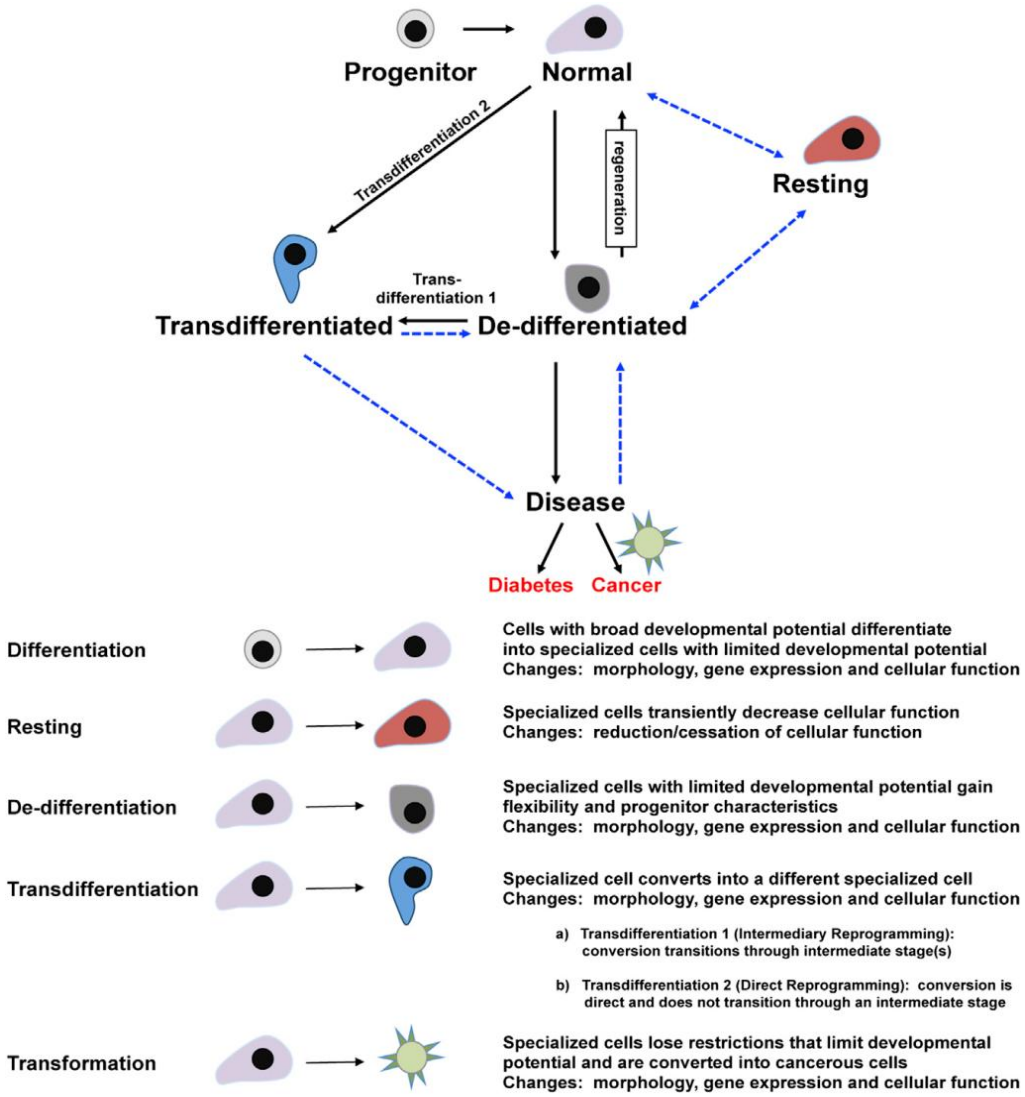

Figure 1.9. Transition states as a result of environmental/genetic manipulation or stress, and relationship to pancreatic disease.
Illustration of differentiation potential of cells, resulting from either stress/disease, or by genetic and/or environmental manipulation. Note that "transdifferentiation" can occur by direct means from "normal" (1), or by prior "dedifferentiation" (2). Regeneration is hypothesized to occur by a return to " normal" from the "dedifferentiated" state.

Reproduced from Puri S, Folias AE, Hebrok M**.** Plasticity and Dedifferentiation within the Pancreas: Development, Homeostasis, and Disease. *Cell Stem Cell*. 2014:1–14.

(182), basic FGF (bFGF) and leukemia inhibitory factor (LIF) (183), epidermal growth factor (EGF) and cholera toxin (179,184), or insulin/ transferring/selenium (ITS) (185), and generally, the use of serum-free media.

Many groups contended that the resultant cells, with extensive proliferation of fibroblast-like cells, had arisen from β-cells, due to the re-expression of insulin (186,187). However, the insulin generated from *in vitro* culture was much lower than would be found *in vivo*. Epithelial-to-mesenchymal transition (EMT) and its subsequent reversal (MET) have been proposed as a requirement for differentiation (188), but there is now substantial evidence that it is not causally related (182,183).

The advent of lineage tracing technology allowed β-cell fate to be followed during *in vitro* culture conditions, and demonstrated that the majority of mouse β-cells were, in fact, eliminated from culture (167,168,182,183,185). A few studies were able to dedifferentiate a small subset (<5%) of adult mouse β-cells (167,168) or adult human β-cells (189) to non-insulin-expressing states, showing some β-cell dedifferentiation potential. Why some protocols have resulted in successful β-cell differentiation and survival, while others did not, has yet to be determined. Moreover, it also remains to be determined why some β-cells are capable of differentiation whereas the majority of them are not. Interestingly, it emerged that human β-cells potentially retain a higher plasticity potential than do mouse β-cells (167,190), which was postulated as due to β-cell "chromatin memory" (167), although the mechanisms behind this have not been elucidated.

Collectively, these data would suggest that replication of pre-existing β-cells accounts for the *de novo* generation of the majority of postnatal β-cells. Indeed, replication of existing β-cells may be the default mode of endocrine mass maintenance; however, the

contribution from putative progenitors, or the situations in which they are required, has not been thoroughly examined to exclude them entirely.

1.5.2 Differentiation of stem/progenitor cells to β-cells

An alternate mechanism for the generation of insulin-producing cells is through directed stem/progenitor cell differentiation (**Figure 1.5** (89)). The use or role of stem/progenitor cells in medicine and tissue repair, especially in the field of diabetes, has been hotly debated and anticipated. Multiple examples of endocrine regeneration have prompted investigation into the role of stem cells. A widely accepted definition of any stem cell is based on functional properties, exhibiting an unlimited capacity for self-renewal and retaining the ability to generate multiple cell types (or, multi-potentiality), which may or may not be in response to injury. A progenitor cell, on the other hand, is a proliferative cell with a limited capability for self-renewal and which is most often uni-potent (191,192). The distinction between these two cell states is often fluid and vaguely indeterminate in the literature, but may yield important and definitive outcomes for medicine. Similarly, subdivisions in definition exist for the somatic, or tissue-specific, stem cells *vs.* embryonic stem cells, namely that the latter demonstrates the capacity to generate progeny across all primary germ layers. However, precedents exist to suggest that this is not always rigidly adhered to, as has been demonstrated by haematopoietic and neural stem cells forming tissues from germ layers other than mesoderm- and ectoderm-lineage, respectively (193–196).

Endogenous stem cell repair mechanisms have been demonstrated following hyperglycemia and during endocrine regeneration. Transplants of mesenchymal stem cells (MSCs) after injury aided in the endogenous regeneration of tissue, instead of direct differentiation from stem cells to β-cells (197). In some cases composite cells from MSCs/endothelial cells were detected (198), which assisted in revascularization of islets or counteracted innate immunity response after transplant (199). This action was replicated by endogenous hematopoietic stem cells homing to injury site and enhancing β-cell mass regeneration through endothelial interaction (161,200). That the stem cells did not directly differentiate into new tissue either *in vivo* or after injecting *ex vivo* was perhaps surprising, implying instead that regeneration occurred through differentiation of tissue-specific stem/progenitor cells, and/or replication of surviving cells, rather than activation of haematopoietic stem cell differentiation.

Bone-marrow-derived progenitor cells purified based on aldehyde deydrogenase (ALDH) activity, a conserved characteristic of multipotent progenitor lineages, were shown to correct hyperglycemia in mouse models of diabetes. When uncultured $ALDH^{hi}$ cells were transplanted to STZ-treated recipients, islet-specific proliferation and pro-angiogenic programs were induced. If the $ALDH^{hi}$ cells were instead cultured prior to transplant, an increase in small islet clusters was noted, and which were associated with the ductal epithelium (201). This indicated that the subtype(s) of stem/progenitor cells presented to injured pancreas modulated distinct endogenous repair mechanisms. Moreover, if these $ALDH^{hi}$ cells were transplanted directly into the pancreas instead of injected intravenously, there was a significantly higher β-cell regeneration capacity. Furthermore, these cells could

be obtained from umbilical cord blood rather than bone marrow, representing an easily obtainable stem cell source (202).

Alternatively, a few groups have created insulin-producing cells directly from differentiation of human embryonic stem (hES) cells (203–205), although these cells had overall limited function. In the past decade, insulin-producing cells have been successfully generated from hES cells in a system that could be reproducibly, and potentially safely applied for future clinical use (206–209). The approach was to use a 5-stage, stepwise protocol which recapitulates endocrine pancreas development, in conjunction with the use of large scale cell production and cell differentiation cassettes for transplants that reduce opportunities for autoimmune destruction (210). The differentiation of hES cells to extra-embryonic and definitive endoderm was shown to be regulated by SOX7 and SOX17, respectively, and importantly, a stable population of endoderm progenitor cells could be generated by the constitutive expression of these transcription factors in hES cells (211). The Melton group has also successfully generated insulin-producing β-cells from hESCs, which were shown to be closer in phenotype to human fetal β-cells than adult β-cells, with poor glucose responsiveness and an immature β-cell transcriptome (212). Importantly, these results provide a novel toolbox of transcription factors where research efforts can be concentrated for future study. However, despite these advances, these "beta-like" cells often require *in vivo* transplantation to be fully glucose-responsive (213), and the associated risks of uncontrolled cell growth and teratoma formation are not to be underestimated, and therefore currently infeasible for human clinical application.

1.5.3 Differentiation of β-progenitor cells found within the ductal epithelium

Besides extraneous sources of stem cells, the search for intra-pancreatic progenitor cells is an attractive option. Significant data has historically suggested that the ductal compartment contains the predominant source of pancreas-derived β-cell progenitors (73,91,134,138,214–218).

Developmentally, pancreatic endocrine cells are derived from cells within the ductal epithelium which share a common pathway via a bipotent progenitor with duct cells (55). As noted earlier, pancreatic duct ligation (PDL) has been shown to be an important mode of endocrine pancreas regeneration. Wang *et al* performed PDL and found a replacement of acinar cells for ductal complexes and significant growth of islet tissue (134). There was a doubling of the β-cell population, an increase in small islets and islet clusters, and an increase in cell proliferation in the ligated portion of the organ, indicating islet neogenesis from cells resident within the duct.

An *in vitro* model of expanded human ductal tissue was used by Bonner-Weir *et al* to effect differentiation to islet structures with the use of FGF-7, nicotinamide, and ITS (73). The authors found that after cellular expansion and transfer to Matrigel (a basement membrane compound containing primarily laminin, entactin, and collagen), small cyst-like structures were formed which expressed insulin as detected by immunohistochemistry, and exhibited a 2.3-fold increase in insulin secretion after glucose exposure as compared to basal levels (73). Similar results were recently demonstrated by Kim and colleagues (219) and by Corritore *et al* (220) The appeal of this approach is that the ductal compartment accounts for ~10% pancreas mass (140), and is entirely discarded after human islet isolation. If ducts indeed contain endocrine progenitors or can differentiate directly into β-

cells themselves, the shortage of available cells for diabetes treatment would be effectively solved.

Indeed, while extensive data has proposed the contribution of cells from the ductal compartment to β-cell mass, the only way to definitively test that new β-cells conclusively arise from ductal epithelium is to perform lineage tracing experiments. However, the major hurdle for determining pancreatic cellular origins is that early ductal cells lack a definitive progenitor marker; while Pdx1 or Ngn3 can be used to assess newly-formed β-cells, and a series of transcription factors, such as NeuroD/Beta2, Nkx6.1, Nkx2.2, Pax4, Pax6, MafA, and MafB, or mature islet markers such as insulin, Glut2, or Gck can be examined, duct cells express the same markers early in development as well as late. Therefore, while transgenic technology has revolutionized the field of developmental biology to assess cellular origins, the use of mature duct-cell markers is a shortcoming in the absence of a more progenitor-specific marker.

In one study, the mature duct cell marker carbonic anhydrase II (CAII) was used to assess cell state during pancreas organogenesis from e14.5 to adulthood in the mouse and human (136). It was found that all duct cells in the adult pancreas of both species expressed CAII, as well as some α-cells in the mouse (72). CAII was notably absent in the β-cells. CAII was expressed in embryonic duct cells as early as e18.5 (72). CAII was subsequently used to lineage trace duct cells using the CAII-Cre or CAII-CreER transgenic mice crossed with ROSA26 (R26R) mice, which adds a LacZ reporter (expressing β-galactosidase (β-gal)) to any cell expressing CAII after induction with tamoxifen (136). Using longitudinal assessment and modelling of β-cell mass, the authors estimated that up to 30% of new β-cells in the 31 day old mouse did not arise from replication of pre-existing β-cells (85). When pancreata of

CAII-Cre;R26R mice were examined at P0 and at P28, LacZ expression was found in ducts, in patches of acinar cells, and in some islets (marking both α- and β-cells), which increased with age, indicating that formation of new islets and acini from ductal progenitors occurred in the neonatal time period (136). The authors then used the inducible (CAII-CreER) strain combined with a duct ligation regeneration model to assess the role of CAII during pancreas remodelling, and found a significant increase in LacZ expression in the ligated portion of the pancreas (136), indicating a role for duct cells during endocrine regeneration.

Xu *et al* (135) sought to address the contribution of pancreatic progenitor cells' contribution to β-cell mass after injury, and used a recombinant lentivirus encoding two Ngn3-specific short hairpin (sh) interfering RNA molecules to tag Ngn3-expressing ($Ngn3^+$) cells with an eGFP reporter protein immediately prior to PDL. The shRNA knocked down the majority of regeneration in the PDL pancreas, indicating that Ngn3 is required for β-cell regeneration. The authors then used the Ngn3-nLacZ mouse, which adds a β-gal reporter to any cell expressing Ngn3 at the time of tagging. It was found that 15% of all $β\text{-}gal^+$ cells were present in the ductal lining 7 days after PDL, and co-expressed cytokeratin (CK). They also found that one third of all $β\text{-}gal^+$ cells expressed hormones, including insulin. The authors further showed that this was due to $Ngn3^+$ progenitor cell differentiation to $hormone^+$ cells, and not mature ($hormone^+$) cell dedifferentiation, by using InsCre;R26R mice to trace mature $insulin^+$ cells during injury. They found after PDL, all $LacZ^+$ cells were $Ngn3^-$, indicating that this was indeed progenitor cell differentiation.

Criscimanna and colleagues similarly found that duct cells were capable of both endocrine and acinar cell regeneration using either PdxCre;$R26^{DTR}$ or ElacreERT2;$R26^{DTR}$ transgenic lines; interestingly, they also found that the mode of regeneration, either

recapitulation of the developmental program, or direct differentiation of duct to acinar cells, was directly associated with the severity of damage (221).

These studies are countered by articles which examined the role of insulin$^+$ cells present in the ducts during aging and after injury. One study lineage traced the early ductal transcription factor Hnf1β using an Hnf1β-CreER;R26R mouse model (59), and found, similar to other reports, that Hnf1β$^+$ cells are precursors for all pancreatic- epithelial cell types (acinar, duct, and endocrine) during early gestation, which exclude acinar cell fates by mid-gestation (59). However, it was reported that the Hnf1β$^+$ cells do not contribute to endocrine cell fate after birth, at 6 months of age follow-up, or following two separate models of endocrine injury using PDL and alloxan treatment. The authors tried to reconcile their findings with the significant data that propose that duct cells are the progenitors for islets, and suggested instead that ducts share a "tissue milieu" that favors the formation of new endocrine cells. They further proposed that while some endocrine cells may reside in the ductal epithelium and expand after regenerative stimulus (such as injury), these cells were not ultimately derived from duct cells postnatally (59).

Another paper sought to determine the origins of insulin$^+$ cells found in the ductal epithelium after injury, and found that insulin$^+$ cells indeed arise from ductal epithelial cells during development, but not after birth (222). Using a model of ductal hyperplasia induced by transforming growth factor-α (TGFα), they lineage traced pancreatic cells and showed that the ducts had arisen in some limited cases from acinar cell trans-differentiation, but not from insulin$^+$ cells. The authors further reported that the majority of insulin$^+$ cells present within the ductal epithelium had arisen from pre-existing β-cells and not duct or acinar cells (222). However, the authors conceded that up to 5% of these insulin$^+$ cells

contained the lineage marker from acinar cells, but concluded that this was due to either/both transgene "leakiness" (and not proof of acinar – or duct - to-endocrine transdifferentiation) or an insignificant contribution from those compartments. This has been independently shown using a mucin1 (*Muc1)*-lineage tracing mouse model (223).

Overall, many of these reports provide evidence in support of current dogma, that β-cells primarily arise from pre-existing β-cells in the *in vivo* adult pancreas (111), during aging and after injury and consequent regeneration, indicating that the potential for contribution from ductal cells and/or ductal progenitors in the postnatal time period is limited. However, to dismiss the substantive data amassed previously would be imprudent, and differential interpretations of phenomena may be required.

1.5.4 Differentiation of stem/progenitor cells within the endocrine pancreas

The search for stem/progenitor cells present within the endocrine pancreas itself has yielded frustratingly little information, as proto-typical "stem" cells have not been readily identified within the pancreas as can be found in other organs, such as the intestine (192). However, recent paradigm shifts have changed the focus to looking within the endocrine pancreas itself as the source of regeneration. Szabat *et al* traced human and mouse β-cells with a dual reporter system, using a lentivirus which differentially labelled Pdx1 and insulin. The authors reported that a subpopulation of adult β-cells from both species exhibited negative/low expression of insulin while maintaining expression of Pdx1 ($Pdx1^{+}Ins^{low}$cells) in relatively high proportion (15-25%), which could differentiate into $Pdx1^{+}Ins^{+}$ cells without cell division, implying differentiation (224). Furthermore, these $Pdx1^{+}Ins^{-}$ cells displayed a progenitor phenotype, lacking expression of Glut2, Gck, and

MafA, whilst being proliferative. Interestingly, these $Pdx1^{+}Ins^{-}$ cells could be sub-divided into two groups: those that matured into insulin-expressing cells, and those that maintained a progenitor phenotype (224).

A separate study examined the role of nutritional insult in early life with regard to β-cell regeneration potential (159).The authors found that there was no effect on $Pdx1^{+}Ins^{-}$ cell number when mice were protein-restricted during gestation, or treated with STZ independently. However, the cells were present in higher proportion in pancreas exposed to STZ and low protein simultaneously, suggesting that the ability of progenitor cells to mature and differentiate to functioning β-cells was mitigated as a result of the *in utero* insults (159).

Data from Liu and colleagues directly contradicted work by Dor *et al* (111), in that lineage tracing of the β-cell, using the same RIPCreER;$Z/AP^{+/+}$ transgenic mouse model, resulted in an increase in $HPAP^{+}$ (reporter) β-cell labelling with age, as well as the presence of $HPAP^{+}$ $insulin^{-}$ cells after aging and STZ-mediated injury (225). They found that $HPAP^{+}Ins^{-}$ cells demonstrated an immature phenotype, with expression of Pdx1 and MafB, and the lack of Nkx6.1 and Glut2. Furthermore, $HPAP^{+}$ Ins^{-} cells were proliferative after STZ as demonstrated by expression of Ki67, and were 2-fold smaller than typical β-cells. The relative proportion of $HPAP^{+}Ins^{-}$ cells after injury was found to be 0.1% of all $insulin^{+}$ (β-) cells. Liu and colleagues concluded that these cells represented β-cell progenitors which, by default of expressing the β-cell reporter, must have exhibited active insulin expression at the time of Cre induction. Only after injury or aging, and hence after differentiation to

proto-typical mature β-cells, was insulin expressed, which accounted for the increase in $HPAP^+$ labelled Ins^+ cells with increased age (225).

Seaberg *et al* found that rare cells within mouse islets and ductal preparations could form clonal spheres with the capacity to generate neural and pancreatic exocrine and endocrine lineages (226); these cells could then be further differentiated into functional β-cells which demonstrated glucose-sensitive insulin release. The group determined that these cells were not derived from embryonic stem cell origin, nor from neural crest, nor from mesoderm-lineage, but instead from "multipotent precursor" (progenitor) cells found within the pancreas (226). A subsequent study from the same group replicated experiments by Dor *et al* (111), and found that $HPAP^+$ cells tagged non-β-cells after a long chase period including endocrine and non-endocrine cells, indicating that cell fate may not be as immutable as was previously stated (227). Moreover, after replicating the clonal sphere assay (226), it was revealed that these multipotent cells had derived from insulin-expressing cells. However, the multipotent cells were found to represent a unique subset of β-cells in that they demonstrated decreased levels of *Nkx6.1* and *Pdx1*, increased levels of *Ngn3*, and importantly, lacked *Glut2*. These cells were present within adult mouse and human islets in a proportion of <1/5,000, and could be differentiated *in vitro* to multiple endocrine lineages (including α, β, δ, and γ cells), exocrine cells, and neural lineage cells including neurons, (neuro)glia, and oligodendrocytes (227). Moreover, after differentiation to β-cells, these were fully capable of ameliorating hyperglycemia after transplant, demonstrating their utility as functional endocrine cells (227).

Based on these reports, there is emerging evidence that β-cell progenitors are present within adult pancreas, although the relative contribution of these cells to normal physiology has not been determined, nor is it known whether they represent a therapeutic target for diabetes.

1.6 Rationale

Clearly, there are multiple routes to generating β-cells, notwithstanding of variable function. As was eloquently stated by AMJ Shapiro, pioneer of the Edmonton protocol, "…given the enormous clinical burden of diabetes, the search for alternative sources of regulated insulin-producing cells must continue, since the current supply from deceased donors cannot meet the demand" (18). Based on the evidence that (i) the generation of new β-cells occurs almost entirely by replication of pre-existing β-cells postnatally; (ii) the replication rate of mature β-cells is extremely low, and (iii) β-cells develop from a bipotent progenitor cell state which shares a common pathway early in development with duct cells, the differentiation of pre-existing β-cells to a ductal phenotype may allow the β-cell population the ability to expand *in vitro.* These cells can then be re-differentiated "back" to insulin-producing cells for use in human transplants given the appropriate environment. Furthermore, since young mammals exhibit higher growth potential and more lineage plasticity than do mature ones, experiments will be conducted using neonatal tissue. Characterization of the cell type(s) capable of differentiation will be consequently accomplished.

1.7 Hypothesis

It is hypothesized that neonatal mouse β-cells demonstrate the ability to dedifferentiate into alternative cell types, including cells sharing characteristics of ductal epithelial cells, which can be subsequently redifferentiated into insulin-producing cells based on *in vitro* environmental cues. Furthermore, the β-cells which retain the capacity for differentiation will be involved during regeneration after pancreatic injury.

1.8 Objectives

To determine the regenerative potential and lineage plasticity of mouse β-cells

1. Demonstrate neonatal mouse islet cell plasticity *in vitro*
    a. Dedifferentiate neonatal mouse islets for the reversion to a proliferative ductal phenotype
    b. Establish if the dedifferentiated cells are capable of redifferentiation to insulin-producing β-cells
    c. Optimize *in vitro* conditions for these processes, and whether the cells are functional after transplant *in vivo*
2. Lineage trace the β-cell *in vitro* using the transgenic mouse model RIPCre;Z/AP to establish whether cell dedifferentiation involves the β-cell
3. Characterize resultant subpopulations of lineage plastic β-cells
4. Assess the analogous population of β-cells within the human pancreas, and how it changes with age

5. Determine why young rodents are capable of endocrine regeneration after STZ-ablation but not in adulthood, using the transgenic mouse model RIPCreER;Z/AP.

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CHAPTER 2

Culture of Neonatal Mouse Islets under Dedifferentiation and Redifferentiation Conditions

Christine A. Beamish, Brenda J. Strutt, Edith Arany, Gediminas Cepinskas, David A Hess, Andrew Pepper, Delfina Siroen, and David J. Hill

2.1 Introduction

The *de novo* generation of insulin-producing cells in the pancreas has been a subject of great research focus, ultimately for β-cell replacement therapy for patients with diabetes. The current gold standard treatment is islet transplantation, but is limited by the availability of cadaveric-sourced tissue. Multiple reports have demonstrated moderate success in the directed differentiation of human embryonic or induced pluripotent stem cells to β-cells (1–4). Alternatively, the dedifferentiation, expansion, and subsequent redifferentiation of pre-existing β-cells as a source of insulin-producing cells has been suggested (5–7). Dedifferentiation is the loss of mature, defining and functional characteristics from a partially or terminally differentiated cell type (8). While transdifferentiation is defined as a change from one differentiated phenotype to another, involving morphological and functional phenotypic markers (9), it can be preceded by cellular dedifferentiation (8). One such protocol involving the use of epidermal growth factor (EGF) and cholera toxin has indicated that human islets may be dedifferentiated to a proliferative monolayer of cells positive for markers of ductal epithelium (10–13). Resultant cells were shown to increase proliferation by 1500%, and demonstrated a precursor-like phenotype. Using a subsequent "redifferentiation" protocol which includes the ductal mitogen keratinocyte growth factor (KGF/FGF-7) and insulin-like growth factor- 2 (IGF-II), "islet-like clusters" could be generated that demonstrated insulin secretion (14–16).

Experiments carried out by Lammert and colleagues showed that explanted e8.5 mouse endoderm grown *in vitro* formed a structure similar to a gut tube, but did not show any pancreatic differentiation, lacking both Pdx1 and insulin expression (17). When this same explanted endoderm was co-cultured with dorsal aortae, both insulin and Pdx1 were

expressed, and pancreatic buds were visible in ~30% of the structures. Moreover, insulin was shown adjacent to the endothelium. Follow-up reports by the same authors illustrated that endothelial cells induce crucial steps in the development of both liver and pancreas (18,19). It has been shown that microvascular loss is a contributing factor in the initial apoptosis and dedifferentiation of islets during transplantation (20), and islets cultured with endothelial cells prior to transplantation demonstrate enhanced survival (21) which can be attributed to decreased immune rejection and increased revascularization in the recipient (22). Furthermore, after pancreatic injury, mesenchymal stem cells have been shown to home to the site of injury and initiate repair, postulated to promote an increase in angiogenesis (23,24). Therefore, the role of endothelial cells in the revascularization of islets after isolation may have a major influence on their capacity for differentiation *in vitro* and *in vivo*. Indeed, multiple studies have demonstrated that the formation of new β-cells or other endocrine cells from embryonic stem cells (25) or human fetal pancreas (26) requires transplantation *in vivo* for full maturation and glucose responsiveness.

Using conditions as described by Yuan and colleagues (10) for islet dedifferentiation, and a combination of factors for redifferentiation including IGF-II, KGF, and Matrigel, we first sought to explore the capacity of neonatal mouse islets to dedifferentiate to a proliferative phenotype, and subsequently redifferentiate back towards functional endocrine cells *in vitro*. We hypothesized that changes in environment would provide the inductive signals for insulin re-expression. The redifferentiation potential was also examined after co-culture with mouse endothelial cells, and after transplantation into immune-deficient mice to assess whether the *in vivo* environment was more effective than that

provided *in vitro* in re-directing the resultant islet-derived cell-aggregates into functional insulin-producing cells capable of ameliorating hyperglycemia.

2.2 Materials and methods

2.2.1 Islet and duct isolation

Animal experimentation was approved by the Western University Animal Use Ethics Committee and in accordance with the Canadian Council on Animal Care. Reagents were obtained from Invitrogen (Carlsbad, CA) unless otherwise specified. Islets were isolated using a modified protocol as described (27). Briefly, postnatal day 7 (P7) C57Bl/6 mice (Charles River Laboratories, Wilmington MA) were euthanized by decapitation and their pancreata excised, starting at the tail of the spleen, and placed in a Petri dish. One millilitre (1 mL) of digestion buffer, consisting of 1 mg/mL collagenase type V (Sigma Chemical, St. Louis, MO) in Hank's Buffered Salt Solution (HBSS), was perfused through each pancreas using a 23 G needle until it expanded, and collected in an additional 1 mL digestion buffer per pancreas. Pancreata were placed in a shaking water bath at 37°C for 20-30 min and additionally shaken by hand gently for 1 min. Digested tissue was passed through a 14 G needle and washed in HBSS + 5% (v/v) fetal bovine serum (FBS). Intact islets were separated from acinar and duct cells using sequential Dextran (MP Biomedical, Solon, OH) gradients at 27, 23 and 11% (v/v), and centrifuged for 25 min at 610 x G. Islets were collected from the 27-23% interface using a 14 G syringe and washed in HBSS + 5% FBS, then placed in a Petri dish with HBSS + 5% FBS and handpicked using an inverted microscope.

Intact ducts were isolated similarly to islets, but the digestion time was decreased to prevent the loss of intact structures, and hand-shaking was omitted. The pancreatic cell

mixture was passed through 2 mesh basins containing filters, one 30 µm and the other 70 µm; large ducts being collected between these two, while intact islets collected at the bottom. The ducts were then handpicked.

2.2.2 Islet dedifferentiation

Isolated islets were cultured in media as described (10), similar in composition to that used for the culture of generalized epithelial cells, including mammary (28), prostate (29), and cornea (30). Such media commonly contain EGF, a corticosteroid, serum, insulin, and cholera toxin, which acts as a cAMP-elevating agent (28). Specifically, the formulation used for pancreatic ductal epithelial culture was composed of DMEM/F-12 (Gibco, Grand Island, New York), 10% FBS, 100 mM Penicillin/Streptomycin (Gibco), 10 mM Fungizone (Gibco), 1 µg/mL insulin (Sigma), 100 ng/mL cholera toxin (Cedarlane Labs, Burlington ON), 1 µM dexamethasone (Sigma), 0.1 mg/mL soybean trypsin inhibitor (Gibco), and 10 ng/mL EGF (ID Labs, London, ON). Islets were cultured on type I (rat-tail) collagen as a basement membrane (10).

2.2.3 Redifferentiation of islet-derived cells

After 1-4 weeks in ductal dedifferentiation culture, collagen was digested using Collagenase XI (Sigma), and the cell monolayer dispersed into a single cell suspension using Dispase and Accutase (BD Biosciences, Mississauga ON) as per manufacturer's instructions. Cells were cultured for endocrine redifferentiation on 0.5 mL Matrigel (BD Biosciences) diluted 1:1 with DMEM/F12, and supplemented with media composed of DMEM/F12, 10% FBS, 100 mM Penicillin/Streptomycin, 10 mM Fungizone (Gibco), 100 ng/mL IGF-II (ID Labs)

and 50 ng/mL KGF (FGF-7) (ID Labs). Alternatively, dispersed and dedifferentiated cells were cultured in media previously shown to promote endocrine maturation as described by Russ *et al* (31), and similar to Seaberg *et al* (32) consisting of 25 mmol/L D-glucose, 1% bovine serum albumin (BSA), ITS (Sigma), N2 and B27 supplements (Gibco), 10 mmol/L nicotinamide (Sigma), 8 nm exendin-4 (Bachem Chemical, Bubendorf Switzerland), and 8 nm activin-A (Cedarlane Labs, Burlington, ON), in 6-well plates coated with Matrigel (1:25 dilution).

2.2.4 RNA isolation and qualitative Reverse Transcription-Polymerase Chain Reaction

For analysis of cells at various stages of development or differentiation, RNA was assessed. Isolated cells were stored in RNAprotect (Qiagen, Venlo, Netherlands), and total RNA extracted using RNeasy Plus Mini/Micro kits where appropriate (Qiagen). One nanogram (1 ng) total RNA was reverse transcribed to cDNA using Superscript II, Oligo(dT)$^{12-18}$ and 10 mM dNTPs. Four microlitres of RT (cDNA) was amplified for specific genes of interest using 20 µM forward and reverse primers (Sigma) as described in **Table 2.1**, with 1 U (0.2 µL) *Taq* DNA Polymerase (UBI Life Sciences Inc, Saskatoon, SK). Annealing temperatures were determined using Fast-PCR software (PrimerDigital Ltd, Helsinki Finland). RT-PCR was performed for islet markers insulin (*Ins*), glucokinase (*Gck*), and glucose transporter 2 (*Glut2*); transcription factors Pancreatic Duodenal Homeobox-1 (*Pdx1),* Neurogenin3 *(Ngn3),* Neurogenic Differentiation1 *(NeuroD),* Paired box gene4 (*Pax4), Pax6,* Homeobox protein Nkx_2.2 (*Nkx2.2), Nkx6.1,* v-Maf musculoaponeurotic fibrosarcoma onco- gene homologue A (*MafA)* and *MafB*; the mesenchymal marker vimentin (*Vim*); ductal (precursor) transcription factors SRY (sex-determining region Y) box 9

Table 2.1. Primer sequences designed for the amplification of mouse cDNA

Gene	Sense primer (5'-3')	Antisense Primer (5'-3')	Band Size
Insulin	ccctgctggccctgctctt	aggtctgaaggtcacctgct	214
Pdx1	tgtaggcagtacgggtcctc	ccaccccagtttacaagctc	326
Gck	gcagatcctggcagagttcca	ggaaggagaaggtgaagccca	408
Glut2	caagatcaccggaaccttgg	attccgcccactgcaaagct	313
NeuroD	cttggccaagaactacatctgg	ggagtagggatgcaccgggaa	230
Ngn3	ggcgcctcatcccttggatg	cagtcacccacttctgcttcg	161
Pax4	gtgagcaagatcctaggacgc	cggggagaagatagtccgatt	379
Pax6	aaacaaacgccctagctctcc	ccgcccttggttaaagtcctc	118
Nkx2.2	ctcttctccaaagcgcagac	aacaaccgtggtaaggatcg	515
Nkx6.1	ttctctggacagcaaatcttcg	ctgagtgattttctcgtcgtca	298
MafA	tcactctgcccaccatcac	tgacctcctccttgctgaag	198
MafB	ggtataaacgcgtccagcag	cgagtttctcgcacttgacc	138
CK19	gtgccaccattgacaactcc	aatccacctccacactgacc	289
Sox9	ttcatgaagatgaccgacga	gtccagtcgtagcccttcag	242
Hnf6	cgcggtgactcagctacaga	atctgtgaccaccattgcag	103
Hnf1β	atctgtgaccaccattgcag	tgcggtgactcagctacaga	178
CAII	agattggacctgcctcaccaa	ttacttaaaggacgctttga	220
β-tub	tcactgtgcctgaacttacc	ggaacatagccgtaaactgc	319
FSP-1	atggcaagacccttggagga	cattgcacatcatggcaatg	263
Vim	aatgcttctctggcacgtct	tcttccatctcacgcatctg	414

Pdx1: pancreatic duodenal homeodomain box 1; Glut2: glucose transporter 2; Gck, glucokinase; Ngn3: neurogenin3; β-tub: β-tubulin; Ck19: cytokeratin 19; Sox9 : SRY (sex determining region-Y) box-9; Hnf6: hepatocyte nuclear factor 6; Hnfβ: Hepatocyte nuclear factor-β; MafA : V-maf musculoaponeurotic fibrosarcoma oncogene homolog A, Nkx2.2: homeobox protein Nkx_2.2; Pax 4: paired box gene 4; NeuroD: neurogenic differentiation 1; CAII: Carbonic Anhydrase II; Vim: vimentin; FSP-1: fibroblast-specific protein 1

(*Sox9),* Hepatocyte nuclear factor 6 *(Hnf6), Hnf1β*, mature duct markers, carbonic anhydrase II (*CAII*), and cytokeratin 19 (*CK19*), and β-tubulin used as control. PCR conditions were established to ensure linear amplification of PCR products using varying cycle numbers (26-40 cycles). Amplified cDNA was run on a 2% agarose gel and visualized under UV-light, counterstained with ethidium bromide.

2.2.5 Immunofluorescent cytochemistry

For imaging of cells grown *in vitro*, collagen was digested using collagenase XI (Sigma) and Accutase (BD Biosciences, Mississauga ON), and Matrigel digested using Dispase (BD Biosciences), and washed in PBS to remove serum. Dispersed cells in PBS were then added to glass-bottomed Mattek dishes (Mattek Corp, Ashland, USA) adsorbed with diluted Cell-Tak (BD Biosciences) for 15 min. Adherent cells were fixed with 4% (v/v) paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA) for 15 min. Whole mount tissues such as intact islets or cell clusters were alternately handpicked and placed on Cell-tak-coated Mattek dishes for 30 min and fixed for 30 min in 4% PFA. Fixed cells adherent on Mattek dishes were stored in PBS at 4°C until immunocytochemistry was performed. Isolated cells were permeabilized with 0.3% (v/v) triton-X-100/PBS for 30 min before blocking with Background Sniper (Biocare Medical, Concord CA) for 8 min. Primary antibodies were incubated in Antibody Diluting Solution overnight at 4°C and secondary antibodies for 2 h at room temperature. A list of antibodies and dilutions is provided in **Table 2.2**. Cell proliferation was determined by the addition of 0.05% (v/v) EdU (5-ethynyl-2′-deoxyuridine)/PBS to the media for 6 h prior to fixation. Cells were stained with EdU Click-It Reaction kit. Nuclei were visualized by counterstaining with 4', 6-diamidino-2-

Table 2.2. Antibodies used for immunofluorescent histochemistry

Protein	Animal	Company	Dilution
Insulin	mouse	Sigma Chemical (St Louis, MO)	1:2000
Insulin	rabbit	Santa Cruz (Santa Cruz, CA)	1:200
Pdx1	rabbit	Abcam Inc (Toronto ON)	1:1000
CK19	rabbit	Abcam	1:100
CK19	mouse	Dako (Glostrup, Denmark)	1:100
E-cadherin	mouse	Abcam	1:100
Ki67	rabbit	Neomarkers (Freemont CA)	1:200
Ki67	mouse	BD Biosciences (Mississauga ON)	1:50
Ngn3	mouse	DSHB *	1:40
Vimentin	rabbit	Santa Cruz	1:100

*DSHB, Developmental Studies Hybridoma Bank, University of Iowa, Department of Biology, Iowa City, Iowa

phenlindole (DAPI, blue), Sytox Green, or ToPro Red. Cells/sections were imaged on a Zeiss Laser Scanning Confocal Microscope (LSCM) (Zeiss, Oberkochen, Germany), and counted manually using the Zeiss LSM Image Browser. The EdU staining kit utilized a far-red fluorophore (647 nm). A beam splitter was placed on the LSCM at 560 nm, allowing a red fluorophore (543 nm/ 555 nm) to be used simultaneously with a far-red fluorophore (647 nm).

2.2.6 Transplantation experiments

NOD/scid IL-2rγ$^{-/-}$ mice were utilized as transplant recipients, as these animals are deficient in T cells, B cells, all lymphocytes, and natural killer (NK) cells, and have been used previously in islet transplant experiments (33). Transplant recipient mice (*n* = 6) were maintained in barrier cages with free access to (sterilized) food and water at St. Joseph Animal Care Facility. Five hundred freshly isolated mouse islets and 1000 clusters from female C57Bl6 mice were transplanted to individual sites under the left kidney capsule of eight-week old NOD/scid IL-2rγ$^{-/-}$ male mice. Sex-mismatched transplants were chosen to distinguish donor from host cells if necessary using a gender-specific biomarker (Star*FISH, Cambio Ltd, Cambridge UK). Mice were anaesthetized using isofluorane, and post-surgical ketoprofen was administered as anaesthetic. Mice were monitored daily after surgery.

Intraperitoneal glucose tolerance tests (IPGTT) were performed 1 month post-transplant to assess functionality. Animals were fasted 4 h prior to injection with 2 g/kg D-glucose. Blood glucose measurements were taken at 0, 5, 15, 30, 60, and 90 min, and 50 µL blood samples were taken at 0, 15, and 90 min. Serum insulin values were assessed using

Ultrasensitive Insulin radioimmunoassay (RIA) (range 0.02-1 ng/mL) (St. Joseph Health Care, London ON).

Mice were euthanized following IPGTT by CO_2. Kidneys and pancreas were dissected and fixed in 4% PFA for 24 h, then washed in PBS for 24 h prior to incubation in 30% (v/v) sucrose overnight, embedded in OCT, and cryosectioned for immunofluorescence analysis. Sections were immunostained for insulin, glucagon, Pdx1, and Ngn3 to determine the degree of maturation of transplanted cells, and proliferation frequency assessed using Ki67 antigen.

2.2.7 Media / Basement membrane recombination

To assess the contribution of basement membrane proteins *vs*. media compositions to differentiation state, islets were cultured in various combinations: (1) dedifferentiation media + type 1 collagen; (2) redifferentiation media + type 1 collagen; (3) dedifferentiation media + Matrigel; and (4) redifferentiation media + Matrigel. After 4 weeks in these conditions, cells were dispersed into single cell suspension and subsequently cultured for "redifferentiation" on Matrigel with redifferentiation media containing IGF-II and KGF as described.

2.2.8 Mouse pancreatic endothelial cell co-culture

Mouse pancreatic endothelial cells (ECs) were isolated from C57Bl/6 mice at P10. Briefly, pancreata were isolated and placed in 0.25 mg/mL type XI collagenase at 37ºC for 15 min. Cells were washed in HBSS + 5% FBS twice, then added to 3 mL DMEM/F-12+ 10% FBS. Cells were then aspirated through a 3 mL syringe, followed by sequential aspiration

through 18 G, 20 G, and 22 G needles. The slurry was filtered through a sterile 100 µm nylon mesh into single cell suspension. EC cell selection was accomplished using PECAM-1 antibody (1:50, CD31) (Hycult, Plymouth Meeting, PA) coated magnetic beads (Dynabeads) and a rotary magnet according to manufacturer's instructions. The isolated ECs were cultured on 0.1% (v/v) Fibronectin (Sigma) cultured with EBM-2 media, with all supplements (Clonetics, Lonza Group Inc, Basel Switzerland). ECs were passaged a maximum of 3 times, splitting the cells 1:5, and media changed twice per week.

Eight thousand ECs were mixed with 500 µl DMEM/F-12, then diluted 1:1 with Matrigel and plated in a 6-well tissue culture dish. ECs formed branching structures within the Matrigel after 7 days and were supplied with EBM-2 media. Alternately, ECs were laid on top of the Matrigel, rather than embedding within it. Differentiated islet-derived cells were then cultured on top of this.

2.2.9 Statistical analysis

Data are presented as percent (%) mean ± SEM, with statistics performed using a one-way ANOVA and Tukey's post-test, or student's t-tests as indicated, and with an acceptable level of significance of $p < 0.05$. Statistics were analyzed using GraphPad Prism 5 software (La Jolla, CA).

2.3 Results

2.3.1 Changes in islet cell morphology

Islets plated in dedifferentiation conditions (**Figure 2.1A**) lost all morphological characteristics of functional endocrine cells (**Figure 2.1B**), forming a heterogeneous,

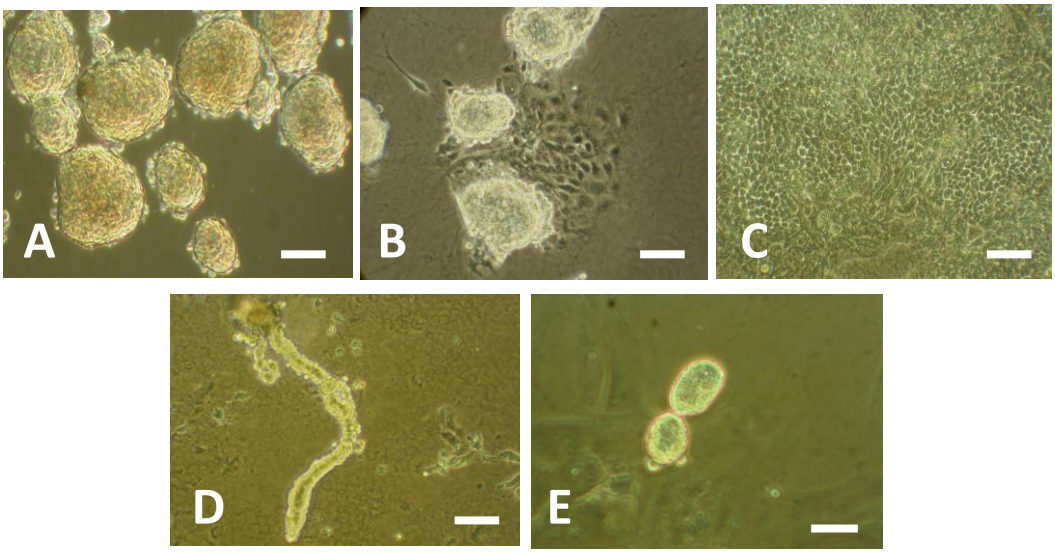

Figure 2.1. Neonatal mouse islets cultured in dedifferentiation and redifferentiation conditions. Neonatal (P7) mouse islets plated on collagen under dedifferentiation culture conditions, shortly after plating (A), after 4 days once monolayer formation had commenced (B), and after 4 weeks (C). Cells cultured in dedifferentiation media and harvested after 4 weeks were re-plated onto Matrigel under redifferentiation culture conditions. The cells formed cellular aggregates which suspended in the media, sometimes exhibiting long, duct-like morphologies (D) or alternatively, small spherical aggregates (E). Size bar denotes 50 µm, $n > 10$ experiments.

proliferative epithelial-like monolayer within 4 weeks (**Figure 2.1C**), consistent with data shown by others (10,34)**.** This monolayer could be passaged multiple times without further change in morphology (not shown). Similar morphologies were found after culture of isolated mouse pancreatic ducts.

When subsequently plated in "redifferentiation" conditions containing IGF-II and KGF, the dispersed islet-derived and duct-derived cell monolayers re-organized within the Matrigel, forming floating cell aggregates. These cell clusters adopted variable morphologies, often forming long, branched tube-like structures which resembled ducts (**Figure 2.1D**), or alternatively forming small spherical clusters (**Figure 2.1E**). These cell clusters could be continuously formed for months after plating.

2.3.2 Changes in islet cell gene expression

Intact islets demonstrated markers of mature islet function, including Ins, Pdx1, and MafA as shown on a composite gel (**Figure 2.2A**). When cultured in dedifferentiation medium these mature markers diminished, instead showing an up-regulation of markers for ductal epithelium such as CK19 and CAII (**Figure 2.2B**), as well as ductal transcription factors Sox9, Hnf6, and Hnf1β (**Figure 2.2B**). MafB expression was maintained while MafA was lost.

After culture in redifferentiation conditions on Matrigel in media containing IGF-II and KGF, the cell aggregates maintained ductal epithelial gene markers (Sox9, Hnf6, Hnf1β, CK19, CAII), but exhibited limited re-expression of some endocrine pancreas markers, such as Pdx1 and Nkx6.1 (**Figure 2.2C**). MafB expression was maintained after cell-cluster formation (**Figure 2.2C**), but Vim expression was lost (not shown). Ins, MafA, and Ngn3 were not re-expressed.

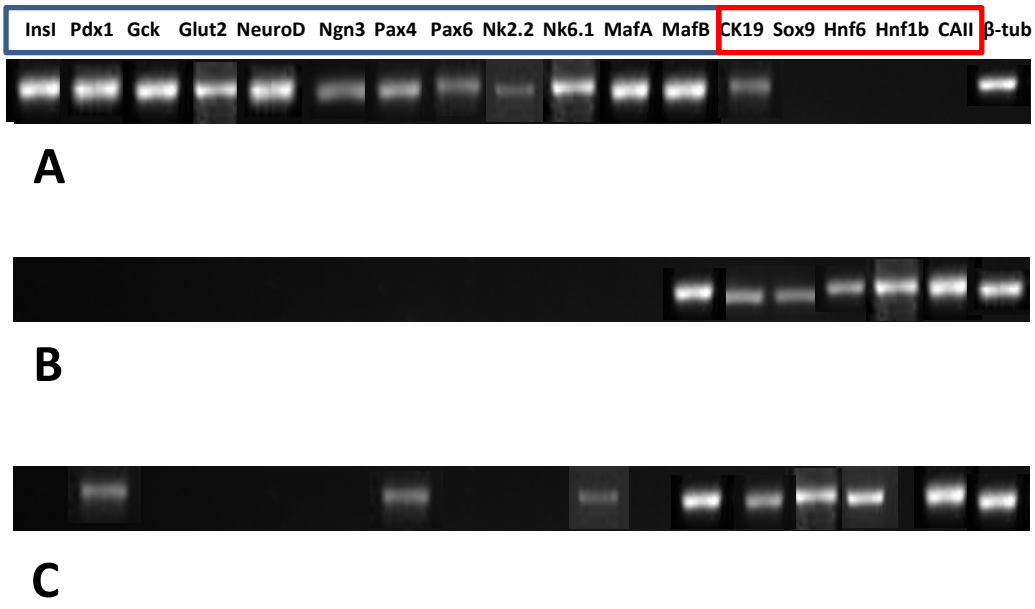

Figure 2.2. Gene expression profile of cells obtained after culture of mouse islets. Expression of cDNA on a composite gel from isolated islets (row A), after four weeks on collagen with dedifferentiation media (row B), and from cellular clusters after subsequent culture in redifferentiation media (row C). Islet-specific markers are surrounded by blue and duct-specific markers in red bar; *n* > 10.

2.3.3 Changes in islet cell protein presence

Assessment of protein presence using whole islet confocal microscopy revealed that isolated islets displayed immunostaining for insulin (**Figure 2.3A**, green), and Pdx1 (**Figure 2.3B**, red), but not CK19 (**Figure 2.3C**, red). The dispersed dedifferentiated monolayer showed a loss of insulin immunostaining (**Figure 2.3D**, green), and an increase in immunostaining for CK19 (**Figure 2.3D**, red), as well as positive staining for e-cadherin (**Figure 2.3E**, green). After redifferentiation culture, intact cell clusters showed that both small spherical and elongated cluster morphologies were cyst-like, demonstrating a hollow core (**Figure 2.3F**, blue, DAPI). These cyst-like cell clusters maintained positive immunostaining for CK19 (**Figure 2.3G**, green), and e-cadherin (**Figure 2.3H**, green). Rarely, the small, spherical clusters demonstrated limited immunostaining for Pdx1 (**Figure 2.3J**, red), which was never seen in the elongated, branching cell clusters (**Figure 2.3I**). There was extremely low cell replication rates as calculated by EdU incorportion within the clusters (<0.1%, not shown). Efforts to promote redifferentiation by addition of other growth factors to redifferentiation media such as EGF, nicotinamide, betacellulin, and gastrin were unsuccessful, in that expression of key markers of redifferentiation were unchanged (not shown). However, when dedifferentiated islet-derived epithelial cells were cultured in established redifferentiation conditions shown to promote endocrine maturation (31,32), rare cells (<1:1000) demonstrated insulin expression by immunostaining (**Figure 2.3K**, red).

2.3.4 Transplantation of islet-derived cells

Donor islets and islet-derived cell clusters within grafts survived after transplantation beneath the kidney capsule of normoglycemic NOD/scid IL-2rγ$^{-/-}$ mice

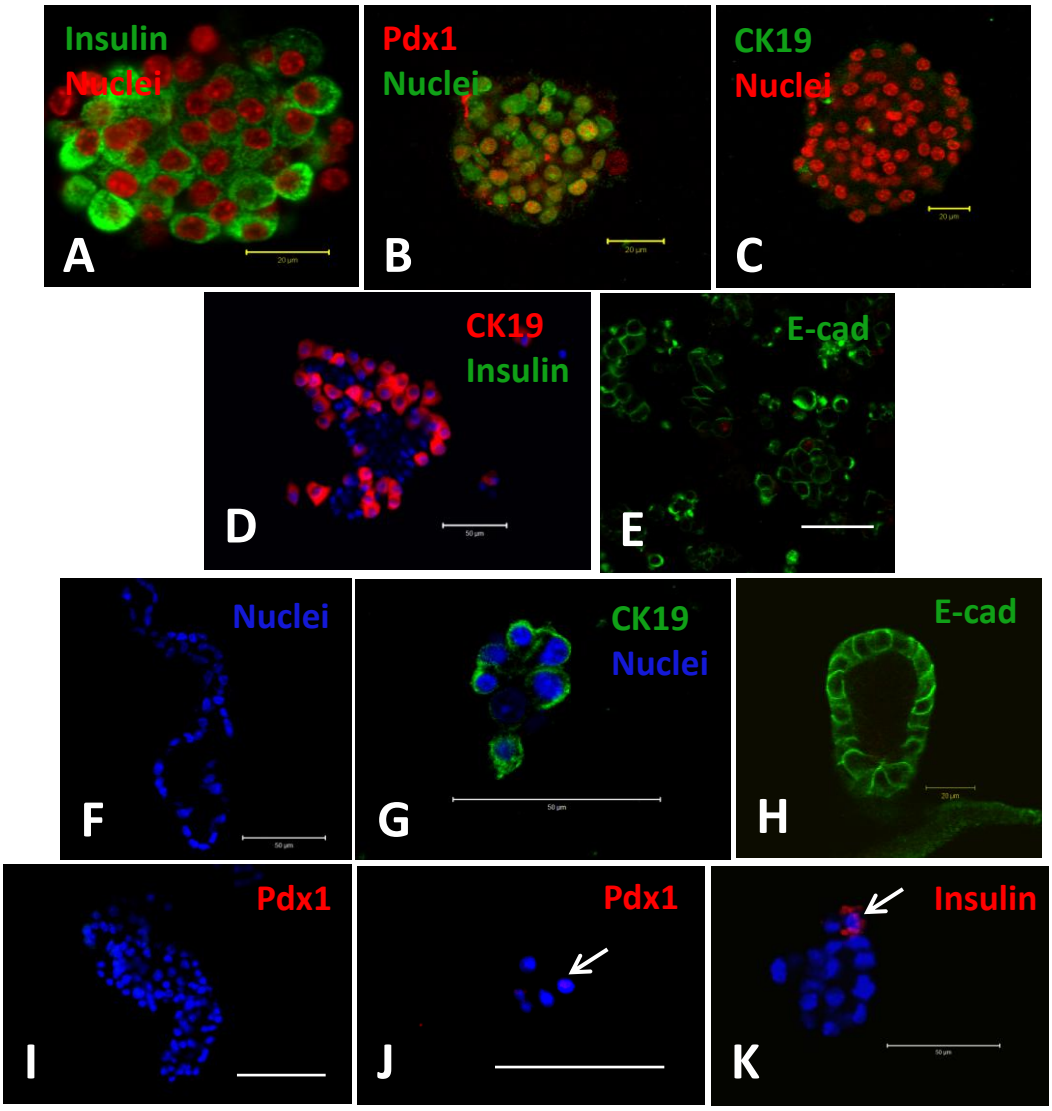

Figure 2.3. Immunofluorescent images of cells obtained after dedifferentiation and redifferentiation culture of mouse islets. Isolated islets were immunostained for insulin (A, green), Pdx1 (red, B) and CK19 (C, green). After dedifferentiation culture to ductal epithelium, there was a significant increase in the proportion of cells positive for ductal markers including CK19 (D, red) and e-cadherin (E, green). Redifferentiation procedures in the presence of IGF-II and KGF yielded cyst-like structures which resembled ducts (F), and which maintained expression of CK19 (G, green) and e-cadherin (H, green) and which was negative for Pdx1 (I, red). Alternative redifferentiation culture in media yielded restricted re-expression of Pdx1 and insulin in the small spherical clusters only (J, and K, red). Size bars represent 50 µm, *n* > 10 experiments.

(**Figures 2.4A-G**). Transplantation of isolated islets demonstrated no loss of expression of insulin (**Figure 2.4A**, green) or Pdx1 (**Figure 2.4B**, red). Transplantation of IGF-II + KGF media redifferentiated cell clusters revealed cells containing immunoreactive Pdx1 after 4 weeks *in vivo* (**Figure 2.4D**, red), as well as cell proliferation shown by staining for Ki67 antigen expression (**Figure 2.4F**, red, not quantified). However, insulin, MafA, and Ngn3 were not re-expressed in any of these grafts. Ductal markers were maintained in the redifferentiated cell grafts, with positive immunostaining for e-cadherin (**Figure 2.4G**, green, arrows). A glucose tolerance test showing normal glycemia after transplant is provided in **Figure 2.4H**, demonstrating that the metabolism of the host mice was not altered following surgery.

2.3.5 Co-culture of dedifferentiated cells with pancreatic endothelial cells

Mouse pancreatic ECs could be isolated and grown on fibronectin-coated culture dishes (**Supplemental Figure S2.1A**), and which could be passaged (**Figure S2.1B**). These cells grew substantially within the first 7 d of culture when isolated and mixed with Matrigel supplemented with EBM-2 medium (**Figure S2.1C**). After 7 d, ECs had formed branching structures (**Figure S2.1D**). When dedifferentiated islet cells were overlaid on top of Matrigel containing EC isolated from mouse pancreas, the dedifferentiated cells grew well in the presence of ECs, although this was not quantified (3 days, **Figure 2.5A**; +7 days, **Figure 2.5B**; +3 weeks, **Figure 2.5C**). The dedifferentiated cell morphology was slightly more disorganized when ECs were cultured on top of Matrigel with dedifferentiated cells overlaying these (**Figure 2.5D**). In both culture conditions however, there was no re-expression of insulin, ngn3, or other β-cell specific markers (not shown).

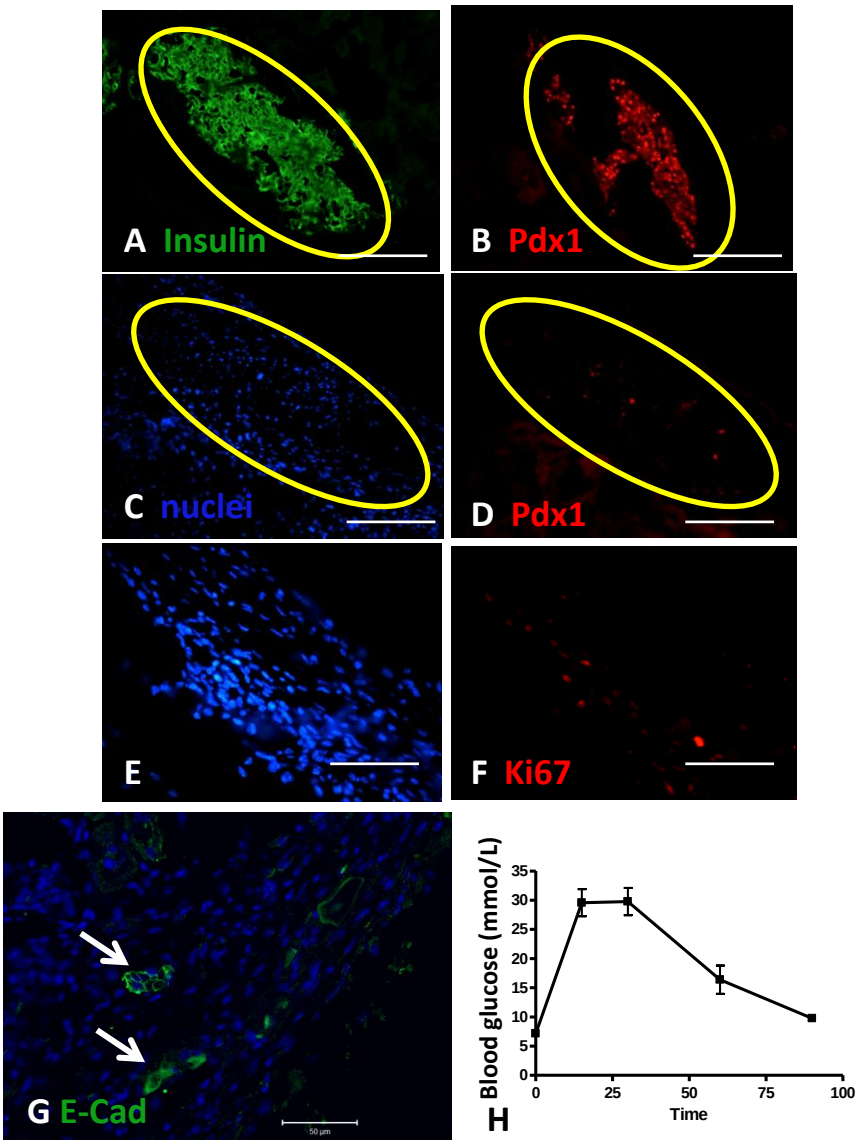

Figure 2.4. Effect of transplantation on cell differentiation state
Islet grafts transplanted under NOD/scid IL-2rγ$^{-/-}$ mouse kidney capsules showing insulin (green, A) and Pdx1 (red, B) immunostaining. Redifferentiated cell cluster grafts (C-F) under the kidney capsule (blue, DAPI, C, E) demonstrated the presence of Pdx1 (D, red) and ki67 (F, red) but retained their ductal phenotype as shown by e-cadherin immunostaining (G, green). A glucose tolerance test of transplant recipient mice is shown in H. Size bar denotes 50 µm, *n* = 6 mice.

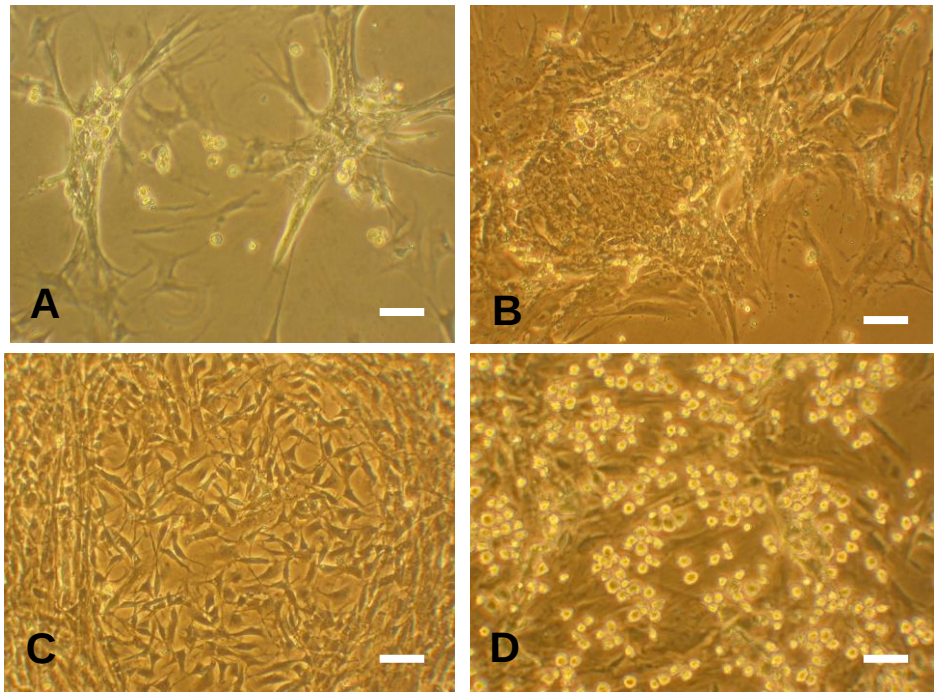

Figure 2.5. Co-culture of islet-derived cells with mouse pancreatic endothelial cells After plating dedifferentiated cells on top of pancreas-derived mouse endothelial cells (ECs), there was significant growth (+3 days, A, +7 days B, + 3 weeks, C). Morphology was less uniform when ECs were plated on top of Matrigel with dedifferentiated cells overlaying these (D). Scale bars represent 50 µm, *n* = 4.

2.3.6 Matrix/media combinations

To determine the effects of matrix and medium on islet dedifferentiation, isolated islets were cultured in either type I collagen or Matrigel with dedifferentiation medium or redifferentiation medium. Islets plated on collagen with dedifferentiation media (combination 1) produced a proliferative, heterogeneous epithelial monolayer (**Figure 2.1A-C**) and islet morphology was lost within the first week in culture, as described above. Islets cultured on collagen with redifferentiation media (combination 2) retained the original islet structure, with an increase in fibroblasts; this was also found when islets were cultured in dedifferentiation media with Matrigel (combination 3), and when islets were grown in redifferentiation media on Matrigel (combination 4) (**Figure 2.6A**). Islets cultured in redifferentiation media with Matrigel became highly disorganized around the original islet structure with areas of epithelial as well as fibroblast growth. After dispersing and culturing all cells for redifferentiation in Matrigel, combination (1) cells formed ductal-like cell clusters which resembled cysts, and which were positive for ductal epithelial markers, as previously described. Cells grown in combinations (2) and (3) both reformed cellular clumps, which were positive for fibroblast markers such as vimentin and fibroblast-specific protein 1 (FSP1), and which maintained expression of insulin and glucagon (not shown). Interestingly, islets grown in redifferentiation conditions from the outset maintained expression of islet markers such as insulin and glucagon, increased expression of fibroblastic markers, and also formed what appeared to be lipid droplets (**Figure 2.6B**) although further validation is required. Islets cultured in dedifferentiation media without cholera toxin did not form an epithelial monolayer and maintained the original islet structure. Overall, these

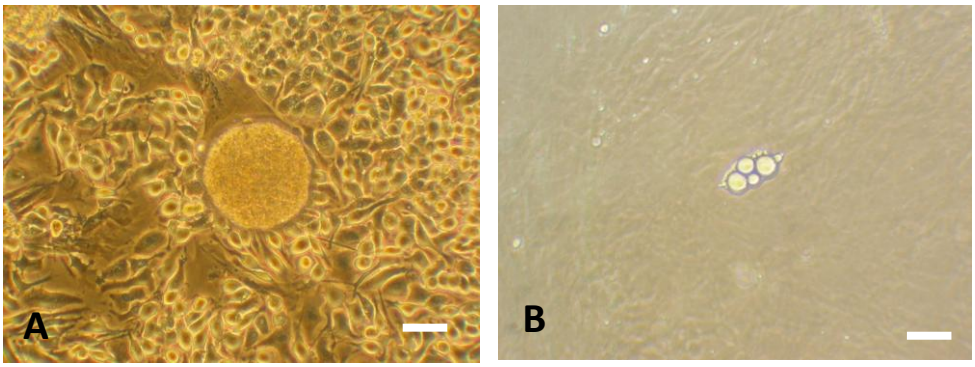

Figure 2.6. Variable cell types arise after culture of mouse islets in altered matrix/media combinations. Changing the combination of media (dedifferentiation or redifferentiation) and matrix (type I collagen and Matrigel) showed that only islets plated on collagen and supplemented with dedifferentiation media caused the islet structure to disappear and a dramatic increase in ductal epithelial markers; all other combinations maintained the original islet structure (A). Cells with adipocyte-like morphology were noted as shown by the appearance of lipid-like droplets when isolated mouse islets were grown in Matrigel with redifferentiation media, then dispersed and re-plated under the same conditions (B). Scale bars represent 50 µm, *n* = 4.

data suggest that the media and matrix combination of type 1 collagen with EGF and cholera toxin promotes the most robust change in islet-derived cellular morphology.

2.4 Discussion

Following islet dedifferentiation to ductal epithelial cells on collagen in the presence of EGF and cholera toxin, a highly proliferative epithelial monolayer of cells was generated. Subsequent culturing of these cells on Matrigel using IGF-II and KGF yielded cell aggregates that maintained ductal genetic markers formed during dedifferentiation, with ductal/cyst-like morphology. Dedifferentiated cells were also plated for redifferentiation in co-culture with mouse endothelial cells, or transplanted under the kidney capsule of immune deficient mice, to promote further cell maturation. These manipulations did not further result in the generation of insulin-producing cells. However, alternative use of redifferentiation medium reported by Russ and colleagues (31) did generate rare insulin-producing cells. It remains to be determined which experimental approach (dedifferentiation *vs* redifferentiation) is the limiting step for the generation of sufficient insulin-producing cells for transplant. Determination of the lineage origin of cells generated in the culture process would provide answers to these questions.

Developmentally, islets are derived from cells within the pancreatic epithelium which share a bipotent progenitor cell state with duct cells (35). Extensive work has centered around the neogenesis of islets or insulin-producing cells from ducts, with compelling evidence suggesting the ductal compartment contains islet progenitor cells (16,36–42). Early lineage-tracing studies using CAII indicated that cells within the ductal epithelium were involved in endocrine regeneration (43), and some $Ngn3^{+}$ lineage-tagged

cells co-localized with duct cells after injury (44). However, recent evidence has indicated that the endocrine cells present within the ductal epithelial layer were not generated postnatally (45,46), thus restricting the contribution from ductal progenitors after birth to endocrine cell replacement.

The media formulation used for the "redifferentiation" protocol is similar in composition to that used in the formation of "islet-like cell clusters" containing IGF-II, KGF, and HGF from human fetal pancreas (14,15), and to that used in the trans-differentiation of ducts to insulin-expressing cells (16). We have previously shown that IGF-II is an islet anti-apoptotic factor (47), and KGF has been shown to be a potent ductal epithelial mitogen (48). Another study dedifferentiated islets to $CK19^+$ cells, and subsequently used nicotinamide, ITS, and HGF for "redifferentiation" to insulin-producing cells (49). The continued use of serum in our model may have limited the ability of mature cells to change differentiation state; however, we found that the cells did not survive long-term culture without the trophic stimulus provided by the serum. Moreover, in the absence of lineage-tracing techniques, it is impossible to definitively conclude that the generation of insulin from ductal preparations did not arise from insulin-producing β-cells present at the outset. Similarly, a recent study by Kim *et al* generated insulin-expressing cells from isolated human ductal cells, using activin-A, exendin-4, and high glucose (50). These same components are present in media used by Russ and colleagues (31), and were shown to generate rare $insulin^+$ cells as demonstrated here. However, Kim *et al* asserted that these cells had derived exclusively from the ductal fraction, as they pretreated the pancreatic cell preparation with 5mM streptozotocin (STZ) to destroy any residual β-cells retained therein (50). Human β-cells have been shown to be generally resistant to the effects of STZ due to the low

expression of GLUT2 as compared to rodent islets (51), and therefore the possibility that pre-existing β-cells present in the ductal cell preparation proliferated and generated functional insulin production has not been thoroughly disproved. In all studies of islet redifferentiation however, the re-expression of insulin was minimal when compared to β-cell secretion in freshly-isolated islets (49,52). Even when directly promoting differentiation of human fetal pancreas, <5% of "islet-like clusters" exhibited positive insulin-expression *in vitro*, although this jumped to ~50% after transplantation (26). This evidence suggests that these *in vitro* manipulations do not provide complete re-maturation of β-cells, let alone islets, thereby questioning the feasibility of this technique for clinical application.

The formation of cysts during islet cell redifferentiation was similarly shown by Bonner-Weir and colleagues during the trans-differentiation of ductal cells (16). While that study reported the generation of considerably more insulin-producing cells, our experiments utilized specific growth factors and serum which created a more mature duct-like phenotype of long, branched structures. Interestingly, a study was performed using embryonic (e11.5) mouse pancreas epithelium to determine the effect of endocrine differentiation on basement membrane selection and mesenchyme inclusion (53). The authors cultured early epithelium in varying culture environments, including collagen I gels, Matrigel, or by transplantation under the kidney capsule of mice, thereby providing a milieu of vascular and dynamic temporal changes by the *in vivo* environment. They found that these early epithelial cells transplanted under the kidney capsule formed islets, whereas the cells grown in Matrigel formed almost exclusively ducts. The cells grown in collagen did not differentiate, and there was no expression of endocrine marker genes. They also concluded that the mesenchyme was only required for acinar cell differentiation (53). Within the

context of the present study, these data indicate that the Matrigel itself preferentially promoted ductal cyst formation. However, since we also performed transplant experiments and co-culture experiments with endothelial cells, and neither generated functional islets nor endocrine hormone-expressing cells, it would suggest that the lineage (re-)specification of intact, P7 islets or insulin-expressing β-cells was greatly determined by previous dedifferentiation on collagen, and that further manipulation of the cell culture environment during "redifferentiation" would have only modest effects.

In addition to ductal-epithelium and fibroblastic cells, islets grown in Matrigel with redifferentiation media were shown to form adipocytes. Culturing pre-adipocytes in the presence of insulin, FBS, a glucocorticoid, and a cAMP-elevating agent has been shown to promote robust adipocyte differentiation (54), but which did not occur when culturing islets. However, when these islet cells were instead grown in Matrigel with media containing IGF-II and KGF, cells with an adipocyte-like appearance were generated. This could be attributed to the presence of laminin in the Matrigel, a strong pro-adipocyte differentiation factor (55), as opposed to the presence of EGF and type I collagen in the dedifferentiation media, which independently exert negative effects on adipocyte growth (55). Clearly, the interactions between media and basement membrane components influence the fate of the cells in question.

The origin of the ductal-derived cell monolayer remains unknown, and there remains the possibility that one or more cell types were capable of ductal differentiation. The α-cell is one candidate cell type shown to exhibit a high level of plasticity (56,57), and may represent a future avenue for exploration. However, as β-cells compose ~80% of the islet compartment by mass, lineage tracing of the β-cell is a top priority for determining the cell

type(s) of origin in the culture conditions purporting to effect dedifferentiation and redifferentiation. Barring significant contributions from δ (somatostatin-expressing), PP (pancreatic polypeptide-expressing) cells, or contaminating duct cells, the present data suggest that the cells obtained after islet growth in epithelial-promoting conditions must have derived from β-cells, or from an unidentified progenitor cell type resident within the islet which could rapidly and reproducibly proliferate. Therefore, determining the contribution of β-cell lineage to the ductal epithelial monolayer will be subsequently undertaken.

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CHAPTER 3

Lineage tracing of the β-cell during Dedifferentiation and Redifferentiation *In Vitro* from Neonatal Mouse Pancreas

A version of this chapter has been previously submitted for publication.

Christine A. Beamish, Brenda J. Strutt, Sofia Mehta, Edith J. Arany, and David J. Hill

3.1 Introduction

It has been proposed that all β-cells in the postnatal pancreas arise from pre-existing β-cells, both during aging and under regenerative circumstances (1,2). However, the contribution of "facultative" progenitor cells, which may require a stimulus of injury or depend on the maturation status of cells, has not been excluded (3). It has been shown that β-cell generation in diabetic mice can be induced by the presence of bone marrow-derived cells (4) and that cells with a functional β-cell phenotype can be generated from embryonic stem cells (5), and multiple groups have demonstrated the generation of β-cells *de novo* from multiple pancreatic cell types (6–8). Since β-cells develop from bipotent trunk cells in the pancreatic epithelium which share a common precursor population with duct cells, we hypothesized that a plasticity of β-cells would exist in early life that would allow for their controlled dedifferentiation to a duct-like phenotype, subsequent population expansion, and finally redifferentiation to a β-cell phenotype. It was previously shown that mouse and human β-cells can be expanded or dedifferentiated *in vitro* (9,10), with dedifferentiation defined here as the loss of mature and functional characteristics from a partially or terminally differentiated cell type. In some cases, this may occur prior to transdifferentiation, defined as the change from one differentiated phenotype to another involving morphological and functional phenotypic markers (11,12). However, lineage tracing of the de- or transdifferentiated cells from those experiments indicated that the proliferation rate of resulting cells was low, insulin expression diminished after culture, and the progeny derived from mouse β-cells were ultimately eliminated from *in vitro* techniques. We adapted a tissue culture protocol to promote dedifferentiation of isolated

mouse islets to ductal epithelium (13,14), and utilized neonatal mice (postnatal day 7, P7) in the expectation that at this age β-cells possess a degree of lineage plasticity. Using a lineage tracing technique where RIPCre;Z/AP$^{+/+}$ transgenic mice (15,16) are genetically tagged with a human placental alkaline phosphatase (HPAP) reporter in the majority of β-cells, we sought to identify the fate of β-cells and their progeny during the dedifferentiation process. Our results indicate that a sub-population of insulin-expressing cells exists within the neonatal mouse pancreas which retains a capacity for multi-lineage plasticity.

3.2 Materials and methods

3.2.1 Animal experimentation

All animal experimentation was approved by the Western University Animal Use Ethics Committee, in accordance with the Canadian Council on Animal Care. The transgenic mouse RIPCre$^{+/+}$ (15) was crossed with a LacZ/HPAP reporter strain (16) (Jackson Laboratories, Bar Harbor, ME). Genotyping of RIPCre;Z/AP$^{+/+}$ mice was accomplished by PCR amplification using primers for the RIPCre (FW – *gcggtctggcagtaaaaactatc* and RV – *gtgaaacagcattgctgtcactt*, 100 bp) and LacZ/HPAP transgenes (FW - *ccgcttcccatatgtggctct* and RV- *gcatgagctcagtgcggttc*, 548 bp) from P5 pups. LacZ/HPAP^{-} littermates were used as controls. Details of genotyping procedures are shown in supplemental methods.

3.2.2 Islet dedifferentiation

Reagents were obtained from Invitrogen (Carlsbad, CA) unless otherwise specified. Islets were isolated from P7 mice as described (17) (**Chapter 2**). Whole islets were plated 25/ well in a 6-well plate coated with 1 mL type I (rat tail) collagen, with media as described (13) (**Chapter 2**). All cell culture media contained 100 mmol/L penicillin/streptomycin and 10 mmol/L fungizone.

3.2.3 Fluorescent immunohistochemistry

Cell monolayers derived after dedifferentiation on collagen were prepared as described in **Chapter 2**. Mice were injected with 2 µl 10 mmol/L EdU /g body weight 24 h prior to dissection, or, for *in vitro* incubation, 0.05% (v/v) was added to culture media for 6 h prior to fixation. To assess pancreatic cells *ex vivo*, mice were euthanized on P7 by decapitation and at later ages by CO_2 asphyxiation, and pancreata were excised and immediately fixed in cold 4% paraformaldehyde (PFA) (Electron Biosciences, Hatfield, PA) for 24 h, washed in PBS for 24 h, incubated in 30% sucrose/PBS for 24 h, then embedded in OCT and snap frozen prior to cryosectioning at 8 nm. The Ki67 antibody required antigen retrieval using 0.1 M Tris-EDTA at 95°C for 30 min. Sections and cells on Mattek dishes were permeabilized with 0.3% triton-X-100 /PBS for 10 min before blocking with Background SNIPER (Biocare Medical, Concord, CA) for 8 min. The HPAP antibody made in mouse (Sigma-Aldrich, St Louis, MO) required the use of a mouse-on-mouse (MOM) staining kit (Vector Labs, Burlingame, CA) to block endogenous immunoglobulins. Other antibodies

utilized are described in **Table 3.1**. Antibodies were diluted in Antibody Diluting Solution and sections/ cells on Mattek dishes incubated overnight at 4°C. Fluorescent secondary antibodies were conjugated to 488/555/647 fluorophores (1/500) appropriately matched to the primary antibody, and sections incubated for 2 h at room temperature in the dark. Rates of cell proliferation were determined by EdU incorporation using the EdU Click-It reaction kit. Cells were counterstained with DAPI for quantification. Slides and Mattek dishes were imaged on a Zeiss LSM 510 Duo Vario (Carl Zeiss Ltd, Oberkochen, Germany) confocal microscope at the Biotron, Western University using Zen software, and cells counted manually using the Zeiss LSM Image Browser (version 4.2).

3.2.4 Quantification of cells

HPAP$^+$ cells were quantified by co-staining with insulin in P7 pancreas sections (> 200 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 / Mattek dish.

Table 3.1. Antibodies used for immunofluorescent histochemistry

Protein	Species	Company	Dilution
Insulin	mouse	Sigma Chemical (St Louis, MO)	1:2000
Insulin	rabbit	Santa Cruz (Santa Cruz, CA)	1:200
Insulin	guinea pig	Abcam Inc (Toronto ON)	1:300
Glut2	goat	Santa Cruz	1:100
Glut2	rabbit	Millipore EMD (Billerica, MA)	1:500
Glucagon	rabbit	Santa Cruz	1:200
Somatostatin	mouse	Santa Cruz	1:200
CK19	mouse	Dako (Glostrup, Denmark)	1:100
CK19	rabbit	Abcam	1:100
Ki67	mouse	BD Biosciences (Mississauga ON)	1:50
HPAP	mouse	Sigma	1:500
HPAP	rabbit	Abcam	1:200
GPm6a	mouse	MBL International (Woburn, MA)	1:100
GFAP	rabbit	Millipore	1:200
β-III tubulin	mouse	Sigma	1:500
Amylase	mouse	Abcam	1:50

Glut2: glucose transporter 2; CK19: cytokeratin-19; HPAP: human placental alkaline phosphatase; GPm6a: glycoprotein m6a; GFAP: glial fibrillary acidic protein.

3.2.5 Redifferentiation of islet-derived cells

Dedifferentiated cells were cultured in redifferentiation media as described by Russ *et al* (18) (**Chapter 2**). Cells were cultured in 6-well plates coated with 1:25 Matrigel (BD Biosciences, Mississauga, ON) and maintained for up to 60 d. Matrigel was dissolved using Dispase (BD Biosciences) prior to downstream cellular analysis.

3.2.6 Fluorescent-activated cell sorting

Excised pancreata were perfused with 2 mL digestion buffer (1.0 mg/mL collagenase V, 0.2 mg/mL BSA, 0.1 mg/mL soybean trypsin inhibitor, in HBSS) and incubated at 37°C, shaking, for 30 min. Cells were further dissociated with 0.25% trypsin/EDTA, shaken in a 37°C water bath for 5 min, then drawn twice through 14 G, 18 G, 20 G, and 22 G needles. Enzymes were inactivated by the addition of 5 mL HBSS + 10% FBS + 0.1 mg/mL DNase I. After washing, the resulting cells were re-suspended in 1 mL Red Blood Cell Lysis Buffer for 1 min, then inactivated by adding 5 mL HBSS + 10% FBS + 0.1 mg/mL DNase I. The slurry was strained through a 40 µm nylon mesh (BD Biosciences). Viability count was taken using 20 µl trypan blue + 20 µl cell mixture. Dispersed cells were immunostained for surface markers using antibodies for Glut2 and GPm6a (19) (**Table 3.1**), with primary and secondary antibodies incubated sequentially for 60 min each. 7-Aminoactinomycin D (7-AAD) was added as a viability marker. Gating strategy is provided in **Figure S3.1**. Live $GPm6a^{+}Glut2^{HI/MID/LO}$ cells were sorted into separate fractions on a Becton Dickinson FACSAria III cell sorter running FACSDiVa software (v. 6.1.2) at the London Regional Flow

Cytometry Facility at the Robarts Research Institute and the University of Western Ontario. Cells were recovered for *in vitro* culture.

3.2.7 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 in (20) using 2.8 and 16.7 mmol/L D-glucose. Insulin release was determined using an Ultrasensitive Insulin RIA (range 0.02-1 ng/mL) (St. Joseph Health Care, London ON).

3.2.8 Neural-lineage differentiation

$Ins^{+}(GPm6a^{+})Glut2^{HI/LO}$ cells recovered from FACS were cultured in Neurocult Neural Stem Cell (NSC) Basal Media with Proliferation Supplement (Stemcell Technologies Inc, Vancouver, Canada) plus EGF and bFGF as per manufacturer's instructions on low attachment plates for 7-14 d. Intact neurospheres were transferred to Matrigel-coated (1:25) Mattek dishes, and cultured with NSC Basal Media with Differentiation Supplement for 7-14 d. Cells were fixed and immunostained for neural-lineage markers β-III tubulin and glial fibrillary associated protein (GFAP) (**Table 3.1**).

3.2.9 Statistical analysis

Data are expressed as mean ± SEM, and comparisons between groups analyzed using a student's t-test or one-way ANOVA where indicated unless otherwise stated, and with a minimum acceptable level of significance of $p < 0.05$. Statistical analysis was performed using Graphpad Prism software (v. 5.01, San Diego CA).

3.3 Results

3.3.1 Loss of islet phenotype after culture

Freshly isolated islets were cultured as described in **Chapter 2** (**Figure 2.1 (A-C)**). Immunostaining for the acinar cell marker amylase was seen in pancreas sections outside of the islet (**Supplemental Figure S3.2A**), and was absent after 1 week in the dedifferentiated cell monolayer, as was insulin immunostaining (**Figure S3.2B**).

3.3.2 Significant increase in CK19 expression during islet culture

Islets within pancreas sections contained insulin as demonstrated by immunofluorescence (IF), and did not express CK19. After islet isolation and 1 week in dedifferentiation medium, 75.9 ± 4.5% of all cells expressed CK19 ($p < 0.001$), and this was maintained throughout the remaining culture period. Cell proliferation was examined by the localization of EdU-positive cells, and within all islet cells was 3.1 ± 0.5%. After

dedifferentiation culture for 1 week, the incidence of cell proliferation was 33.1 ± 8.2% (p < 0.01); and thereafter remained at 6.3 ± 0.4%.

3.3.3 Lineage tracing illustrates rare β-cell dedifferentiation

We sought to determine the extent to which Ins^+ cells contributed to islet cell dedifferentiation *in vitro* using the HPAP lineage tracer localized by immunohistochemistry using RIPCre; $Z/AP^{+/+}$ mice. The HPAP reporter protein (**Figure 3.1B**, red) identified insulin-expressing β-cells (**Figure 3.1A,** green) within the islet (**Figure 3.1C**, merged) with an efficiency of 85.3 ± 1.2% (**Figure 3.1F**). The staining frequency of HPAP (**Figure 3.1E**) in extra-islet β-cell aggregates (BCA) containing <5 β-cells was significantly lower at 39.0 ± 6.9% (p < 0.01) (**Figure 3.1D**, insulin, arrows; **Figure 3.1F**). These BCA were not included in the whole islet analysis as they were not cultured due to the physical limitations of handpicking islets. RIPCre;$Z/AP^{-/-}$ littermates did not exhibit HPAP immunostaining within pancreas sections (**Figure 3.1H**). CK19-positive cells were identified around ducts (**Figure 3.1G**, red) but not within $HPAP^+$ islets (**Figure 3.1G**, green) in P7 pancreas sections. In this system, β-cells constituted 78.9 ± 4.4% of total islet cells; thus 67.3 ± 3.9% of total islet cells expressed HPAP initially.

After islet isolation and 1 week *in vitro*, the dedifferentiated monolayer displayed a total loss of insulin immunostaining (**Figure 3.2A**, red), and the frequency of HPAP expression dropped significantly to <0.1% of remaining cells (**Figure 3.2B**, green, arrow, 67.3 ± 3.9% *vs* 0.04 ± 0.01% $HPAP^+$ cells/total cells, p < 0.0001, islet *vs* 1 week), representing 14-

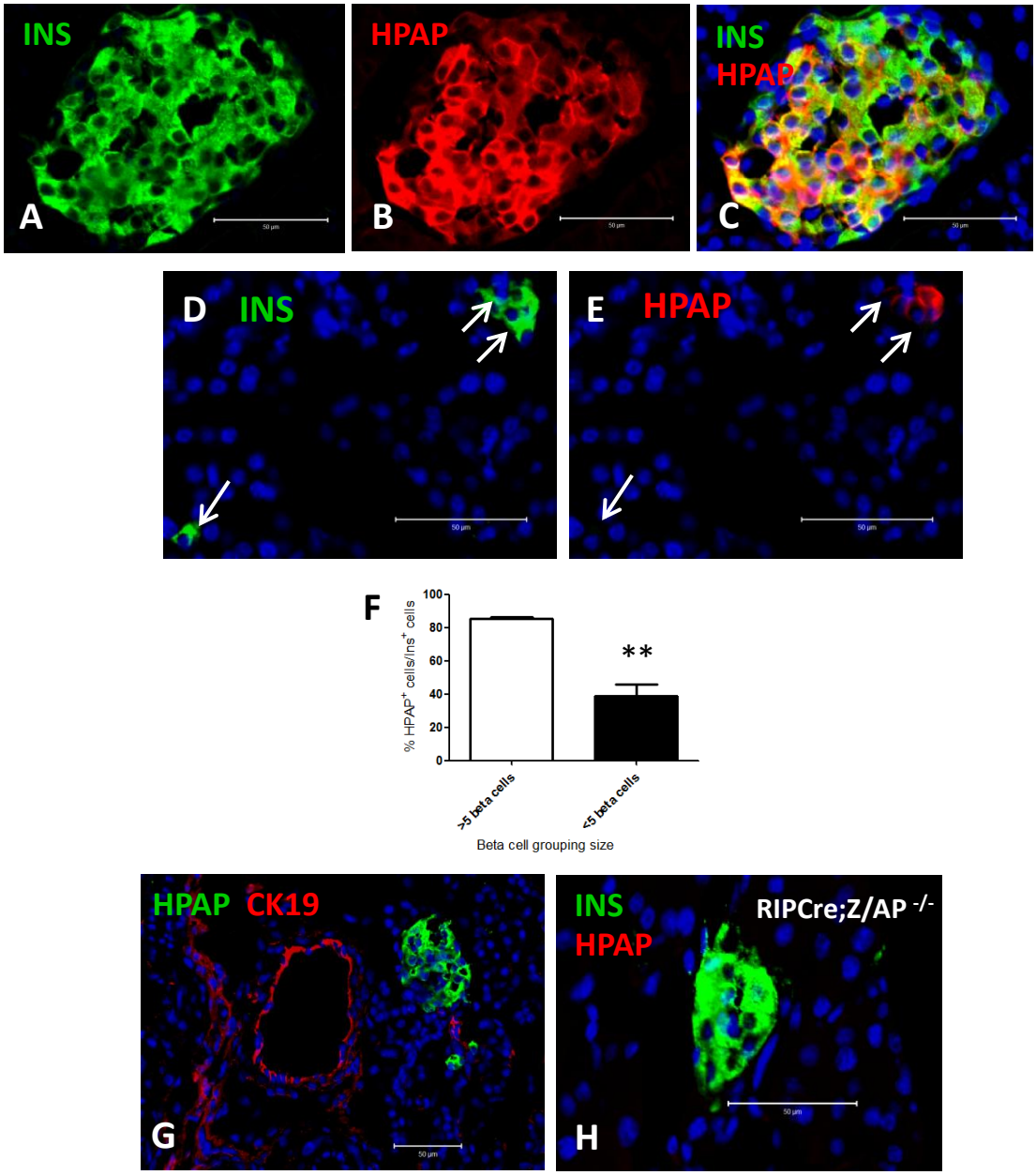

Figure 3.1. Specificity of HPAP to the β-cell using RIPCre;Z/AP$^{+/+}$ mice
(A) RIPCre;Z/AP$^{+/+}$ transgenic mouse model showing insulin^{+} β-cells (green, A) tracked by the reporter protein HPAP (red, B, and merged, C), and quantified in (F). The HPAP expression in β-cell clusters containing fewer than 5 β-cells (D, E) was significantly reduced (F). HPAP^{+} β-cells in pancreas sections (G, green) did not co-express CK19 (G, red). RIPCre;Z/AP$^{-/-}$ mouse beta cells (H, green, insulin) did not exhibit HPAP immunostaining (H, red). Scale bar denotes 50 µm, $n > 5$. Data are represented as % Mean ± SEM, $^{**}p < 0.01$.

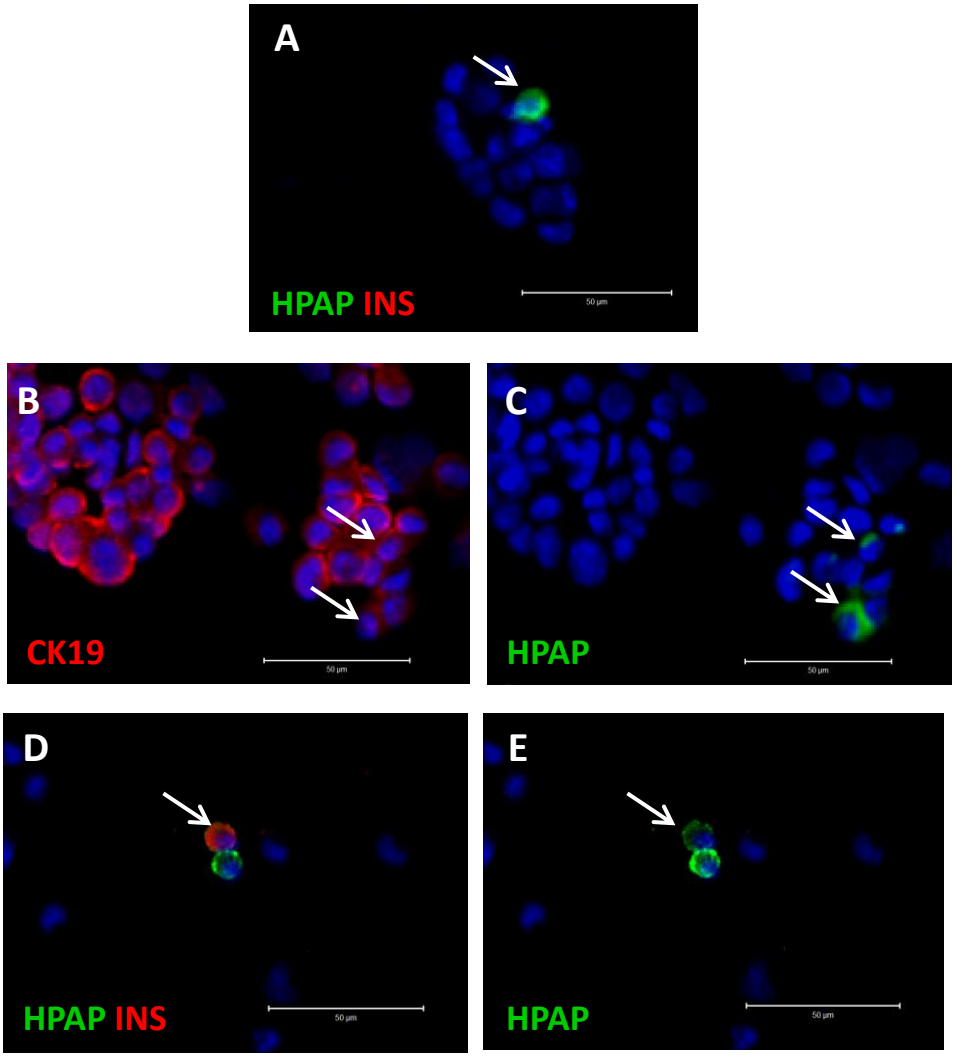

Figure 3.2. Lineage tracing of the β-cell during *in vitro* islet dedifferentiation. After islets were cultured in dedifferentiation media for 7 d, all insulin expression was lost (A, red), and only rare cells retained HPAP expression (A, green). CK19 (B, green) expression increased drastically, and the rare remaining HPAP$^+$ cells also adopted CK19 expression (C, green, arrows). After replating the resultant epithelial-like monolayer of cells in redifferentiation media, the majority of the rare remaining HPAP$^+$ cells (D, E, green) re-expressed insulin (D, red). Scale bar denotes 50 µm, $n > 5$ replicates.

95 HPAP$^+$ immunostained cells retained per dish (n > 6), and equivalent to ~1/2,000 cells deriving from β-cells after 1 week *in vitro*. However, of the surviving HPAP$^+$ cells (**Figure 3.2C**, green), 87.5 ± 3.9% expressed CK19 (**Figure 3.2C**, red) after 1 week in culture (islet *vs* all other time points, p < 0.0001). Proliferation of the remaining HPAP$^+$ cells increased significantly at 1 week in culture, from 2.7 ± 0.9% to 25.6 ± 1.8% (p < 0.001) by EdU immunostaining, then decreased thereafter.

To confirm that this represented loss of β-cells and not simply loss of insulin immunostaining, we examined β-cells early in culture. We found that insulin expression had diminished consistently with time, such that after 3 days, 48.1 ± 1.2% of cells present were insulin$^+$, and by day 5, only 18.4 ± 2.1% of the cells were insulin$^+$, and that HPAP tracked these cells at 85% consistently (**Figure S3.3**).

The dedifferentiated cells were subsequently cultured in media shown to promote endocrine redifferentiation (18), and greater than 90% of rare remaining HPAP$^+$ (**Figure 3.2E** & **F**, green) cells re-expressed insulin (**Figure 3.2E**, red, arrow) over 4 weeks of culture.

3.3.4 Classification of β-cell subpopulations by Glut2 and GPm6a expression

We examined if the β-cells retained after dedifferentiation (i.e., CK19$^+$HPAP$^+$ cells) had a distinct phenotype. Seaberg *et al* reported that rare, pancreas-derived multi-potential progenitor (PMP) cells exist within mouse islets which can generate progeny in both pancreatic and neural lineages (21). It was subsequently reported by Smukler and colleagues that these PMPs expressed insulin, albeit at reduced proportions as compared to

proto-typical β-cells. Furthermore, these cells displayed a progenitor character, with decreased expression of *Nkx6.1* and *Pdx1*, increased expression of *Ngn3*, and a lack of *Glut2* (22). We hypothesized that $HPAP^{+}CK19^{+}$ β-cell progeny in dedifferentiated cultures may have derived from such $insulin^{+}$ PMPs. As the proportion of these progenitor cells in islets was reported to be only 1-5/10,000 (22), flow cytometric sorting of β-cells from whole pancreas from RIPCre;$Z/AP^{+/+}$ mice was initially used to optimize yield. Cells were selected using antibodies for GPm6a (19), a cell surface marker shown to identify the β-cell, and Glut2. GPM6a (red) immunostained insulin-expressing β-cells (green) as shown in **Figure S3.4**. **Figure 3.3A** illustrates $GPm6a^{HI}Glut2^{HI}$ cells (red, phenotypically mature β-cells) and $GPm6a^{+}Glut2^{MID/LO}$ cells (green); these cells were split into $GPm6a^{+}Glut2^{MID}$ and $GPm6a^{+}Glut2^{LO}$ groups and collected separately. Insulin immunostaining was more prominent in the $GPm6a^{+}Glut2^{HI}$ cells than in the other populations (**Figure 3.3C**, red). $GPm6a^{+}Glut2^{HI}$ β-cells were immunopositive for Glut2 (**Figure 3.3C**, 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). Measurement of insulin secretion demonstrated that $GPm6a^{+}Glut2^{HI}$ cells secreted 18.6 ± 1.8 ng insulin when stimulated with 16 mmol/L D-glucose, whereas secreted insulin from $GPm6a^{+}Glut2^{LO}$ cells was below the threshold for detection (*n* = 3, 6000 cells/experiment).

When cultured in ductal dedifferentiation conditions, the majority of $GPm6a^{HI}Glut2^{HI}$ cells did not survive; the few resilient cells being non-proliferative, and failing to stain for CK19. Conversely, $GPm6a^{+}Glut2^{MID/LO}$ cells dedifferentiated *in vitro* and immunostained for CK19 (red) and HPAP (green) (**Figure 3.4A**, arrows and circle). The $GPm6a^{+}Glut2^{LO}$ cells

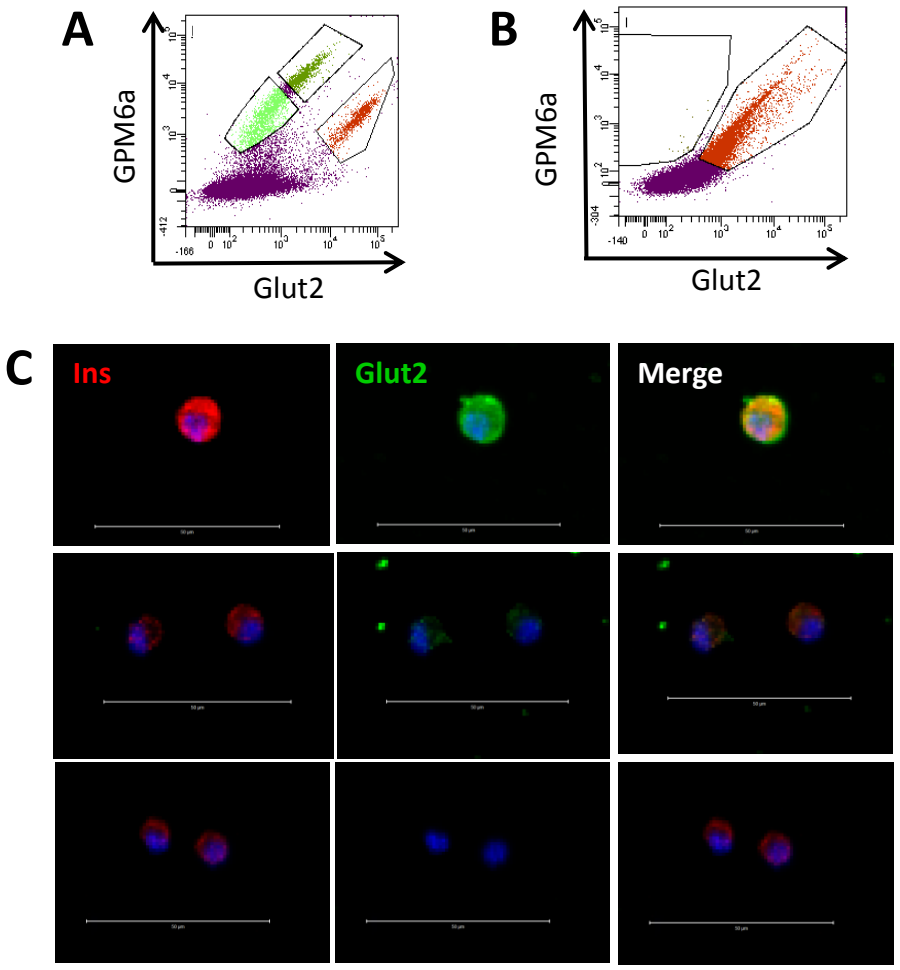

Figure 3.3. Pancreatic β-cells flow-cytometrically sorted by Glut2 and GPM6a. Whole pancreas (A) and isolated islets (B) were dispersed and flow-cytometrically sorted into distinct populations of insulin$^+$ (GPM6a$^+$) cells according to differential Glut2 expression: GPM6a$^+$Glut2$^+$ (red) and GPM6a$^+$ Glut2$^{MID/LO}$ (green), further split into GPM6a$^+$Glut2^{LO} (A, green, lower, and B, left population) and GPm6a$^+$Glut2^{MID} (A, darker green, upper). FAC-sorted GPm6a$^+$Glut2^{LO} cells isolated from neonatal islets (B) represented only 0.3 ± 0.07% of total insulin$^+$ cells. GPm6a$^+$Glut2^{MID} cells were not recovered from isolated islets (B). Cells recovered after sorting were immunostained for Glut2 (green) and insulin (red); insulin staining (C, red) was more prominent in typical β-cells (GPm6a$^+$Glut2^{HI}, top panel) as compared to GPm6a$^+$Glut2$^{MID/LO}$ cells; furthermore, Glut2 immunostaining (green) was diminished in the GPm6a$^+$Glut2^{MID}cells (middle), and was absent in the GPm6a$^+$Glut2^{LO} cells (green, bottom). Scale bars denote 50 µm, *n* = 5.

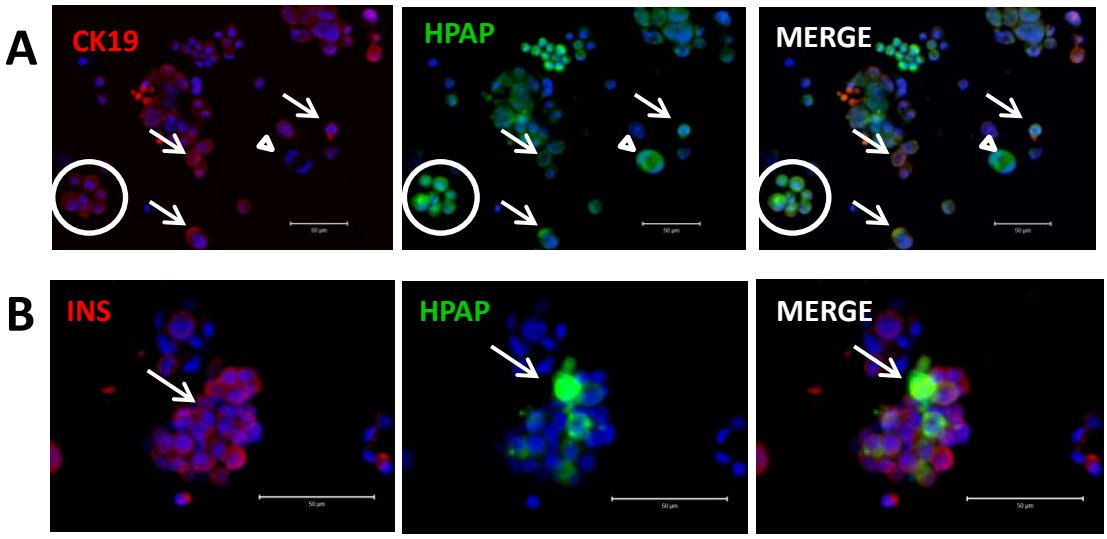

Figure 3.4. Dedifferentiation and redifferentiation of pancreatic GPM6a^{+} Glut2^{LO} β-cells. After flow sorting and 1 week in dedifferentiation culture, the majority of Ins^{+}Glut2^{LO} cells co-expressed CK19 (red) and HPAP (green) (A, arrows and circle). (B) Redifferentiation of dedifferentiated ductal-like cells after 4 weeks of culture, showing the β-cell lineage reporter HPAP (green) co-localizing with insulin (red) (arrow). Scale bar denotes 50 µm, *n* = 5.

exhibited ~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, $p < 0.01$, $n = 5$).

When subsequently cultured for redifferentiation, $HPAP^{+}$ cells (**Figure 3.4B**, green) demonstrated insulin expression (**Figure 3.4B**, red); however, the redifferentiated β-cells were functionally immature and did not demonstrate robust insulin secretion at low or high glucose concentrations (14.1 ± 0.4 pg *vs* 18.9 ± 0.4 pg insulin, respectively, $n = 3$, 5×10^4 cells/experiment). These results are consistent with human stem cell differentiation to β-cells (23), which demonstrated an immature phenotype *in vitro*.

Flow cytometric sorting was performed on β-cells recovered from isolated islets (**Figure 3.3B**), but $GPm6a^{+}Glut2^{MID}$ cells were not recovered in contrast to their presence within whole pancreas (**Figure 3.3A**). $GPm6a^{+}Glut2^{LO}$ cells in isolated islets represented only 0.3 ± 0.1% of total cells by flow cytometry, indicating that the majority of $GPm6a^{+}Glut2^{LO}$ cells exist outside of the islet (**Figure 3.3B**).

3.3.5 $Ins^{+}Glut2^{-}$ cells are primarily found outside of islets

To identify the anatomical location of the insulin-positive but Glut2-negative cells, pancreas sections were immunostained for insulin, Glut2, and EdU. The majority of these cells were found to reside outside of islets; a representative $Ins^{+}Glut2^{-}EdU^{+}$ cell being shown in **Figure 3.5A**. As a convention, we use the nomenclature "$Glut2^{LO}$" to represent isolated β-cells identified by FACs, and "$Glut2^{-}$" to denote β-cells identified by IF analysis within

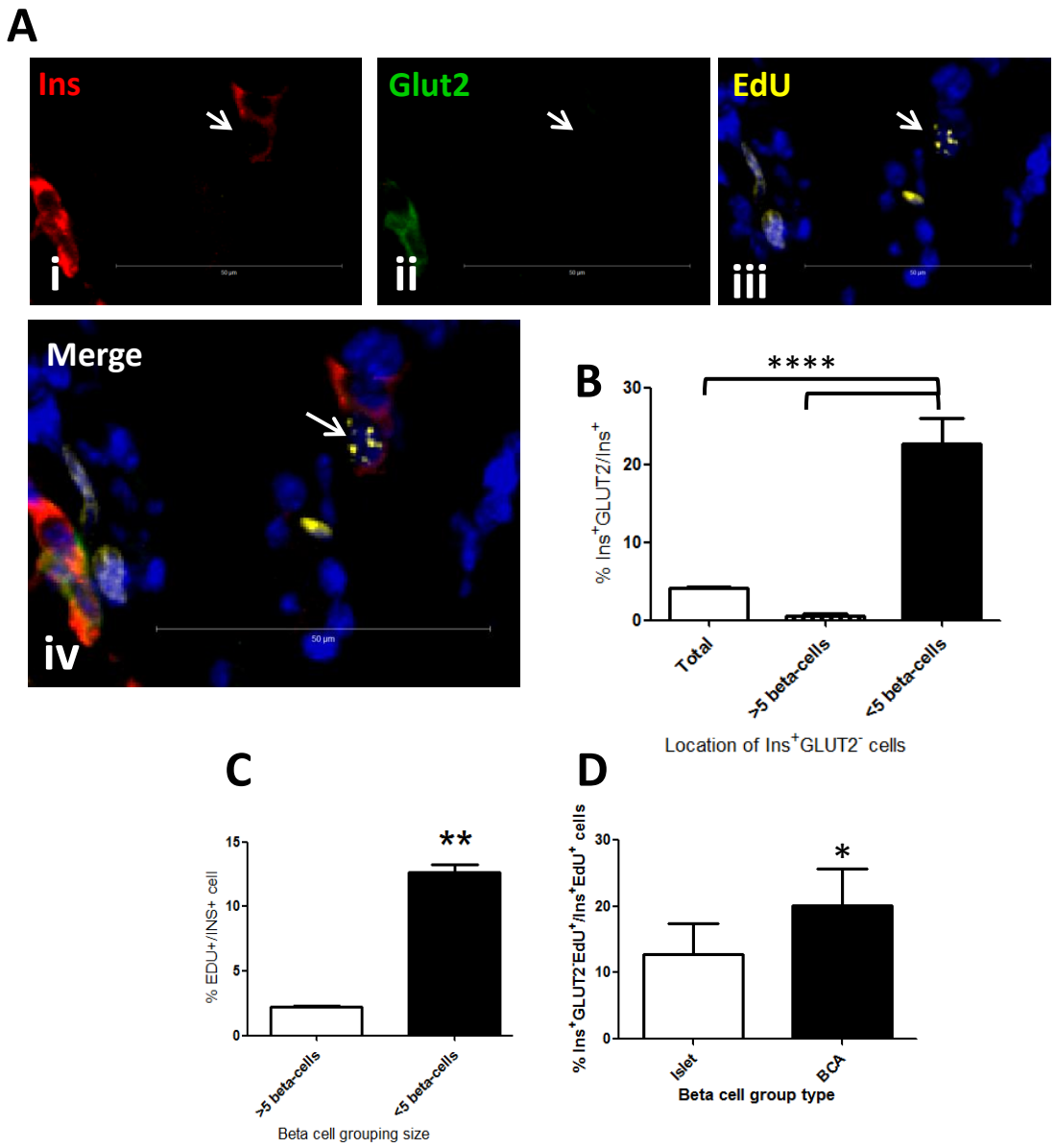

Figure 3.5. Location and proliferation of neonatal mouse Ins$^+$Glut2$^-$ β-cells
(A) Immunofluorescent images of a mouse P7 pancreas section immunostained for insulin (i, red), Glut2 (ii, green), EdU (iii, yellow), and merged images (iv), located in an extra-islet β-cell aggregate (iv, BCA), demonstrating an Ins$^+$Glut2$^-$EdU$^+$ cell (arrow). (B) Ins$^+$Glut2$^-$ cells were primarily located in BCA as compared to islets (B, ***$p < 0.001$ total *vs* <5 β-cells, ****$p < 0.0001$ >5 β-cells vs <5 β-cells). (C) The proliferation rate of Ins$^+$ cells was higher in BCA than in islets (**$p < 0.01$, Mann-Whitney test). (D) Ins$^+$EdU$^+$Glut2$^-$ cells can be found in high proportion in both BCA and islets (D, *$p < 0.05$). Scale bar denotes 50 µm, $n > 5$. Data are represented as % mean ± SEM.

pancreas sections. $Ins^{+}Glut2^{-}$ cells represented 3.5 ± 0.2% of all insulin-expressing cells in the P7 pancreas by fluorescent immunocytochemical staining (**Figure 3.5B**, 1,935 cells, *n* = 5 pancreata). When separated for quantification into β-cells found within islets (>5 β-cells) or within extra-islet β-cell aggregates (<5 β-cells, BCA), $Ins^{+}Glut2^{-}$ cells represented 1.2 ± 0.3% of $insulin^{+}$ cells within islets, and 20.5 ± 3.6% of $insulin^{+}$ cells within BCA (**Figure 3.5B**, 23/1703 cells and 45/232 cells, respectively). The proliferation rate of Ins^{+} cells was higher in BCA than in islets at P7 (12.6 ± 0.7% *vs* 2.8 ± 0.2% respectively, **Figure 3.5C**). These proliferating $insulin^{+}$ cells were examined further, and showed that 12.8 ± 4.1% of $Ins^{+}EdU^{+}$ cells lacked Glut2 within islets, and 20.1 ± 5.6% within BCA (**Figure 3.5D**), illustrating that at P7 $Ins^{+}Glut2^{-}$ cells are proliferative both within and outside of the islet as compared to $Ins^{+}Glut2^{+}$ β-cells.

3.3.6 HPAP+ cells tag non-insulin-expressing-cells after extended time in vivo

In the young RIPCre/Z/$AP^{+/+}$ mouse (<14 d), $HPAP^{+}$ cells that immunostained for proteins other than insulin were extremely rare (**Figure 3.6C** and **D,** white bars**)**. However, when older (>1 yr) RIPCre;Z/$AP^{+/+}$ mice were examined, the proportion of $HPAP^{+}$ cells (green) labelling non-insulin expressing cells increased, including glucagon (**Figure 3.6A, red)** and somatostatin (**Figure 3.6B, red**), Quantification of these events is presented in **Figure 3.6C** and **D** (black bars).

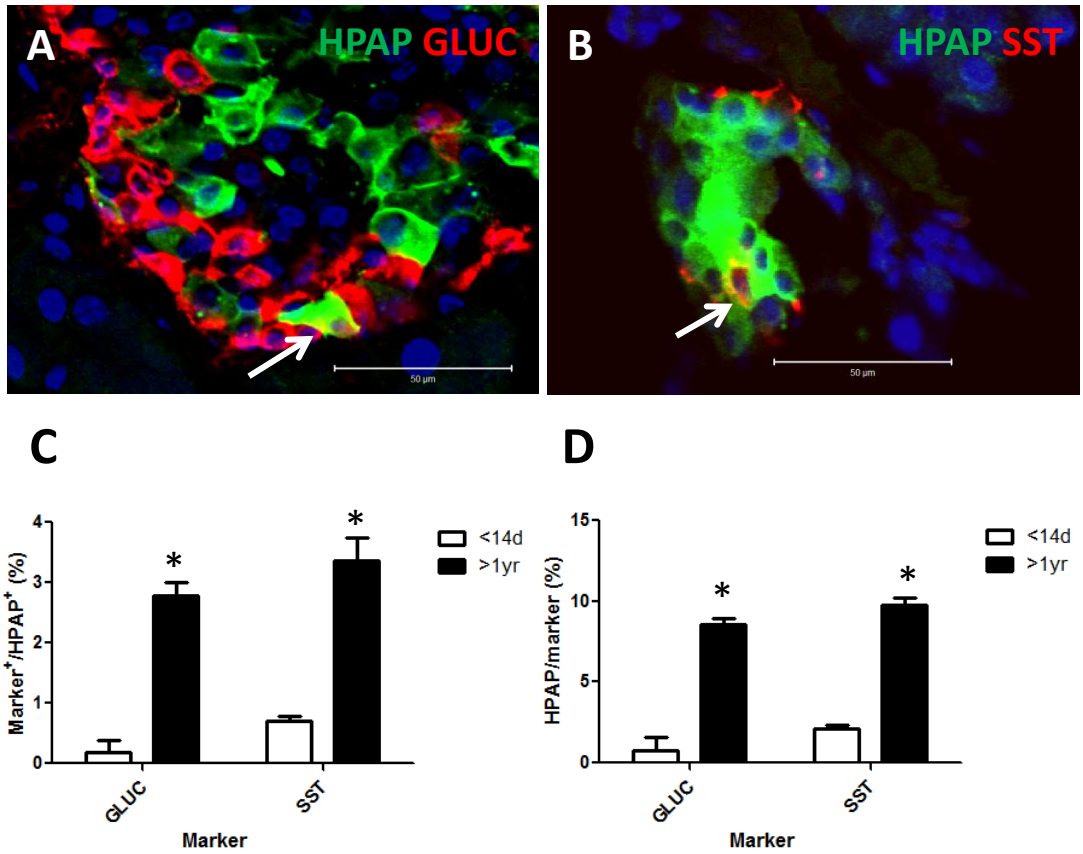

Figure 3.6. Alternate cell fates of HPAP$^+$ cells *in vivo*.
Images from RIPCre;Z/AP$^{+/+}$ mice demonstrating that some HPAP-tagged cells (A, B, green) express non-β-cell markers after a long time period (>1 yr, n = 3 mice), including glucagon (A, red) and somatostatin (B, red) as compared to young mice (<14 d, n = 5). Arrows indicate dual stained cells. These were quantified into marker+/HPAP+ cells (C) or HPAP+/marker+ cells (D). Scale bar denotes 50 µm. Data are represented as % mean ± SEM, C and D, >1 yr *vs* <14 d, t-test, * $p < 0.05$.

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

To assess the capacity for cellular plasticity, insulin-expressing cells detected using GPm6a were recovered after FACS and cultured in neural differentiation conditions. A subset (~1/200) of these GPm6a^{+}Glut2^{LO} cells proliferated and formed neurospheres (**Figure 3.7A**); Ins^{+}Glut2^{HI} cells did not proliferate (**Figure 3.7B**). Once plated for neural differentiation, the Ins^{+}Glut2^{LO}-derived neurospheres formed cells with neuronal-like processes (**Figure 3.7C**, **D**). These cells demonstrated immunostaining for the neuronal marker β-III tubulin (**Figure 3.7E**, **G**, green) and the astrocyte marker glial fibrillary acidic protein (GFAP) (**Figure 3.7F**, **H**, red). Both of these resulting cell phenotypes expressed the β-cell reporter HPAP (**Figure 3.7F**, **G**, red, and **Figure 3.7 G**, **H**, green), demonstrating the lineage plasticity of Ins^{+}Glut2^{LO} cells. Furthermore, all neurospheres generated both neuronal and astrocyte cell types.

3.4 Discussion

Islet dedifferentiation, expansion, and subsequent redifferentiation have long been proposed as a source of insulin-producing cells to facilitate transplantation for the reversal of diabetes. Based on a model which postulated “islet trans-differentiation” to a hormone-negative phenotype with similarities to ductal-epithelium (13), we hypothesized that the resultant cell monolayer was derived, in part, from β-cells that could be tracked using a lineage tracing technique utilizing HPAP expression. We found that the majority of β-cells from isolated islets did not survive *in vitro*; however, ~1/2,000 of the cells present had

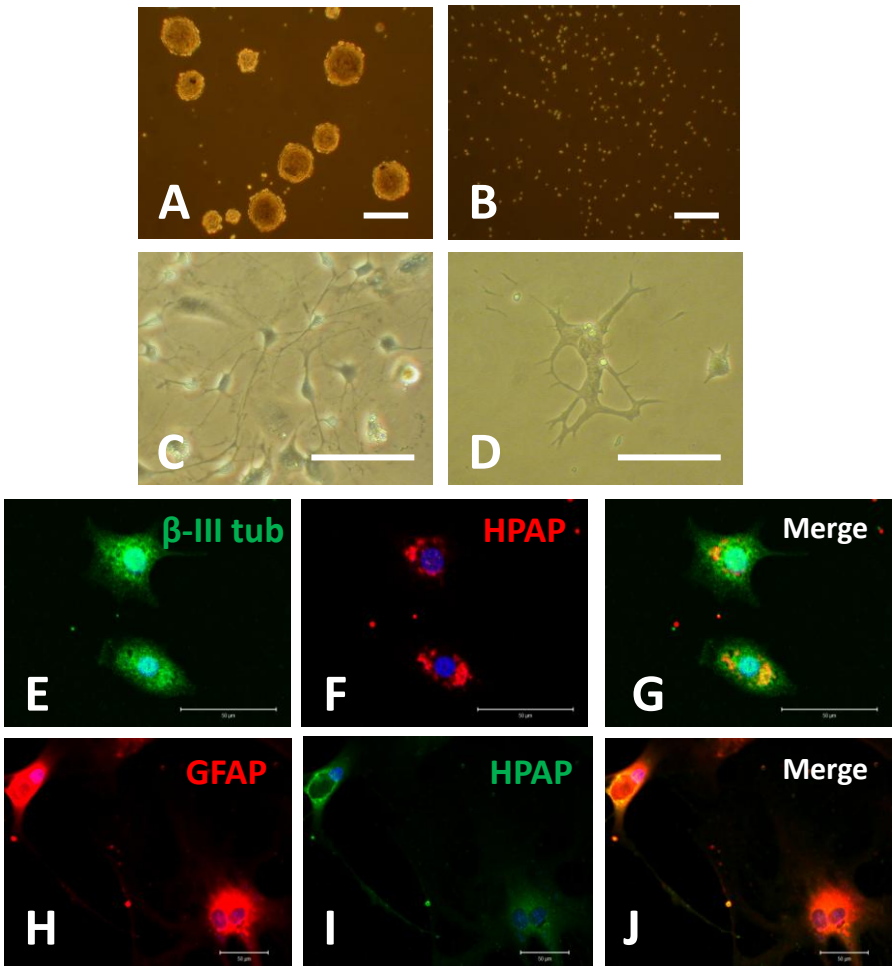

Figure 3.7. Pancreatic Ins^{+}Glut2^{LO} β-cells are multi-potential and can differentiate into neural cell lineages.

Insulin^{+} cells recovered from whole mouse pancreas after FAC-sorting based on Glut2 expression were placed in neural proliferation conditions. Neurospheres (A) were generated from GPM6a^{+}Glut2^{LO} cells, compared to GPM6a^{+}Glut2^{HI} cells (B). After replating in neural differentiation conditions, the neurospheres generated multiple morphologies, including neuron-like processes (C, D). When immunofluorescently stained, neurons were visualized by β-III tubulin (E, G green), and astrocytes by GFAP (H,J, red). These neural cells co-stained with HPAP (red, F, G; green, I, J). Scale bar denotes 100 µm (A, B), and 50 µm (C-J), *n* = 5.

derived from β-cells and dedifferentiated to become $CK19^+$ cells. We subsequently hypothesized that these $HPAP^+CK19^+$ cells represented the progeny of Ins^+Glut2^- cells, reported to denote a pancreatic multipotent progenitor cell pool (22). We have shown that these PMPs can be isolated from neonatal mouse pancreas, and which are capable of differentiation to a variety of cell types *in vitro*. Importantly, while Ins^+Glut2^- cells can indeed be found in intact islets as reported (22), a higher proportion are located in scattered β-cells outside of islets, and which have a significantly greater proliferative capacity at P7 than the Ins^+Glut2^+ cells.

More than one cell type populated the islet-derived dedifferentiation cultures, with only a minority derived from β-cells. Other groups have demonstrated similar results by lineage tracing experiments, with 1.4% and 0.5% mouse β-cells dedifferentiating *in vitro*, respectively (9,10), and <5% of β-cells dedifferentiating from human islets using similar culture conditions (24). These findings indicated higher rates of dedifferentiation than found by us; however, those groups did not further characterize the rare β-cells capable of dedifferentiation. As our initial cellular preparation contained multiple cell types, it is conceivable that some of the CK19-expressing cells generated during dedifferentiation culture were derived from adherent ductal cells present at the time of islet isolation and which subsequently expanded *in vitro*, diluting the cellular contribution from β-cells.

The concept 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

for mature marker presence in stem cell populations exist, such as neural stem cells expressing the astrocyte marker GFAP (25). We have shown that $Ins^{+}Glut2^{-}$ 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 being similar to that found by Smukler *et al* (22). However, a striking 3.5% of insulin-expressing cells within whole pancreas at P7 lacked Glut2, with up to 20% of these cells being found within BCA. This suggests that the highest source of β-cells capable of demonstrating lineage plasticity is found outside of the islet, and these extra-islet BCAs are most abundant in the neonatal period and thereafter decline with advancing age (26). The absence of Glut2 expression is likely not the sole identifier of a β-cell progenitor however, since the co-expression of other markers such as Ngn3 and Nkx6.1 has been proposed by others (22,27). 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* (6,28). 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 maturation (28). Mouse β-cells in the immediate postnatal period are immature, with poor GSIS and lower expression of Glut2 compared to adult β-cells (29–31). It was recently reported that neonatal β-cells require a glucose threshold for maturation, and that β-cells within BCA of young mice are functionally different than those in islets (20). Furthermore, infant human β-cells demonstrate some plasticity in insulin secretory machinery as compared to adult β-cells (32). Our data suggests that a lack of

Glut2 expression may reflect not only β-cells with decreased function, but also a subset of progenitor cells. Interestingly, Stolovich-Rain *et al* (33) showed that after β-cell ablation, surviving β-cells in the mouse pancreas displayed significantly decreased levels of Glut2, purportedly due to glucose toxicity. 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 an increased proliferation in the $Ins^{+}Glut2^{-}$ cells, it is conceivable that these could clonally expand to replace β-cells after injury, with subsequent maturation to functional β-cells exhibiting high Glut2 expression.

Russ *et al* (9) showed that human β-cells were able to dedifferentiate much more readily than mouse β-cells *in vitro*. Despite distinctions between human and rodent islets with respect to architecture (34) and higher prevalence of GLUT1 utilization over GLUT2 (35–39), Smukler *et al* (22) demonstrated that $Ins^{+}Glut2^{LO}$ cells from adult human islets had a similar phenotype, occurrence, and lineage plasticity to those found in mice. Moreover, GLUT2 has been recently implicated in human neonatal diabetes (40) and its importance for glucose transport into human β-cells has been demonstrated (41). If $Ins^{+}GLUT2^{-}$ cells are as abundant in the BCA 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 transplantation.

Dor *et al* showed that β-cells in adult mice within the small extra-islet clusters had a similar labelling index with a genetic lineage tag as did those in larger islets, and concluded

that these simply represented small islets, or islets with β-cells undergoing apoptosis, rather than reflecting a new source of β-cells derived from a progenitor population (1). Our experiments differ in that; 1) we used a non-inducible transgenic model which results in the labelling 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 labelling with HPAP in β-cells located within the BCA than in anatomically mature islets in P7 mice (39% *vs* 85%), which is likely to reflect the generation of new β-cells from progenitors within the BCA during early postnatal life. While the number of extra-islet BCA decreases with age in rodents, they are still found in adulthood (26). Chintinne *et al* (42) 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. 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 (43), the study by Smukler *et al* (22) 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 are tagged, and which has been recently suggested to inappropriately label a fraction of acinar cells over an extended chase, raising concern over RIPCreER transgene stringency (44). 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. As the amount of lineage-tagged β-cells retained was only ~0.05% after 1 week *in vitro*, we propose that this

number does not over-represent the surviving β-cell population. Furthermore, while the lineage-label eGFP was used for sorting β-cells in that study (22), the non-fluorescent HPAP reporter in our model required us to employ a proxy β-cell surface marker, GPm6A (19). We have shown that $Ins^{+}Glut2^{-}$ cells can be detected and collected using different techniques (FACS and immunofluorescence), various markers for β-cells (insulin, GPm6A, and HPAP), and two separate antibodies for Glut2, indicating that these findings are unlikely to represent artefact. That a sub-population of Ins^{+} cells can reproducibly create $CK19^{+}$ duct cells, subsequently re-express insulin, or can alternatively differentiate into neural-lineage cells indicates a substantial plasticity potential. It has also been questioned whether <0.1% of β-cell progenitors within islets could reasonably account for β-cell mass renewal (43). We have shown that the majority of $Ins^{+}Glut2^{-}$ cells are found in the scattered BCA of the pancreas, which have received little experimental attention. These $Ins^{+}Glut2^{-}$ cells are proliferative, and as speculated by Smukler *et al*, could represent the rare, proliferative β-cell "clones" described previously (45); findings contrary to studies which indicated that all β-cells exhibit an equal proliferative potential (2,46). However, given the scarcity of the $Ins^{+}Glut2^{-}$ cells within adult islets, they could have been overlooked. Of note, 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, which were found to regenerate the pancreas, and which had derived from $Ngn3^{+}$ cells within small β-cell clusters (47).

This work builds on evidence that the mouse and human pancreas contain Ins^{+} multipotent pancreatic progenitor cells, which can be identified by the absence of Glut2

(21,22). 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.

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CHAPTER 4

Ontogeny of $Ins^{+}Glut2^{-}$ Progenitor β-cells Over the Human and Mouse Lifespan

Christine A Beamish, Brenda J Strutt, Sofia Mehta, Subrata Chakrabarti, Piotr Witkowski, Michael Millis, Manami Hara, and David J Hill

4.1 Introduction

A replacement of insulin-producing β-cells is required for the reversal of diabetes, and may be theoretically obtained by utilizing endogenous pancreatic β-cell progenitors. However, the presence of these β-cell progenitors is controversial, with as many publications supporting their existence (1–4) as those that reject it (5–7). The debate may not be as polarized as appears, since the presence of facultative stem cells (8), age (9–11), pregnancy (12–14), obesity (15–17), release from cell-cycle regulators (18), altered glucose metabolism (19), and the type of pancreatic injury (20,21) may each contribute to the milieu of factors putatively capable of effecting regeneration of the endocrine pancreas. This debate is further complicated by evidence that rare pancreatic progenitor cells present in human and mouse islets are capable of multi-potential differentiation and express insulin (22), usually considered to be a marker of a mature β-cell. Importantly, while these cells expressed insulin, they lacked Glucose-transporter 2 (Glut2), another feature of mature β-cells, and furthermore displayed progenitor characteristics, with increased expression of early β-cell transcription factors. Recently, we (**Chapter 3**) demonstrated that these $Ins^{+}Glut2^{-}$ cells were present within neonatal (postnatal) P7 mouse islets which demonstrated multi-lineage plasticity, and displayed a loss of glucose responsiveness, indicating that these β-cells are atypical. Importantly, there was a higher proportional presence of these in the small pancreatic β-cell aggregates of cells (BCA, <5 β-cells) than in islets. BCA have been presumed to be of little physiological importance. The relative abundance of the cells capable of multi-lineage differentiation ranged from 1/2000 β-cells within P7 mouse islets as found by us, to 1/10, 000 in the adult human and mouse islet as

found by Smukler *et al* (22). It was established by immunohistochemistry that up to 1% of all β-cells present in the P7 mouse pancreas – the majority of them extra-islet β-cells - expressed insulin but not Glut2, but that only 1/200 were capable of differentiation; the bulk then possibly representing immature and/or glucose-unresponsive β-cells. The present study therefore sought to explore two main avenues of interest: first, to identify whether $Ins^{+}GLUT2^{-}$ cells were also present in human samples, especially within BCA; and second, to determine how the location and abundance of these $Ins^{+}GLUT2^{-}$ cells changed with age in both mouse and human pancreas.

4.2 Materials and methods

4.2.1 Human and mouse pancreas sample collection

Twenty-five human pancreas samples were assessed, obtained as formalin-fixed and paraffin-embedded tissue sections from the Department of Pathology at the University of Western Ontario, Schulich School of Medicine (London ON), and the University of Chicago (Illinois), with approval from the University of Western Ontario Research Ethics Board #103167, and Lawson approval number R-12-501. Inclusion criteria were that pancreas samples be selected from cadaveric tissues, taken from donors with no known metabolic disease, and body weight within established healthy BMI standards (18-25 kg/m^2). Tissues utilized were representative of diverse ethnic backgrounds, and evenly split between male and female samples (48% female, 12/25 samples). Paraffin blocks were chosen from mid-pancreas whenever possible.

All animal procedures were approved by the Western University Animal Use Ethics Committee, in accordance with the Canadian Council on Animal Care. Mouse pancreas samples were collected from wild-type littermates and retired breeders from our existing transgenic colonies present at the Lawson Health Research Institute, St Joseph Health Care, London ON, and with a C57Bl/6 background. Mouse pancreata were fixed in 4% paraformaldehyde (PFA, Electron Microscopy Sciences (Hatfield PA)) for 24 h, washed in PBS for 24 h, incubated in 30% sucrose for 24 h, and frozen in OCT at -80°C prior to sectioning at 8 nm.

Tissues from various ages were chosen to illustrate major developmental stages, including early endocrine pancreas development, infancy, childhood, adolescence/sexual maturation, adulthood, and late life; human samples are shown in **Table 4.1**, and a schematic of life stages for both species are shown in **Figure 4.1**. Tissue samples were age-matched within each life stage and were collected from both sexes.

4.2.2 Fluorescent immunohistochemistry

Fluorescent immunohistochemistry was performed using antibodies for insulin (human anti-mouse, Sigma-Aldrich (St Louis MO), 1/2000, and rabbit anti-human, Santa Cruz Biotechnologies (Santa Cruz, CA) 1/200), Glut2 (goat anti-human, Santa Cruz, 1/200, and rabbit anti-human, Millipore (Billerica, MA) 1/500), and Ki67 antigen (mouse anti-human, BD Biosciences (Mississauga, ON), 1/50). Secondary antibodies were matched to the species of the primary antibodies, and conjugated to 488/555/647 fluorophores (Life Technologies Inc (Carlsbad CA) 1/500). DAPI (Life Technologies, 1/1000) was used to

Table 4.1. 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	M	36 years	Young Adult
18	F	41 years	Young Adult
19	M	50 years	Adult
20	F	52 years	Adult
21	M	54 years	Adult
22	M	56 years	Adult
23	M	65 years	Late Adult
22	F	72 years	Late Adult
24	M	72 years	Late Adult
25	M	79 years	Late Adult

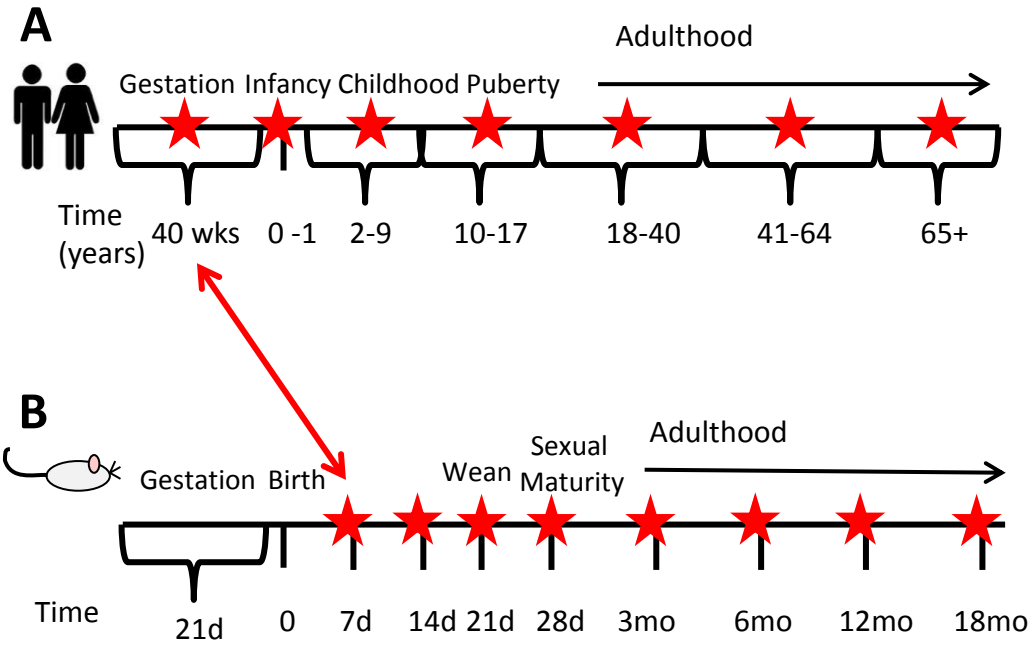

Figure 4.1. Lifespan of the mouse and human. The entire lifespan was examined in human (A) and mouse (B), with ages selected to illustrate major developmental stages, including early development, infancy, childhood/weaning, puberty/sexual maturation, and adulthood, as represented by each red star. When possible, developmental points were matched. A significant proportion of mouse islet development occurs postnatally, such that 20 weeks gestation in humans is analogous to postnatal d8 in mice. $n \geq 4$ mice, and ≥ 3 humans/star.

counterstain nuclei of all tissues. GLUT2 immunostaining in human pancreas samples required the use of tyramide signal amplification (23) using a kit (Perkin Elmer, Waltham, MA) conjugated to a Cy3 fluorophore. Ki67 signal visualization required antigen retrieval at 95°C in Tris-EDTA buffer for 30 min. All tissues were permeabilized for 10 min with 0.3% triton-X-100 and blocked with Background SNIPER (Biocare Medical, Concord CA) for 8 min.

Tissues were imaged by confocal microscope using a Zeiss LSM 510 Duo Vario (Carl Zeiss Ltd, Oberkochen, Germany) at the Biotron Facility, Western University, and counted manually using Zen software. Minimum cell counts were >250 insulin$^+$ β-cells/section for mice (≥2 sections per pancreas), and >1500 insulin$^+$ β-cells/section in human pancreas (1 section per pancreas from 1 block). Controls were determined by cutting replicate sections from the same block (>100 μm apart), and variance around the mean was below 9%.

4.2.3 Statistical analysis

There were at least quadruplicate samples per cohort per life stage for mouse samples and triplicate pancreata analyzed for human samples (**Table 4.1**). Data are presented as percent (%) mean ± SEM of insulin$^+$ β-cells, with statistics performed using a one-way ANOVA and Tukey's post-test, or student's t-tests as indicated, and with an acceptable level of significance of $p < 0.05$. Statistical analysis was performed using Graphpad Prism software (v. 5.01, San Diego CA).

4.3 Results

4.3.1 β- cell Aggregate (BCA) proportion decreases with age in mouse and human pancreas

Pan & Wright (24) showed that mice display significant postnatal endocrine development, such that P8 mouse endocrine pancreas is analogous to 22 week gestation fetal pancreas in humans; thus, we used these time points to begin our analysis. At 7d, 16.1 ± 0.8% of mouse β-cells were located in BCA (**Figure 4.2A**, white bar). This proportion decreased by 18 months of age to only 2.1 ± 0.7%; the majority instead being present in proto-typical islets (**Figure 4.2A**, black bar, 18 mo *vs* 7d, $p < 0.005$).

In contrast to mice, 41.6 ± 3.3% of human β-cells were located in BCA during the fetal period (G22-30w) (**Figure 4.2B**, white bar, and **Figure 4.2C**, immunostaining positive for insulin (green)). This proportion was significantly larger than the equivalent population in mice during the earliest time point studied (G22-30w *vs* 7d, $p < 0.0005$, t- test, not shown). The proportion of BCA in human pancreas dropped during infancy (0-1 y, hatched bar) and childhood (2-9 y, hatched bar) to 28.4 ± 2.2% (**Figure 4.2B**, 0-1 and 2-9 *vs* G22-30w, $p < 0.05$), then significantly decreased at puberty to 5.5 ± 1.1% (**Figure 4.2B**, hatched bar, G22-30w *vs* 10-17, $p < 0.0005$, and **4.2D**). Interestingly, there was an equivalent proportion of BCA at the time of sexual maturation between mouse and human samples (28d mouse *vs* 10-17 yr human, t-test, $p = 0.68$, not shown). The majority of human β-cells were thereafter maintained throughout adulthood within islets (**Figure 4.2B**, black bars, and arrows, **Figure 4.2D**). There was a statistically significant difference in the BCA proportion between human female and male samples in early adulthood, with 11.0 ± 0.3% *vs* 4.3 ± 0.7% respectively

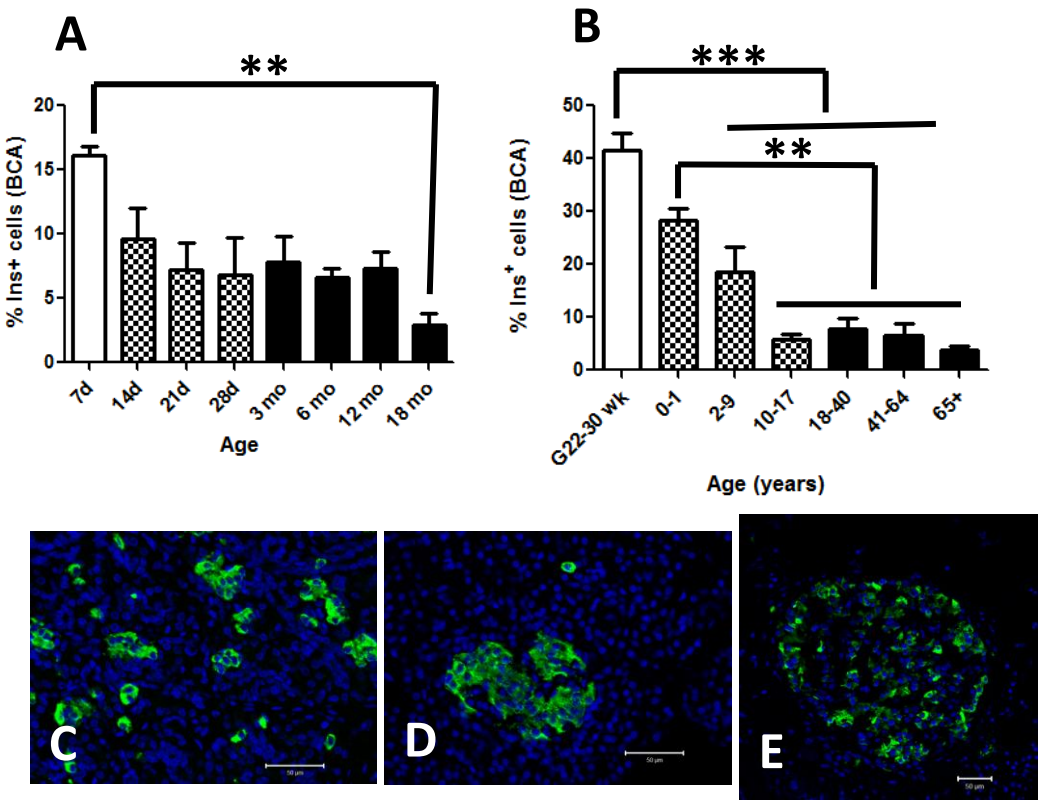

Figure 4.2. Beta cell aggregate proportion decreases with age in both mouse and human pancreas. Similar developmental points were grouped (7d (mouse) and fetal (human) samples = white bars, pre-weaning and sexual maturation (14-28d) (mouse) and infancy/childhood/adolescence (human) samples = hatched bars, and all adult ages = black bars. β-cells were counted and assigned to either the BCA compartment (<5 β-cells) or islet (>5 β-cells). (C-E) Representative images from human pancreas immunostained with insulin (green) illustrate changes during the maturation of islet structures, from fetal age (C, 30 week gestation female) through puberty (D, 14 year old female), and late adulthood (E, 72 year female). Graphs are shown as proportion (%) mean ± SEM of total β-cells counted; 1-way ANOVA * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. Size bar denotes 50 µm, $n > 3$.

(18-41, p <0.05, t-test). By late adulthood, there were very few BCA present within sections of human pancreas (**Figure 4.2E**, arrows). Mouse and human pancreata displayed no difference in BCA proportion during adulthood and late adulthood. It must be acknowledged that if islets were sectioned at the periphery, then the size would theoretically be equivalent to that of the BCA; however, this would be a relatively rare event. Furthermore, if that were true, then these data would not change with age as seen here.

4.3.2 Ins$^+$Glut2$^-$ cell proportion decreases with age

We examined the population of Ins$^+$Glut2$^-$ cells over the lifespan of both human and mouse. Human pancreas sections demonstrated immunostaining for insulin (green) and GLUT2 (red) as shown in **Figure 4.3**. A representative image of human Ins$^+$GLUT2$^-$ cells is shown in **Figure 4.3B** (arrows) within the BCA compartment. In mice, the proportional presence of total Ins$^+$Glut2$^-$ cells decreased significantly from 3.8 ± 0.8% at 7d to 0.6 ± 0.2% at 3 mo (**Figure 4.4A**, 7d *vs* 28d-12mo, $p < 0.05$), which was further exaggerated by 18 mo (**Figure 4.4A**, $p < 0.005$). The decrease in this population was also significant from pre-weaning to late adulthood (**Figure 4.4A**, 14d, 21d *vs* 18 mo, $p < 0.05$). There was a similar trend found in humans, with total Ins$^+$GLUT2$^-$ cells decreasing from the fetal time period to adulthood (**Figure 4.4B**, G22-30w *vs* all others, $p < 0.0005$) , as well as from childhood to late adulthood (**Figure 4.4B**, 2-9 *vs* 65$^+$ $p < 0.05$). There were twice as many total Ins$^+$GLUT2$^-$ cells in human pancreas than in mouse at the initial time point studied (7.8 ± 0.9% G22-30w *vs* 3.8 ± 0.8% 7d, $p < 0.05$).

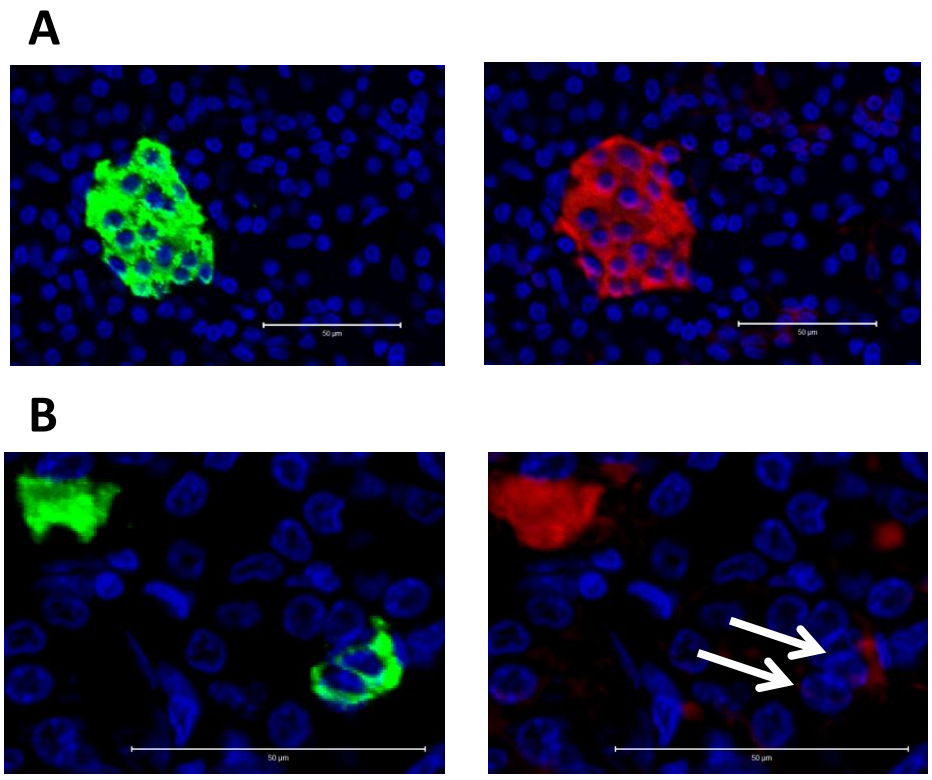

Figure 4.3. Insulin and GLUT2 immunostaining in human pancreas. Representative images of an islet (A) and beta cell aggregate (B) stained for insulin (green) and GLUT2 (red) in human pancreas. $Ins^{+}GLUT2^{-}$ cells are shown in B (arrows). Size bar represents 50 µm.

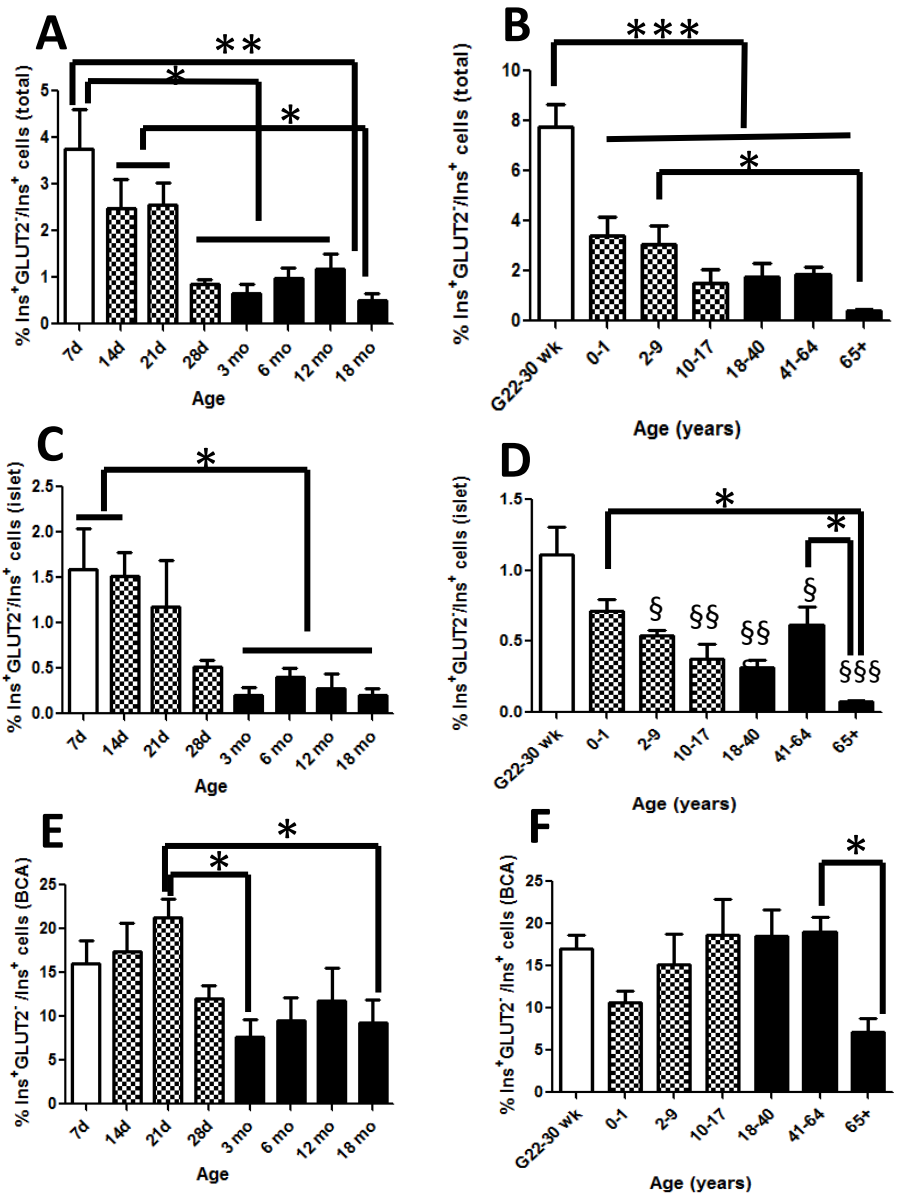

Figure 4.4. Ins⁺Glut2⁻ cell proportion decreases with age.

Ins⁺Glut2⁻ cell proportion from total β-cells (A, B), islets (C, D), and BCA (E,F) as found in mouse (A, C, E) and human (B, D, F) pancreas samples. Graphs are shown as proportion (%) mean ± SEM of total β-cells counted; 1-way ANOVA with tukey's post-test or student's t-test, §,* $p < 0.05$; §§ $p < 0.005$; §§§, *** $p < 0.0005$.

The anatomical location of $Ins^{+}Glut2^{-}$ cells was then examined. **Figure 4.4C** shows that 1.6 ± 0.4% of mouse β-cells lack Glut2 at 7d within the islet (white bar), which is consistent with previous findings (**Chapter 3**). As hypothesized, this proportion significantly decreased by early adulthood (range 0.2 ± 0.1% – 0.4 ± 0.1%, 7d *vs* 3 mo, $p < 0.05$).

In humans, 1.1 ± 0.2% of the β-cells within the islet lacked GLUT2 during fetal life, which decreased significantly by childhood, and further thereafter (**Figure 4.4D**, G22-30w *vs* 10-17, $p < 0.005$). There were few $Ins^{+}GLUT2^{-}$ cells present in islets by late adulthood in human pancreas (**Figure 4.4D**, 1.1 ± 0.2 *vs* 0.07±0.01%, G22-30w vs 65^{+}, $p < 0.0005$). There was a trend towards a decrease in $Ins^{+}GLUT2^{-}$ cells within the islets in early adulthood, and a subsequent increase in the population during mid-adulthood (**Figure 4.4C** and **D**, black bars; 0.6 ± 0.1% *vs* 0.07±0.01%, 41-64 *vs* 65^{+}, $p <0.05$).

The proportion of $Ins^{+}Glut2^{-}$ cells within the BCA compartment was markedly different than that within the islet, with 16.1 ± 2.6% of all mouse insulin-expressing cells lacking Glut2 within BCA at 7d (**Figure 4.4E**). This proportion increased at the immediate postnatal time period (14d, 21d), but decreased thereafter at 3 mo and 18 mo (**Figure 4.4E**, 16.1± 2.6% *vs* 9.2 ± 2.7%, 7d *vs* 18 mo, $p <0.05$).

In the human pancreas, a similar proportion of $Ins^{+}GLUT2^{-}$ cells were found in BCA in the fetal time period as in the 7d mouse (17.0 ± 1.7% *vs* 16.1 ± 2.6%, G22-30w *vs* 7d, ns). $Ins^{+}GLUT2^{-}$ cell proportional presence in the BCA was maintained throughout most of life, with a significant decrease in $Ins^{+}GLUT2^{-}$ cells in BCA only in late adulthood (**Figure 4.4F**, 19.1 ± 1.7% *vs* 7.1 ± 1.6%, 41-64 *vs* 65^{+}, $p < 0.05$).

4.3.3 β-cell proliferation decreases with age

The proliferation index of total insulin$^+$ β-cells was examined in mouse by Ki67 immunostaining, and was found to be highest at 7d compared to all other time points (**Figure 4.5A**, 3.3 ± 0.1% *vs* 0.7 ± 0.1%, 7d *vs* 3mo, p <0.0005). Beta-cell proliferation had decreased by weaning (2.0 ± 0.2% at 14d and 1.6 ± 0.2% at 21d), and thereafter proliferation was maintained at ~0.6% (**Figure 4.5A**, black squares). In the human pancreas, a similar trend was noted, with the highest total β-cell proliferation found in fetal life, which dropped thereafter (**Figure 4.5B**, 1.4 ± 0.5% *vs* 0.2 ± 0.1%, G22-30w *vs* 65$^+$, p <0.05). There was significant variability in the pre- and perinatal samples, ranging from 0.9 – 2.3% and 0.2 – 0.9%, respectively (**Figure 4.5B**).

Proliferation of β-cells within islets followed similar patterns as was found in total β-cell proliferation, with rates of replicating β-cells highest in mice at 7d, and which decreased significantly by early adulthood (**Figure 4.5C**, 2.8 ± 0.2% *vs* 0.6±0.1%, 7d *vs* 6mo, p < 0.005). In the human, a similar trend was noted as in mice, with proliferation of β-cells being highest during the fetal time period, and decreasing thereafter (**Figure 4.5D**, 1.3 ± 0.5% *vs* 0.2 ± 0.03%, G22-30w *vs* 65$^+$, p < 0.05). There was considerable variability in the β-cell proliferation rate within the islet in the fetal and infant samples, ranging from 0. 6-2.2% and 0.1-1.2%, respectively. After adolescence, β-cell proliferation within the islet was maintained <0.5% (**Figure 4.5D**).

Within BCA, β-cell proliferation dropped significantly in mice from 7d to 28d and at 3mo (**Figure 4.5E**, 7.7 ± 1.0% *vs* 1.5 ± 0.03%, 7d vs 28d, p < 0.05). A similar trend occurred in the human BCA compartment, with a significant decrease in proliferation from mid-gestation to adolescence (**Figure 4.5F**, 1.4±0.3% *vs* 0.0 ± 0.0%, G22-30w *vs* 10-17, p < 0.05).

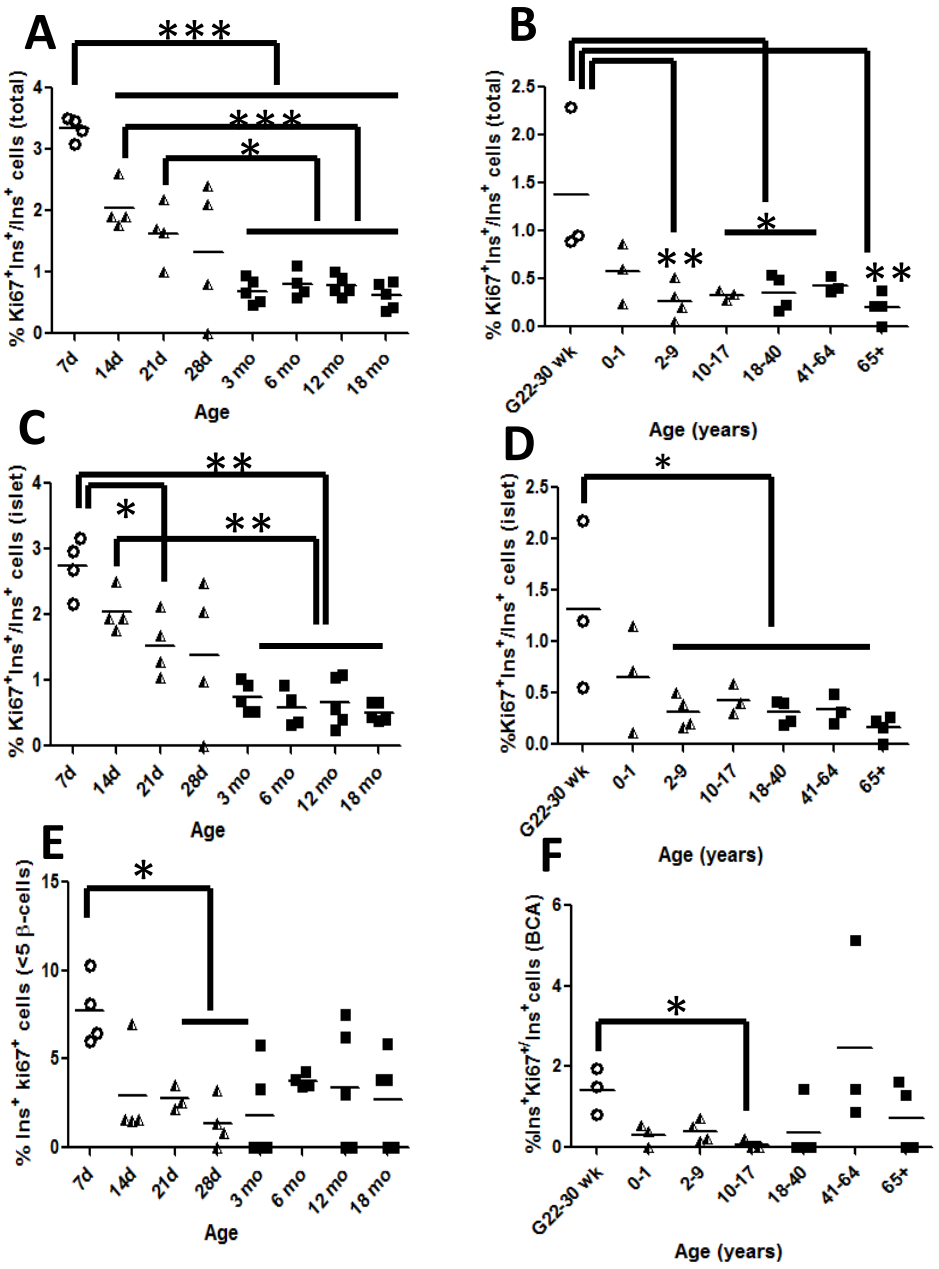

Figure 4.5. Frequency of β-cell proliferation decreases with age. Proliferation of insulin⁺ β-cells by Ki67 presence is shown in total β-cells (A, B), islets (C, D), and BCA (E,F) within mouse (A, C, E) and human (B, D, F) pancreas samples. Scatter graphs show the spread of data points between individual pancreata, and % mean of β-cells counted is indicated by 1-way ANOVA; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

We have previously shown that $Ins^{+}Glut2^{-}$ cells displayed increased proliferation in the mouse at P7, with 12.8 ± 4.1% of $Ins^{+}EdU^{+}$ cells lacking Glut2 in the islet, and 20.1 ± 5.6% of those cells lacking Glut2 in the BCA (**Chapter 3**). We examined this same population of $Ins^{+}Glut2^{-}Ki67^{+}$ cells throughout the lifespan of mice and humans. Consistent with our previous findings, 11.9 ± 5.0% of proliferating β-cells lacked Glut2 in the islet, and 23.2 ± 4.7% in the BCA at 7d in the mouse. At the equivalent age in humans (G22-30 w), only 1/3 of pancreas samples exhibited $Ins^{+}GLUT2^{-}Ki67^{+}$ immunostaining. The total proportion of proliferating β-cells which lacked GLUT2 increased at infancy to 14.4 ± 9.9% (0-1 yr, 2/3 samples), and during childhood to 10.4 ± 6.2% (2-9 yrs, 2/4 samples); in all cases, the cells being predominantly found in BCA. After 7d in the mouse and childhood in the human, the proportion of $Ins^{+}GLUT2^{-}Ki67^{+}$ cells diminished greatly, with only rare cells found at all subsequent ages in BCA, and none in islets.

4.4 Discussion

Over the mammalian lifespan, the endocrine pancreas undergoes significant changes in terms of cellular maturity, function, replication ability, and cell macro-organization (25). Previous studies have shown that a population of $Ins^{+}Glut2^{-}$, lineage-plastic putative progenitor cells can be identified in adult human and mouse islets (22), and which are proportionally increased in the small, extra-islet clusters of β-cells of neonatal mice (**Chapter 3**). Hence, we sought to determine if this population was also present in BCA in the human pancreas, as well as to assess how this cell population changes with age. We have found that human pancreas also contains a significant proportion of $Ins^{+}GLUT2^{-}$ cells both

within and outside of the islet, and this population decreases with advancing age in both species.

Some novel insights may be gleaned from this ontological study of human pancreas, specifically regarding pancreas maturation and remodelling at key developmental time periods, namely birth and adolescence. First, a significant proportion of endocrine cell mass is present in the young human pancreas outside of proto-typical islet structures, with nearly 45% of β-cells present in small clusters of cells (<5 β-cells) during mid-gestation, ~30% during infancy, and ~20% during childhood. This trend has been shown previously (26), however it was not quantified *per se*. Indeed, there is a lack of consistency in nomenclature and size metrics when quantifying these small β-cell aggregates or clusters, with an "islet" being identified as 4 (insulin^{+}) β-cells together (26,27), as compared to "β-cell clusters" constituting <10 β-cells (5), or <20 β-cells (3) when quantified by cell number, or alternatively <50 μm (28,29) or <60 μm(30) when determined by β-cell area grouping size. Within the confines of our model, the proportion of β-cells present as single cells/small clusters (i.e., BCA, <5 β-cells) outside of the islet only drops substantially around puberty, and is thereafter maintained in the adult at ~5%. This dramatic decrease was not seen in mouse, which showed a more gradual decline after weaning. However, in both species, β-cell proliferation in the BCA compartment was extremely low (mouse)/ absent (human) at this developmental stage, which may indicate a shift in cell plasticity. The low rate of β-cell proliferation during adolescence has been noted previously (26), although not specifically in reference to β-cells outside of islets. It is of interest to note that the incidence of type 1 diabetes in humans peaks between 10-14 years (i.e., puberty/adolescence) (31), which

coincides with the developmental shift to larger islets and fewer β-cell aggregates as demonstrated here. We propose that in addition to the hormonal demands and somatic growth necessary at this age (26), adolescence may be an especially vulnerable window for diabetes if this reserve pool of β-cells is not available for endocrine tissue remodelling. It is currently unknown if the $Ins^{+}GLUT2^{-}$ β-cells are equally susceptible to autoimmune destruction as mature β-cells. It is speculative to hypothesize that these cells may represent the small clusters of β-cells retained in the pancreata of older "Medalist" donor tissues (32).

Second, it has been shown that human pancreas contain a higher proportion of small islets relative to large islets than is found in mice (30). Interestingly, there is evidence that small islets are functionally superior to large ones following transplant (33,34). While the physiological relevance of extra-islet β-cells in human pancreas remains to be determined, the implications of this data are clear: as only intact islets are isolated for transplant, a significant proportion of β-cell mass is currently discarded during human islet transplant preparations, specifically from young donors. It was recently shown that islet transplants from young donors generate superior results, although islet yields are poorer compared to those from adults (35). Furthermore, we contend that this population of cells contains the highest proportion of $Ins^{+}GLUT2^{-}$ β-cell progenitors.

There was a surprisingly high degree of correlation between human and mouse in the cellular population of interest, given that humans predominantly use GLUT1 (23,36,37) and to a lesser extent GLUT3 (23) compared with GLUT2 for glucose transport when compared with mice. Regardless, there is clearly a role for the glucose transporter-2 in human pancreas. In both species, the population of $Ins^{+}Glut2^{-}$ cells was present in high proportion within the BCA compartment, decreasing only in late life. This implies that as

long as there are BCA present, there is a capacity for tissue remodelling, and by extension, tissue regeneration potential. Within the context of the complete BCA data however, the $Ins^{+}Glut2^{-}$ cell population does decrease in absolute terms as the total population of BCA decreases with advancing age.

Evidence for β-cell proliferation in the human has, until recently, been limited to few publications. Kassem *et al* demonstrated a low rate of proliferation, even in prenatal life between 17-32 weeks gestation, the rate of human β-cell replication being only 3.2%, and dropping further to 0.1% after 6 months of age (38). Detailed analyses by Meier *et al* showed similar rates of human β-cell proliferation at birth of 2.5% (26,39). Interestingly, that group also found higher rates of proliferation in small islets and β-cells found scattered throughout the pancreas, and which decreased with advancing age. This same trend was also shown by Jo *et al*, who concluded that large islets were created both from proliferation as well as the coalescence of small clusters (27), and this cluster aggregation decreased with age (40). By adulthood, there is the consensus that β-cell proliferation is extremely low, at ~0.2-0.5% (26,41–45). Rates of β-cell proliferation in rodents have been shown to be similar to those in humans, with 1-3% β-cells replicating in the young rat (46), and only ~0.2% by adulthood (45,47). Our results are consistent with the reported rates of human β-cell proliferation, as well as the observed variability between young human pancreata. However, we have demonstrated the mouse β-cell proliferation rate to be higher than has been reported by others.

A limitation to this study was availability of human tissue of specific ages. Mouse pancreas showed some subtle, but potentially important, differences in the $Ins^{+}Glut2^{-}$ cell population within the BCA compartment during the immediate postnatal time period and

up to weaning at 21d. However, the human samples we were able to analyze at a similar developmental stage were limited to the month around birth only. Assessing human pancreata around the time of weaning could potentially yield more information, although given the variability in human genetics, lifestyle, and diet which is not found in laboratory mice, this is conjecture. Direct comparison between human and mouse developmental time-points contains inherent limitations, but even despite these restraints, the data demonstrated high correlation. Due to constraints of sample size, we were not able to fully analyze gender-specific differences in human pancreas. Moreover, we did not have information regarding whether the human female subjects we studied had ever been pregnant. We did not find, however, any differences in any parameter studied (BCA proportion, $Ins^{+}Glut2^{-}$ cell population, or proliferation rate) between nulliparous and multiparous female mice (48).

A key goal is to determine the physiological function of the $Ins^{+}Glut2^{-}$ cells.. We have demonstrated that these cells are present in substantial number during early postnatal life, and that they decline with age. We postulate that this represents a reserve pool of cells available for use in times of metabolic need and during endocrine pancreas maturation. However, it is theoretically conceivable that β-cells instead undergo a normal cycle of developmental transition in glucose-responsiveness, expressing Glut2 in high and low proportion, although this phenomena would not likely change with age as shown here. Our data would suggest that some of these $Ins^{+}Glut2^{-}$ cells mature into $Ins^{+}Glut2^{+}$ cells, similar to data proposed by Szabat *et al* (49), which showed that β-cells can transition between $Pdx1^{+}Ins^{lo}$cells to $Pdx1^{+}Ins^{+}$ cells.

The regenerative capacity of the endocrine pancreas decreases with advancing age. This coincides with the decline in both $Ins^{+}Glut2^{-}$ (putative progenitor) cells, and the location in which they are predominantly found. Determining the role of these cells during pathological circumstance or in times of metabolic demand will further elucidate a potential role for this cell population in human health and pancreas development.

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CHAPTER 5

$Ins^{+}Glut2^{-}$ β-cell Progenitors Contribute to Endocrine Pancreas Regeneration after STZ Ablation in the Neonatal Mouse

Christine A. Beamish, Sofia Mehta, Brandon Cheuk, Brenda J. Strutt, and David J. Hill

5.1 Introduction

The replication of pre-existing β-cells has been shown to be the prominent source of cell turnover with age (1,2). However, the contribution of progenitor cells to β-cell mass postnally is less understood, especially within the context of induced regeneration, such as following pancreatic duct ligation (PDL) (3,4), or administration of the β-cell toxin Streptozotocin (STZ) in the young rodent.

The glucose-transporter 2 (Glut2) is a transmembrane carrier protein that enables glucose movement across cell membranes in liver, kidney, and pancreatic β-cells (5). The existence of insulin-positive, Glut2-negative (Ins^+Glut2^-) cells was identified within the pancreas over two decades ago (6), but a potential role for these cells was only recently elucidated, with their characterization as pancreatic multi-potential progenitor cells in both adult mouse and human islets and ducts (7). We have recently shown a significantly higher number of these Ins^+Glut2^- cells in neonatal (postnatal day 7, D7) mice, within islets and especially in extra-islet β-cell aggregates (BCA, <5 β-cells) (**Chapter 3**). These cells are proliferative *in vivo* and *in vitro*, and a subset retain the capacity to differentiate into pancreatic endocrine and ductal lineages. Moreover, these Ins^+Glut2^- cells are present in the highest proportion during the neonatal time period and decrease with age in both mouse and human pancreas (**Chapter 4**).

We (8–12) and others (13–15) have shown that young rodents retain the capacity to partially regenerate β-cell mass after STZ ablation, but this regenerative potential is mitigated in adulthood (15–17). As a glucose mimetic, STZ accesses the β-cell *via* Glut2 (18–20) and causes β-cell necrosis within 24 h of administration by DNA alkylation and

fragmentation (21); Glut2$^{-/-}$ mice then are resistant to the effects of STZ (20). Given the numerous Ins^{+}Glut2^{-} progenitor cells present in BCA, and because the number of BCA are highest in the neonatal time period and decrease with age (22) (**Chapter 4**), it is hypothesized that neonatal rodents are able to regenerate their β-cell mass after STZ administration in part from Ins^{+}Glut2^{-} cells spared exposure to the toxin. These Ins^{+}Glut2^{-} cells then contribute to the regeneration of the endocrine pancreas. Using a pulse-chase experiment in which a proportion of insulin^{+} cells are tagged with a human placental alkaline phosphatase (HPAP) reporter followed by STZ administration and EdU incorporation, we sought to lineage trace the fate of β-cells after ablation and during regeneration, and to determine the contribution of Ins^{+}Glut2^{-} cells to this process.

5.2 Materials and methods

5.2.1 Animal experiments

All animal experimentation was performed with the approval of the animal use ethics committee of Western University, London, ON and in accordance with guidelines of the Canadian Council on Animal Care. Mice were maintained at the Animal Care Facility at Lawson Health Research Institute, St Joseph Health Care, with free access to food and water. *(Ins2-cre/ERT)1Dam/J* (RIPCreER) (23) mice was crossed with a LacZ reporter strain, tagged with the human placental alkaline phosphatase (HPAP) gene (24) as a lineage tracing technique to follow the β-cell. LacZ/HPAP$^{+/-}$ and RIPCreER$^{+/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Mice were genotyped using ear/tail tissue samples on D4. Identification of double-positive mice was accomplished by PCR

amplification using primers (Sigma Chemical, St Louis, MO) for the transgenes RIPCreER (FW- *aacctggatagtgaaacaggggc*, RV- *ttccatggagcgaacgacgagacc*, 410 bp), and LacZ/HPAP (FW- *ccgcttcccatatgtggctctgtcc*; RV- *gcatgagctcagtgcggttccacac*, 548bp). Transgenic control experiments are shown in **Supplemental Material** and **Figures S5.1** and **S5.2**, and details of transgenic mouse strain development is described by Dor et al (23).

To induce β-cell tagging with the HPAP reporter, RIPCreER;Z/AP$^{+/+}$ pups were injected on D4 and D5 with 0.1 mg/g body weight tamoxifen (Sigma), from 20 mg/ml stock prepared in corn oil (Sigma). Detail of the dosing strategy for neonatal mice is described in **Supplemental Material** and **Figure S5.3**.

5.2.2 Induction of hyperglycemia by STZ

Streptozotocin (Sigma) was injected on D7 (100 μg/g body weight, in 0.1 M citrate buffer). Transgene-negative litter mates were used as controls, and injected with corn oil and 0.1 M citrate buffer (sham). Both STZ-treated and control mice were pulse labelled with EdU on D8 (Invitrogen, Carlsbad, CA) (2 μl/g body weight of 10 mM stock) to assess β-cell proliferation immediately following STZ and the transmission of the retained DNA label in subsequent progeny. Glucose measurements were taken prior to sacrifice by tail vein blood samples using a One Touch Ultra glucometer (Lifescan Inc, Milpitas, CA). An experimental schematic is shown in **Figure 5.1A,** and the lineage-tracing paradigm in **Figure 5.1B.**

Mice were euthanized on D9, 14, 21, or 28. Pancreata were excised and prepared for cryosectioning as described in **Chapter 3.**

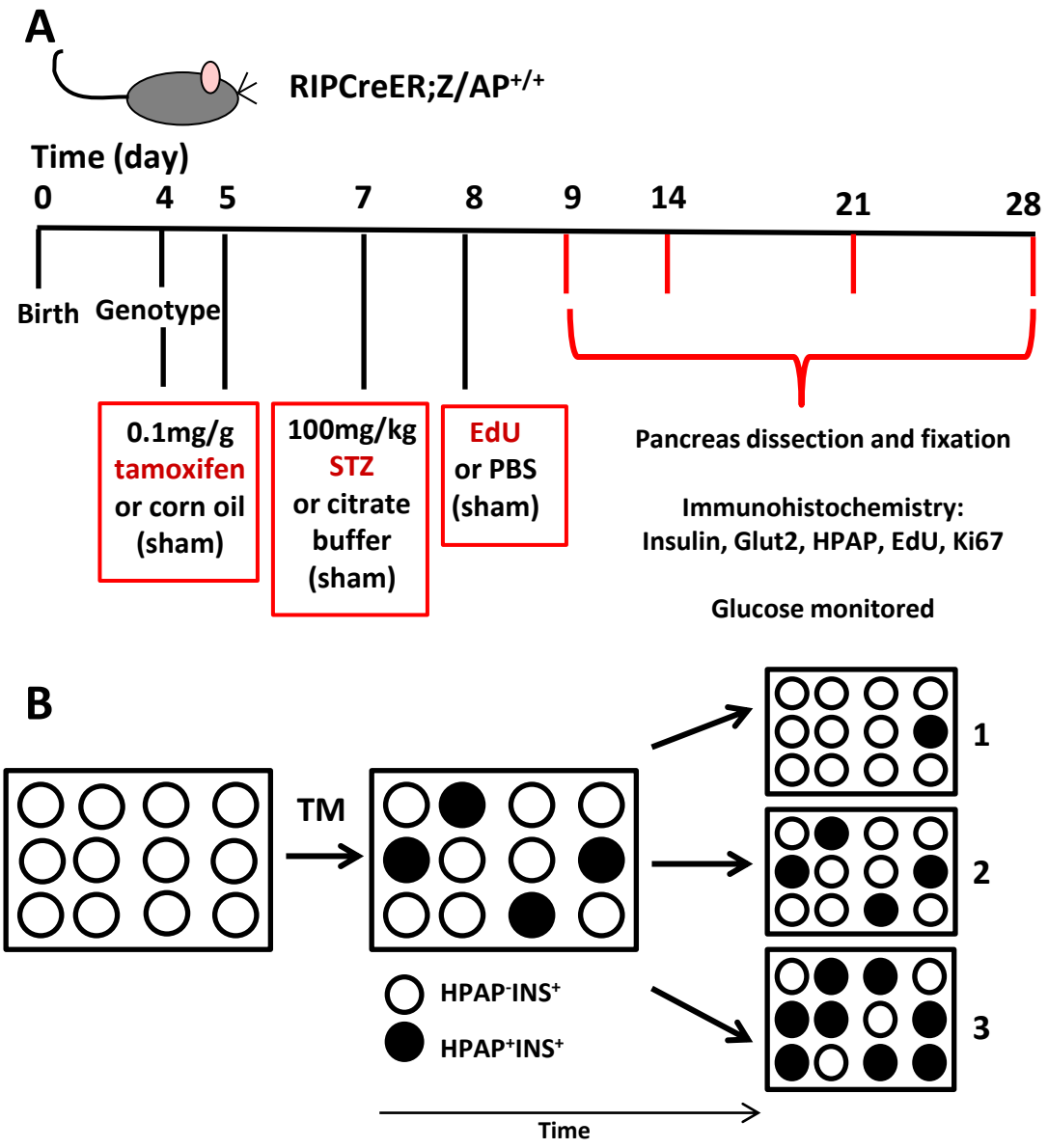

Figure 5.1. Experimental schematic and lineage tracing paradigm.
(A) Transgenic RIPCreER;Z/AP$^{+/+}$ mice were induced to express the reporter protein HPAP after 2 injections of tamoxifen (TM) on postnatal days (D)4 and 5. Streptozotocin was injected 2 days later (D7) to knock down a proportion of insulin-expressing β-cells. EdU was injected on D8 to track the cells which proliferated immediately following injury. Starting on D9 and up to 3 weeks later, pancreata were dissected and analyzed for endocrine regeneration by the presence and location of cells expressing of insulin, Glut2, HPAP, EdU, and Ki67. **(B) Lineage tracing paradigm using a pulse-chase method.** Squares represent islets, and circles represent unlabelled (white) and labelled (HPAP, black) β-cells. After induction of HPAP by TM, β-cells were tracked during aging or following injury. A decrease in the labelling index of HPAP indicates that β-cells form from cells not expressing insulin initially, i.e., stem cell source (option 1). Conversely, maintenance of the labelling index of HPAP indicates that β-cells form by self-duplication exclusively (option 2).

5.2.3 Immunofluorescent histochemistry

Pancreas sections on slides were subjected to antigen retrieval using 95°C in 0.1 M Tris-EDTA buffer for 30 min. Sections were permeabilized with 0.3% triton-X-100/PBS for 10 min before blocking with Background SNIPER (Biocare Medical, Concord, CA) for 8 min. $HPAP^+$ cells were identified using a mouse anti-HPAP antibody (Sigma, 1/500) or with a rabbit anti-HPAP antibody (1/200, Abcam, Cambridge, UK); the mouse antibody required the use of a mouse-on-mouse (MOM) staining kit (Vector Labs, Burlingame, CA) to block endogenous immunoglobulins. Other antibodies utilized include mouse anti-human insulin (Sigma, 1/2000), rabbit anti-human insulin (Santa Cruz Biotechnology, Santa Cruz CA, 1/200), and goat anti-human Glut2 (Santa Cruz, 1/100). Antibodies were diluted in Antibody Diluting Solution (Life Technologies Inc, Carlsbad CA) and sections incubated overnight at 4°C. Fluorescent secondary antibodies were conjugated to 488/555/647 fluorophores (Life Technologies, 1/500) appropriately matched to the primary antibody, and sections incubated for 2 h at room temperature in the dark. Rates of cell proliferation were determined by EdU incorporation using the EdU Click-It reaction kit (Life Technologies), and by Ki67 immunostaining using a mouse anti-human Ki67 (BD Biosciences, Mississauga, Canada, 1/50). Cells were counterstained with DAPI for quantification. Slides were imaged on a Zeiss LSM 510 Duo Vario (Carl Zeiss Ltd, Oberkochen, Germany) confocal microscope at the Biotron Institute, Western University using Zen software, and cells were counted manually using the Zeiss LSM Image Browser.

5.2.4 Beta cell morphometry

Two pancreas sections per mouse were immunostained with insulin as described previously (10), and imaged and analyzed using Northern Eclipse software (v7.0; Empix Imaging, Mississauga, ON, Canada). Total pancreas area was traced at 2.5x magnification, and β-cell area traced at 40x magnification by insulin immunoreactivity. β-cell mass was calculated by multiplying the fractional β-cell area by the weight of the pancreas (mg).

5.2.5 Statistical analysis

Data are expressed as percentage of insulin$^+$ β-cells (% mean ± SEM). Changes between means were determined using Student's t-test or one- or two-way ANOVA, followed by Tukey's post-test where appropriate, and with an acceptable level of significance of $p < 0.05$. Statistical analysis was performed using Graphpad Prism software (v. 5.01, San Diego CA).

5.3 Results

5.3.1 Hyperglycemia and β-cell mass moderately regenerate after neonatal STZ exposure

Hyperglycemia was noted 2 days after neonatal STZ treatment (i.e., D9), but blood glucose recovered by D28 (**Figure 5.2A**, STZ *vs* control, $p < 0.05$). The presence of hyperglycemia correlated with the extent of β-cell mass loss (**Figure 5.2B**), being greatest at D14 and moderately recovering by D28 (**Figure 5.2B**, Control *vs* STZ, D14 and D21, $p < 0.05$).

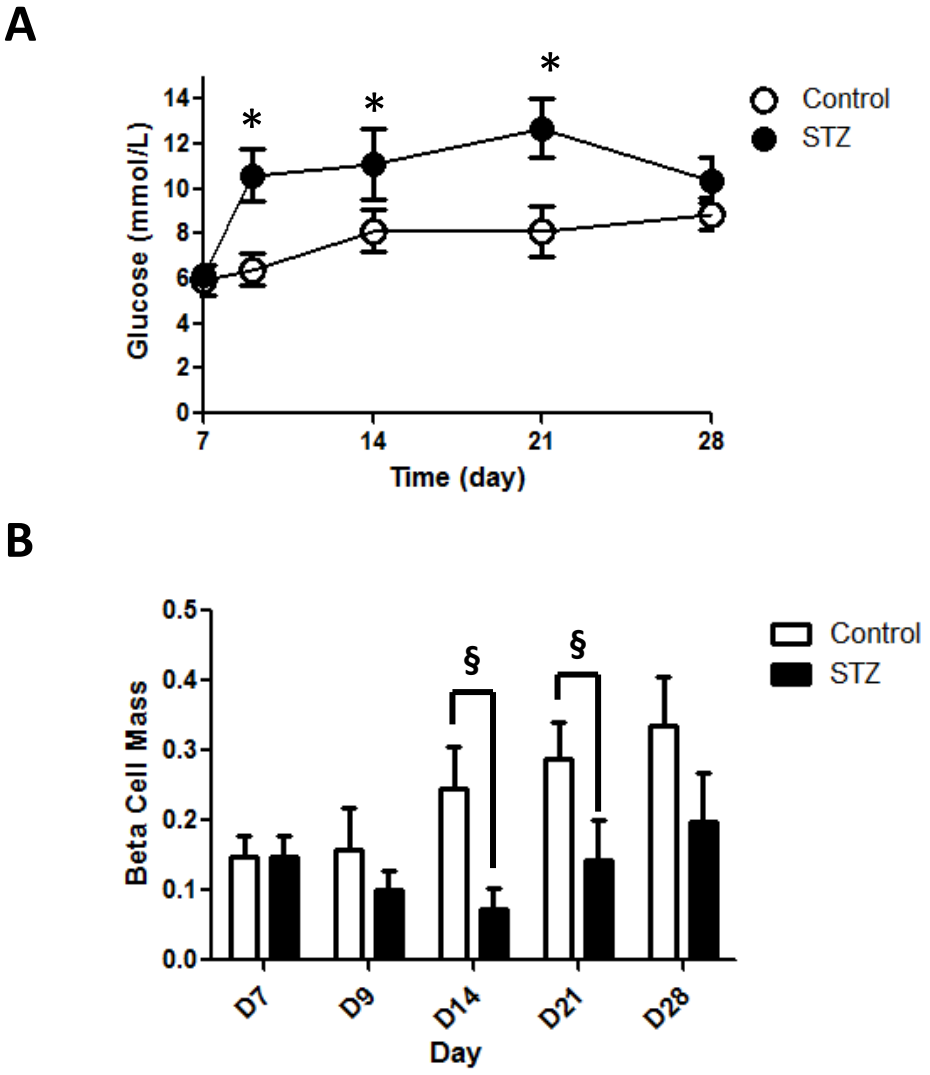

Figure 5.2. Induction of diabetes by STZ.
Glycemia (A, mmol/L) and β-cell mass (B) were measured in control (white bars) and streptozotocin (STZ)-treated (black bars) mice over the first 4 weeks of life. Data were analyzed using 1-way ANOVA and Tukey' s post-test or student's t-test. Data shown represent % Mean ± SEM. *, § $p < 0.05$, $n > 5$.

5.3.2 Changes in the proportional presence of Ins⁺ cells after STZ

In control mice we previously showed that the proportional presence of Ins^+ cells within BCA (<5 β-cells) scattered throughout the pancreas significantly decreased with advancing age (**Chapter 4**). Consistent with those results, the proportion of BCA decreased in this model from D9-28 (**Figure 5.3A**, white bars, $p < 0.05$, D9 *vs* D14, 21, and 28). In small islets (5-15 β-cells, **Figure 5.3B**) and large islets (>15 β-cells, **Figure 5.3C**) there was no significant proportional change in the distribution of Ins^+ cells with age over this range.

After STZ administration, the relative proportion of Ins^+ cells present within small islets significantly increased at D14 (**Figure 5.3B**, black bars, $p < 0.05$, D14 *vs* all other ages, and $p < 0.001$, D14 C *vs* STZ). This correlated with a corresponding decrease in the proportion of Ins^+ cells present within large islet at D14 (**Figure 5.3C**, black bars, $p < 0.05$, D14 *vs* D21). Unlike controls, there was no decrease in the proportional presence of Ins^+ cells in BCA following STZ treatment after D9, and instead there was an increase in the proportional presence of Ins^+ cells in both BCA (**Figure 5.3A**, $p < 0.05$ D9 and D14 *vs* D28 black bars and D28 STZ *vs* Control) and small islet proportion at D28 (**Figure 5.3B**, $p < 0.05$, D28 STZ *vs* Control). Thus, while the maximal destruction of β-cells seen at D14 following STZ administration is likely to have caused the proportional shift of Ins^+ cells from large to small islet categories, there was significant regeneration of β-cell mass by D28 occurring in the BCA and small islet compartments. In addition, the normal age-related decrease in the proportion of Ins^+ cells located in BCA was prevented following STZ treatment, suggesting that an early expansion of Ins^+ cell number occurred in BCA shortly after administration of STZ.

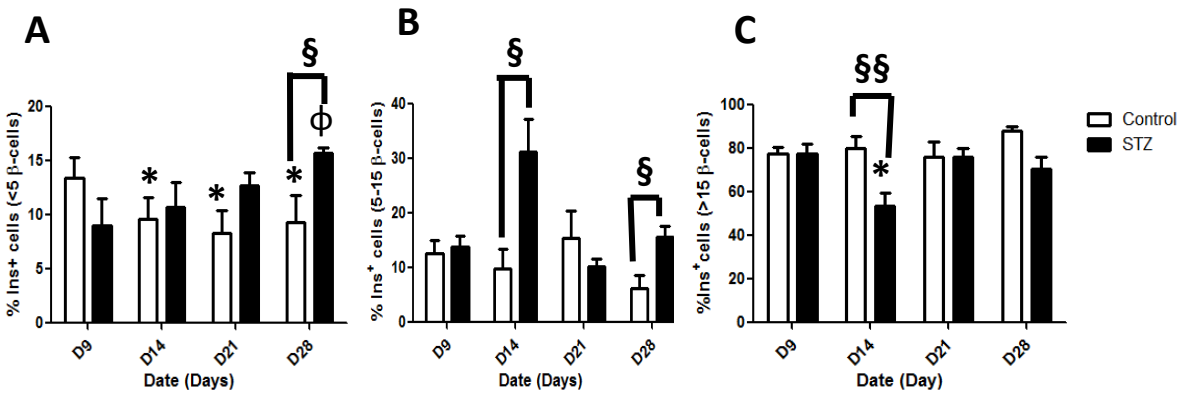

Figure 5.3. **Proportional β-cell location changes after STZ administration.** Proportional presence of β-cells within β-cell aggregates (BCA, <5 β-cells, A), small islets (5-15 β-cells, B), and large islets (>15 β-cells, C) in control (white bars) and streptozotocin (STZ)-treated mice (black bars) over the first 4 weeks of life. Data shown represent % Mean ± SEM, §,*, ϕ $p < 0.05$, §§,**$p < 0.005$, $n > 5$.

5.3.3 β-cell proliferation increases after injury and during regeneration

The presence of β-cell proliferation shortly after STZ administration was measured by injection of EdU on D8. The labeling of Ins^+ cell nuclei with EdU was tracked using immunohistochemistry over the course of the study on the assumption that the pulse label of EdU would be detectable in subsequent β-cell progeny at later time points (**Figure 5.4A**). In control mice, the percent labeling of β-cells with EdU was greater in BCA and small islets than in large islets, and a significant peak in the population of EdU-tagged cells was observed at D21 (**Figure 5.4Ai**, $p < 0.0005$, D21 *vs* other ages, and **Figure 5.4Aii**, $p < 0.005$, D21 *vs* other ages), before declining at D28. No significant changes in the relative proportion of EdU-labeled β-cell population were observed in large islets with age (**Figure 5.4Aiii**).

After treatment with STZ there was a trend towards an increase in the percent EdU labeling of Ins^+ cells in the BCA at D14, a week earlier than was shown to occur in control pancreata, although this did not reach significance (**Figure 5.4Ai**, black bars). There was a significant increase in Ins^+EdU^+ cells within small islets at D21, which disappeared by D28, similar to controls (**Figure 5.4Aii**, black bars, D21 *vs* D28). A surge in β-cell proliferation within BCA following STZ treatment might be expected to result in many of the BCA expanding to be counted as small or large islets at later time points, thus explaining the apparent paucity of EdU-labeled cells in the BCA fraction at later ages. In support of this possibility, a significant increase in the proportional presence of EdU^-labeled Ins^+ cells was also observed in large islets following treatment with STZ at D28 (**Figure 5.4Aiii**, black bars,

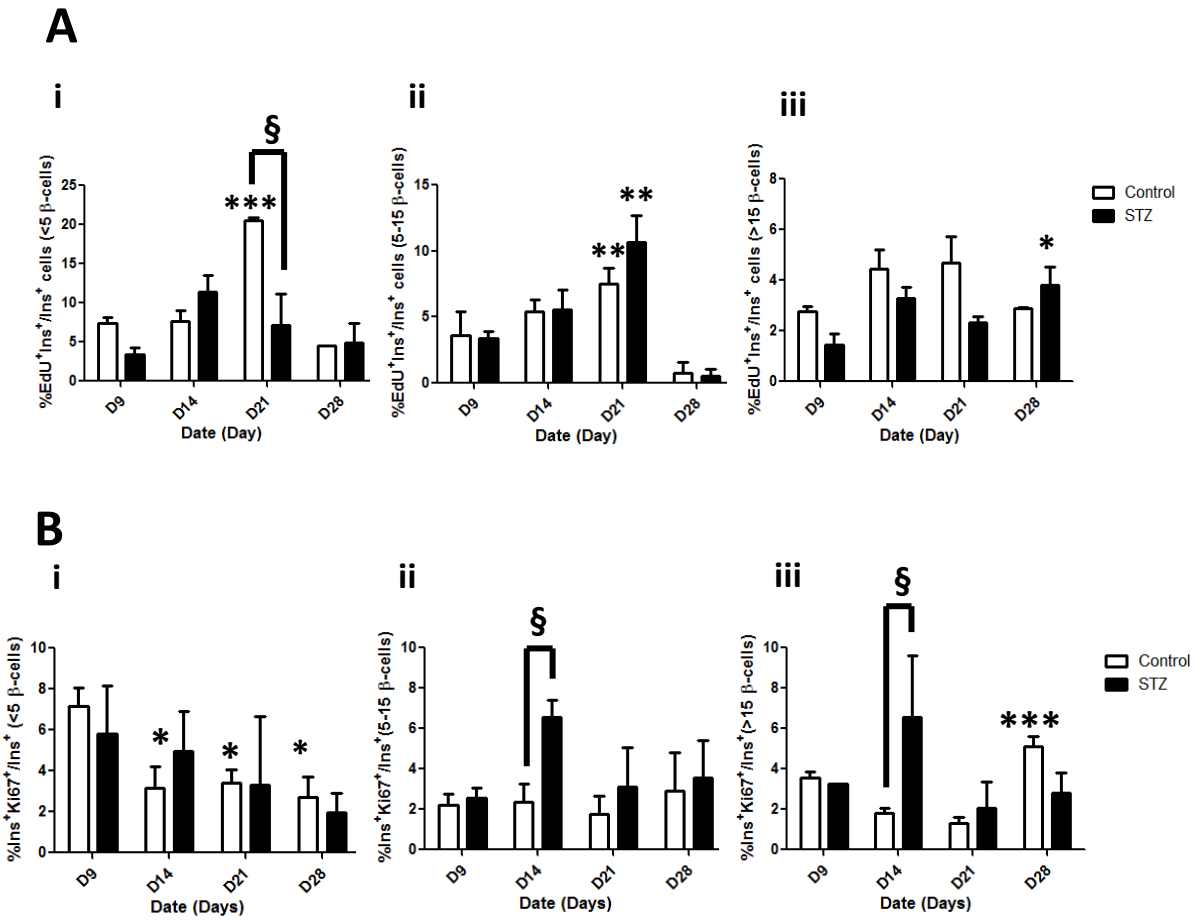

Figure 5.4. Beta cells proliferate to regenerate endocrine cell mass following damage.

Proportional presence of β-cells within β-cell aggregates (BCA, <5 β-cells, i), small islets (5-15 β-cells, ii) and large islets (>15 β-cells, iii) in control (white bars) and streptozotocin (STZ)-treated mice (black bars) over the first 4 weeks of life.

(A) EdU. β-cell proliferation immediately following injury was measured by EdU incorporation, and retained in cells during aging and regeneration **(B) KI67**. β-cell proliferation at the time of euthanasia was measured by Ki67 immunostaining within Ins+ cells. Data were analyzed using 1-way ANOVA and Tukey's post-test or student's t-test. Data expressed as % Mean ± SEM, §,*$p < 0.05$, **$p < 0.005$, ***$p < 0.0005$.

D28 *vs* D9). This suggests that the pulse label of EdU within β-cells could be 'chased' into larger islets with time during the recovery of β-cell number after STZ treatment.

Assessment of β-cell proliferation at the time of euthanasia was measured by Ki67 immunoreactivity within Ins^+ cells. In control animals, β-cells within BCA demonstrated a greater proportional presence of $Ki67^+$ cells than either small or large islets at D9 (**Figure 5.4Bi-iii**, white bars, $p < 0.05$), and which declined at later ages (**Figure 5.4Bi**, white bars, $p < 0.05$ D9 *vs* D14-28). There was also an increase in Ins^+Ki67^+ cells within large islets at D28 relative to other ages (**Figure 5.4Biii**, white bars, $p < 0.005$), likely due to a remodelling of the endocrine pancreas following weaning.

One week after STZ administration (i.e., D14), β-cell proliferation as detected using Ki67 presence had increased in both small and large islets by 2.6- and 2.1-fold, respectively relative to controls (**Figure 5.4Bii** and **Figure 5.4Biii**, $p < 0.05$, D14, STZ *vs* control). In BCA, Ki67 presence remained in high proportion until after D21, although this was not significant (**Figure 5.4Bi**, black bars). Combined with the EdU proliferation data, the increased rate of β-cell proliferation detected in small and large islets after STZ could have resulted, in part, from the prior expansion of the β-cell population within at least some BCA. To determine if this new population of β-cells could also have arisen from a progenitor cell population not previously expressing insulin within either BCA or islets we next employed lineage-tagging of β-cells, either with or without STZ treatment.

5.3.4 The proportion of β-cells genetically tagged with HPAP increases with postnatal development, and is altered after STZ

In control mice, 22.2 ± 3.2% of β-cells within BCA (**Figure 5.5A,** horizontal bar) expressed HPAP at D9 (baseline), compared with 28.5 ± 3.1% of β-cells in small islets (**Figure 5.5A,** hatched bar) and 31.0 ± 2.8% of β-cells within large islets (**Figure 5.5A,** vertical bar). Representative images are shown in **Figure 5.5B**, with β-cells immunostained for insulin (green) and HPAP (red). In control mice, the proportion of HPAP-tagged β-cells in all compartments more than doubled over the early postnatal time period (**Figure 5.5C**, BCA, white bars, $p < 0.05$, D28 *vs* D9, D14; **Figure 5.5D**, small islets, $p < 0.05$, D21 *vs* D9; **Figure 5.5E**, large islets, $p < 0.05$, D21 & D28 *vs* D9).

Following STZ administration, the proportion of HPAP tagged β-cells in BCA did not change throughout the study period, and by D28 was significantly lower than in controls (**Figure 5.5C**, black bars, $p < 0.001$ D28 STZ *vs* C). A similar profile was seen in both small and large islets (**Figure 5.5D** and **5.5E**) with a reduced presence of HPAP-tagged cells by D21 and D28 after STZ treatment (**Figure 5.5C**, $p < 0.001$ D28 Control *vs* STZ; **Figure 5.5D**, $p < 0.05$, D21 Control *vs* STZ; **Figure 5.5E**, $p < 0.05$, D28 Control *vs* STZ). This implies that 3 weeks after administration of STZ, a greater proportion of β-cells were present in each of the three cellular compartments that had not derived from pre-existing β-cells, relative to control animals. This would be consistent with the regeneration of β-cells being derived in part from non-insulin or low-insulin expressing progenitors within both BCA and islets.

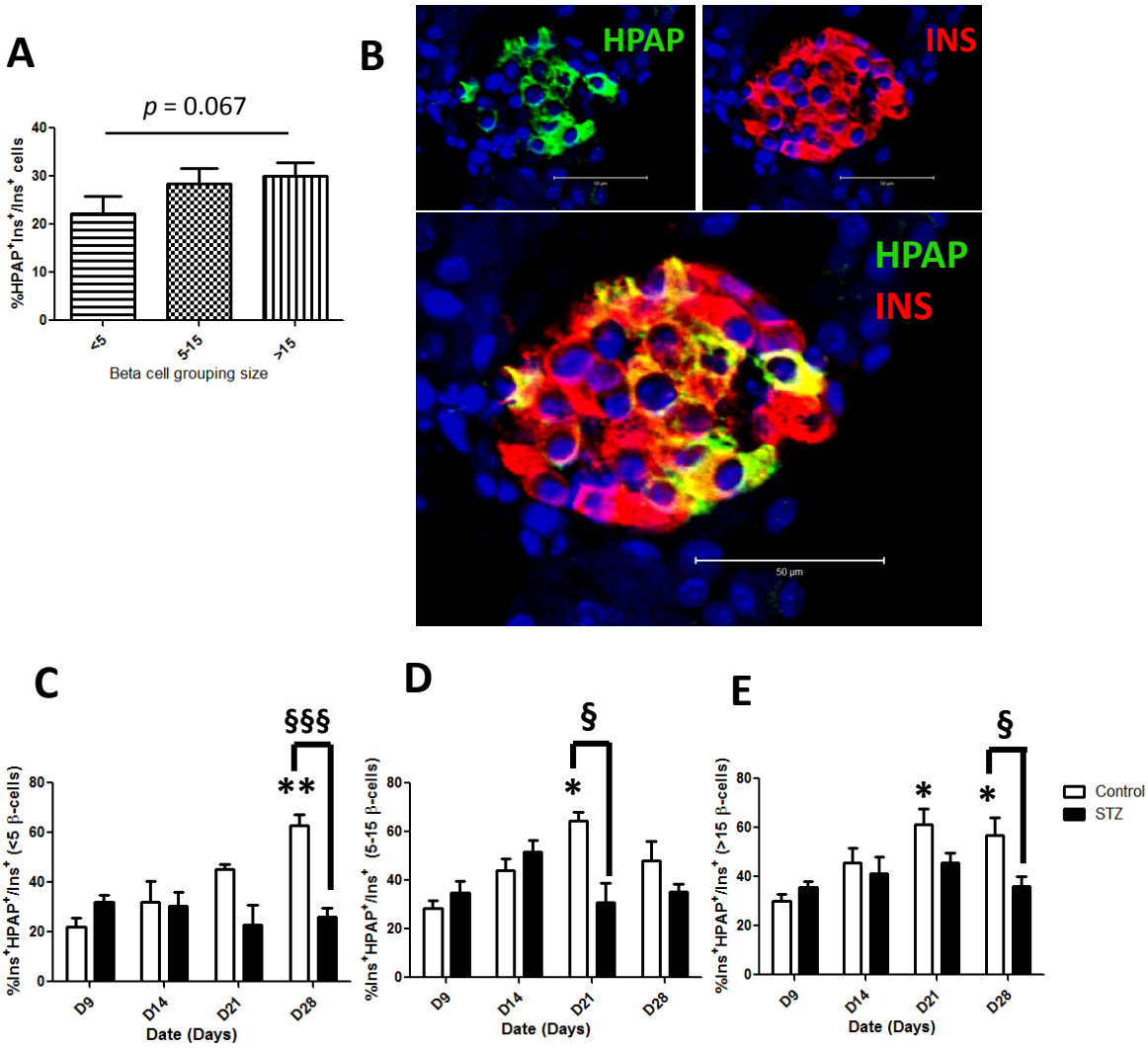

Figure 5.5. Lineage tracing of the β-cell during postnatal development and regeneration after STZ.

Tagging proportion of the reporter protein human placental alkaline phosphatase (HPAP) within β-cells at D9 (A) and over time (C-E) within β-cell aggregates (BCA, <5 β-cells, A, horizontal bars, and C), small islets (5-15 β-cells, A, hatched bars, and D) and large islets (>15 β-cells, A, vertical bars, and E). Changes in proportional presence of HPAP-tagged β-cells within Control (white bars) and Streptozotocin (STZ)-treated mice (black bars) were noted over the first 4 weeks of life depending on size compartment (C-E). Representative image of HPAP- tagged (B, green) β-cells (insulin, red) in a D28 large islet. Data were analyzed using 1-way ANOVA and Tukey's post-test or student's t-test, and data expressed as % Mean ± SEM. (C) control **D28 *vs* D9 and D14, §§§ D28 control *vs* STZ; (D) control *D9 *vs* D21, § D21 control *vs* STZ; (E) control *D21 and D28 *vs* D9, § D28 control *vs* STZ. §,*$p < 0.05$, **$p < 0.005$, §§§ $p < 0.0005$. Size bar denotes 50 µm, $n > 3$.

5.3.5 Ins$^+$Glut2$^-$ cell proportion increases following STZ treatment

We previously showed that the proportional presence of Ins$^+$Glut2$^-$ cells decreased with age, representing ~3.6% of total β-cells at D7 but <0.7% by 3 months, with the majority of these cells present in BCA (**Chapter 4**). In the present model, and consistent with earlier findings, the proportion of Ins$^+$ cells that did not show the presence of Glut2 was 10-fold greater in BCA than in islets (**Figure 5.6A**), and was maintained at this proportion until D28 when there was a significant decline (**Figure 5.6A**, $p < 0.05$ D21 *vs* D28). There was a non-significant, gradual decline in this population in large islets over the same range (**Figure 5.6C**, white bars, D9-28).

Following treatment with STZ the proportional presence of Ins$^+$Glut2$^-$ cells within BCA did not alter, but in small and large islets their presence significantly increased relative to controls at D14 (**Figure 5.6B**,**C,** black bars, $p < 0.05$ D14 *vs* D28). Thereafter, the population disappeared by D28 in small and large islets (**Figure 5.6B, C**, D28, Control *vs* STZ) but was maintained in BCA (**Figure 5.6A**, D28, Control *vs* STZ). This implies that the Ins$^+$Glut2$^-$ cells were spared STZ-mediated destruction in all β-cell size compartments, and contributed to regeneration in islets at D14, presumably by the differentiation into Ins$^+$Glut2$^+$ β-cells. Within BCA however, the lack of increase in cell proportion may have been overshadowed by the complex and dynamic changes occurring during both development and regeneration after injury. These data are consistent with a return to euglycemia in STZ-treated mice by D28 and a moderate recovery of β-cell mass.

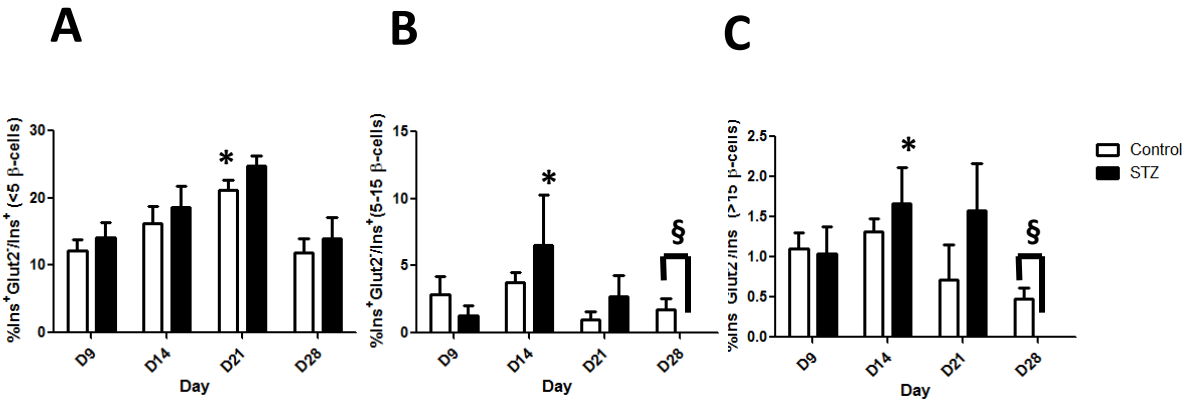

Figure 5.6. Ins^{+}Glut2^{-} cell proportion increases after STZ administration.
Proportional presence of Ins^{+}Glut2^{-} cells within β-cell aggregates (BCA, <5 β-cells, A), small islets (5-15 β-cells, B) and large islets (>15 β-cells, C) in control (white bars) and streptozotocin (STZ)-treated mice (black bars) over the first 4 weeks of life. Data were analyzed using 1-way Anova and Tukey's post-test or student's t-test. Data expressed as % Mean ± SEM. (A) white bars, *D28 *vs* D21. (B)(C) black bars, D14 *vs* D9; (B)(C) D28 C *vs* STZ. §,* $p< 0.05$, $n > 3$.

5.4 Discussion

It was demonstrated here that modest endocrine regeneration is possible in the neonatal rodent pancreas. Consistent with data presented by others, it was shown that most β-cells indeed arise from pre-existing β-cells after endocrine pancreas damage induced by STZ. It was further demonstrated that some of the β-cells that recovered during endocrine cell regeneration arose from a subtype of pancreas progenitor cell. These progenitor cells express insulin but lack Glut2. The finding that these cells normally diminish in number with advancing age provides a basis that may explain the enhanced β-cell regenerative capacity in the young which is not evident in older mice.

For many decades, STZ has been used extensively as a model for type I diabetes research and has been the agent of choice for the induction of β-cell damage due to its relative stability during administration (25). While newer damage models such as those using tetracycline-dependent diphtheria-toxin (26,27) have recently gained favour, we have learned much information from STZ-administration regarding β-cell death (and its corollary, regeneration), pancreas development, and diabetes progression. Furthermore, while the diphtheria-toxin based ablation method exhibits higher specificity for β-cell death without confounding effects from inflammation or non-pancreatic organ damage, diabetes itself presents as an inflammatory condition at the time of onset, and in this way STZ administration in mice mimics disease progression in humans more closely than other methods. A limitation of the use of STZ as a means of effecting diabetes is that due to differences in human pancreatic glucose transporter utilization of GLUT1/3 over GLUT2, STZ

administration in rodents cannot identically parallel diabetes progression in humans, as human endocrine pancreata are less susceptible to the effects of the drug (28,29).

The ability to recover β-cell mass after STZ is age-related, with moderate regeneration after subtotal ablation in young, but not old, rodents. We (**Chapter 4**) and others (22,30) have shown that the proportion of β-cells present in single cells and small clusters decrease with advancing age, concomitant with islet maturation and coalescence into large islets. This coincides with a transition in the proportion of $Ins^{+}Glut2^{-}$ β-cells during neonatal life, a time of significant endocrine pancreas remodelling (31–33). Indeed, the finding that there was an increase in BCA and small islet proportion after STZ demonstrates the validity of the diabetes model: these cells were spared STZ-mediated death, with the majority of β-cells instead dying from within islets [where mature β-cells reside (34)]. As others have also illustrated, rodents exposed to STZ during neonatal life consequently (re)develop hyperglycemia in adulthood (10,17). This is perhaps explained by the loss of the putative "reserve β-cell pool" (i.e., the $Ins^{+}Glut2^{-}$ cells, present in early life), which were recruited to maintain normoglycemia following STZ-mediated injury, and were therefore unable to contribute to normal tissue turnover thereafter. Conceivably, in humans similar mechanisms may contribute to misalignment of β-cell supply and demand, as observed in instances such as obesity, pregnancy, or autoimmunity.

β-cell proliferation is the primary mode of β-cell replacement in the postnatal pancreas. In this model, Ki67 expression doubled within Ins^{+} cells present in small and large islets 7 days following STZ administration. These data are, although slightly lower, broadly aligned with data shown by others using older mice (15). The difference may be resulting

from both increased (baseline) proliferation in early life, and some β-cell regeneration from $Ins^{+}Glut2^{-}$ cell maturation, and not cell proliferation exclusively. Results from the Ki67 analysis were otherwise as expected, but data from EdU incorporation yielded some interesting insights. First, it was shown that the β-cells present in BCA and small islets which proliferated early in development peaked at D21 (i.e., weaning), and the population thereafter declined. This indicates that these cells either died, or were incorporated into larger, proto-typical islets. After STZ however, and in agreement with the regeneration model, these EdU^{+} β-cells peaked at D21 in small islets and thereafter disappeared from this compartment. Subsequently there was a peak of EdU^{+} β-cells at D28 in large islets, indicating that the proliferative cells present in the early postnatal pancreas could be chased into large islets.

Based on the lineage tracing paradigm established by others (23,35), it was predicted that if β-cells arose from stem cells, there would be a dilution in lineage tag (HPAP) over the course of study. The corollary being that if there was no change in lineage tagging frequency, then β-cells arose from pre-existing β-cells present at the time of induction. Interestingly, in the present model, HPAP expression increased during early endocrine development, which may be interpreted as β-cells primarily arising from self-duplication postnatally. Conversely, after STZ exposure and during recovery, the reporter protein was maintained at the initial tagging proportion in all β-cell grouping compartments, indicating that a significant proportion of β-cells arose from cells not tagged initially (i.e. new β-cells).

Differential interpretations of this phenomenon are that (i) the reporter labelling continues longer than the two-day window between tamoxifen injection and STZ exposure

(36); (ii) that β-cells arose from cells that previously did not greatly express insulin at D4-5 and therefore did not label with HPAP, but could still have been an immature β-cell subtype; (iii) STZ preferentially destroyed HPAP-labelled β-cells; or (iv) that the HPAP-tagged a highly proliferative sub-population of β-cells present in early postnatal life. We can dismiss scenario (i), as this would be true only if there was an increase in reporter protein proportion shortly after tamoxifen administration. Since the reporter protein continued to increase thereafter in control, but not STZ-treated mice, we can conclude this is not the cause. Evidence from Reinert *et al* showed that after high doses of tamoxifen, there was indeed continued reporter labelling long after the expected window for cell tagging; however, our dosing regime was similar to the low dose model in that report (37), and which could be expected to account for only ~5% increase in reporter labelling shortly (<1 week) after administration. In the second and third options, considering that only mature β-cells demonstrate active transcription of the insulin promoter, these options are theoretically conceivable, as there was a difference in labelling index between BCA and islets at D9, albeit non-significantly. HPAP itself would not (theoretically) render those β-cells more susceptible to STZ-mediated damage, but by the fact that mature cells would tag with HPAP and therefore express high Glut2 presence, these are *de facto* linked. The fourth option is also theoretically possible, given the increase in β-cell mass concomitant with somatic growth during this stage in life, and could be in fact due to the $Ins^{+}Glut2^{-}$ cell population, although this was not confirmed. As evidenced here, the established lineage-tracing paradigm did not originally allow for the possibility that the putative stem cell source could also express insulin.

These lineage-labelling trends are partly consistent with aspects of other studies. Liu *et al* found that there was a substantial increase in reporter protein frequency with aging, as was shown here, but also after STZ (35). That study showed an increase in $PLAP^+$ [HPAP] Ins^+ cells, which the authors contend arose from β-cell progenitor cells which lacked insulin but retained the β-cell reporter HPAP. The authors found that these $HPAP^+Ins^-$ cells demonstrated an immature phenotype, with positive expression of *Pdx1* and *MafB*, but being negative for both *Nkx6.1* and *Glut2*, and which were proliferative after STZ administration. The relative proportion of these cells remaining after injury was found to be 0.1% of all $insulin^+$ (β-) cells. Taken together, the authors concluded that these cells represented β-cell progenitors which, by virtue of expressing the β-cell reporter, must have exhibited active insulin promoters at the time of induction. Only after injury or aging, and hence after differentiation to proto-typical mature β-cells, was insulin re-expressed, and which accounted for the increase in $HPAP^+$ labelled Ins^+ cells with increased age.

The data presented here also partly align with a study by Dor *et al*, which demonstrated no change in lineage reporter expression during normal aging, nor after insult induced by partial pancreatectomy (23). All three studies used the same mouse strain for analyses, but demonstrated differences in data trends and interpretation. We assert that these differences are directly related to age, as those previous papers are used adult mice, and as such, the population of β-cells available was largely established and functional. One point of discrepancy is the difference in function between β-cells located within and outside of islets. Multiple groups have previously purported differences in function based on location of β-cells (38–43), but the definitive evidence from lineage-tracing experiments by Dor *et al*

demonstrated no differences in β-cell lineage labelling frequency as stratified by location (23), thus ending the debate. The defining difference in our study is the examination of β-cells not just by location, but crucially also at a young age. We have shown that the β-cells located in small clusters outside of the islet to be substantially different than those located within it, in terms of proliferation rate, lineage-label tagging frequency, and Glut2 expression.

While multiple papers have sought to determine the role of (unidentified) stem cells to endocrine regeneration, we questioned specifically the contribution of $Ins^{+}Glut2^{-}$ cells during this process. As has been shown previously, the β-cells that can be targeted by STZ must express Glut2 (20), and as previously demonstrated, the population of $Ins^{+}Glut2^{-}$ cells decrease with age (**Chapter 4**). We show that under control (non-diabetic) conditions, the proportion of $Ins^{+}Glut2^{-}$ cells present in both BCA and islets decrease in the first 4 weeks of life. In agreement with our hypothesis, these cells increased in relative proportion in both islet compartments in the immediate time-course following STZ exposure indicating that they were spared destruction. This was followed by a dramatic reduction in the cell population in islets after weaning, which we speculate to illustrate a maturation of these cells into functional, glucose-responsive β-cells to compensate for the loss after damage. However, this assumption requires a conclusive assessment of cells present at the time of damage versus during regeneration, and thus correlating the HPAP tagged β-cells with the (lack of) expression of Glut2 would theoretically illustrate this phenomenon. Attempts to track the $Ins^{+}Glut2^{-}$ cells using HPAP were unsuccessful, as the expected frequency of

HPAP^{+}Ins^{+}Glut2^{-} cells in control mice at P7 was only ~0.3% of total β-cells in islets and <3% in BCA; furthermore, the BCA proportion decreases shortly thereafter in control mice.

We sought to answer three questions in this study: first, do Ins^{+}Glut2^{-} cells survive STZ mediated death?; second, do these cells contribute to endocrine regeneration following damage?; and third, what is the origin of these Ins^{+}Glut2^{-} cells? However, limitations arose which made full assessment of all three questions difficult. The first is that the baseline (control) conditions are in fact, constantly changing. During the first month of life in rodents, there is significant growth of β-cells concomitant with growth of the pancreas, and which has demonstrated an even more pronounced change around the time of weaning. Overlaying this rapidly changing phase with severe insult and subsequent regeneration yields a considerable number of variables.

The second limitation was that the pharmacological agents used to (independently) tag and destroy β-cells are likely targeting different populations of cells. We anticipated that the Ins^{+}Glut2^{-} cells would have a decreased β-cell-tagging frequency and would be selectively spared ablation by STZ. However, this lineage tracing technique relies on general trends only and which has made interpretation difficult, especially in the absence of a definitive lineage label for the Ins^{+}Glut2^{-} cells. It would be useful to examine a cohort of mice weeks or months after STZ ablation to determine if the Ins^{+}Glut2^{-} cells are replenished in adulthood, and if so, from which compartment. It would be also informative to determine if the lineage-label frequency continues to increase after D28.

Despite these limitations however, we show substantial changes in proliferative subpopulations and in BCA proportion supporting our hypothesis that some β-cells have

both survived STZ-mediated death, and subsequently matured from $Ins^{+}Glut2^{-}$ cells into $Ins^{+}Glut2^{+}$ cells. It was also demonstrated that these $Ins^{+}Glut2^{-}$ cells were primarily located in BCA, and coalesced from the BCA into islets. Therefore, the temporally-defined nature of STZ-mediated partial regeneration in the young rodent can be attributed in part to the presence of $Ins^{+}Glut2^{-}$ β-cells, which normally diminish with age and are therefore unable to aid in the rescue of the endocrine pancreas if β-cells are ablated after this window of time.

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CHAPTER 6

Summary and Perspectives

6.1 Summary of findings

Given the improvements in islet transplant procedures, with many patients experiencing >3 years post-transplant without exogenous insulin injections, a sustained improvement in glycemia, and a reduction in diabetes-related complications (1), we sought to find alternate sources of cells, given the paucity of β-cells available from cadaveric donors. We dedifferentiated isolated islets to duct-like cells based on an established protocol (2), with the intention of enhancing cell number while in the dedifferentiated state, then redifferentiating the cells back to an insulin^{+} condition.

Our results were consistent with those found by Yuan *et al* (2), demonstrating a substantial increase in the number of cytokeratin-19 (CK19) expressing cells, as well as a loss in islet morphology, insulin hormone presence, and β-cell and islet-specific transcription factors (**Chapter 2**). However, and similar to findings by others, we were unable to effect significant redifferentiation to insulin^{+} cells using Matrigel, insulin-like growth factor –II (IGF-II) and keratinocyte growth factor (KGF), or following the addition of trophic factors such as gastrin, nicotinamide, epidermal growth factor (EGF), nor with other established redifferentiation protocols (3,4), co-culture with endothelial cells *in vitro*, or after transplantation of the cells into immune-deficient mice. This was surprising, given the substantial production of ductal cells. As β-cells compose ~80% of mouse islet cell proportion, we next sought to determine the cell type of origin during *in vitro* dedifferentiation and hypothesized that it would involve the β-cell.

Lineage tracing of the β-cell using the non-inducible RIPCre;Z/AP mouse model revealed that during the dedifferentiation process only a rare minority of β-cells survive

culture and adopt a duct-like cell phenotype (**Chapter 3**), which explained the relative rarity of insulin-producing β-cells generated after redifferentiation techniques. We sought to characterize those rare β-cells capable of differentiation, and found evidence in support of a progenitor cell population present in mouse and human islets which express insulin, albeit at lower hormone levels than is present in fully mature cells. These cells were previously described as having decreased expression of key transcription factors such as *Nkx6.1* and *Pdx1*, increased expression of *Ngn3*, and, importantly, they can be identified by the lack of the glucose-transporter 2 (*Glut2*) (5). We showed that these $Ins^{+}Glut2^{-}$ cells are present in the neonatal mouse islet and provided evidence that the same cells were responsible for *in vitro* differentiation to duct-like cells, and could alternatively be differentiated to neural-lineage cells (**Chapter 3**). Importantly, the relative proportion of these cells is higher in the neonatal islet than is present within the adult islet, and furthermore, that there is a significant population of these cells in the β-cell aggregates (<5 β-cells, BCA) that exist outside of the proto-typical islet (**Chapter 3**). Not all $Ins^{+}Glut2^{-}$ β-cells were capable of multi-lineage differentiation however, with many more $Ins^{+}Glut2^{-}$ β-cells present in the neonatal pancreas than can demonstrate the capacity for altered cell fate. This result provides further evidence that β-cells exist within a spectrum of functional and developmental heterogeneity.

We consequently sought to identify whether the population of $Ins^{+}GLUT2^{-}$ cells could be identified in the human pancreas, specifically within the BCA compartment, and how the population changed with age in both mouse and human. We showed that $Ins^{+}GLUT2^{-}$ β-cells were present in very high proportion in human BCA, and that in both

human and mouse, these cells decreased with age, concomitant with a decrease in BCA proportion and proliferation rate (**Chapter 4**). Our analysis of β-cell proliferation in the human and mouse pancreas was consistent with data from previous publications, and showed that the proliferative potential of the $Ins^{+}GLUT2^{-}$ cells declines significantly before adulthood is reached, further contributing to the loss of regenerative potential with age. While this immunohistochemical study did not directly illustrate the functional capacity of the $Ins^{+}GLUT2^{-}$ in terms of multi-potential fate decisions, data from the van der Kooy group do show that these cells are retained in (at least) islets during adulthood (3,5), and provide a clear direction for future research.

We finally sought to assess the role of $Ins^{+}Glut2^{-}$ β-cells in the context of a diabetes model. It has been long known that β-cells can moderately recover their cellular mass after exposure to the β-cell toxin streptozotocin (STZ) in the neonatal time period, but not as adults. We theorized that because STZ accesses the β-cell via Glut2, and because there is a significant proportion of $Ins^{+}Glut2^{-}$ β-cells in the neonate and not in the adult pancreas, that neonatal endocrine pancreas could regenerate after STZ exposure because a significant component of β-cell mass is not damaged and can subsequently re-establish normoglycemia. We therefore examined the changes in β-cell mass, β-cell proliferation, and proportional changes in $Ins^{+}Glut2^{-}$ cells during the first month of life, with and without STZ, while lineage tracing the β-cell using the inducible RIPCreER;$Z/AP^{+/+}$ mouse model.

We found that there was indeed modest β-cell regeneration in the early postnatal time period after neonatal STZ administration. We also showed that there was retention of BCA, and an increase in proportion of specifically $Ins^{+}Glut2^{-}$ cells within islets during the

regenerative phase, which significantly decreased in islets 3 weeks after damage, which is likely to indicate a maturation of these progenitor cells into glucose-responsive β-cells. Interestingly, the origin of the β-cells present after 3 weeks in the early postnatal mouse pancreas indicated self-duplication as their main source in the normal developmental program, but not during regeneration as indicated by the altered trends in β-cell labelling with HPAP. Explanations of these opposing trends point to the presence of immature β-cells contributing to regeneration after β-cell loss, but not during normal postnatal development. Their contribution to β-cell regeneration seems to be age-related, as these trends only partially align with similar experiments performed in adult mice (6,7). However, further analysis at later ages may yield further insights. Overall, this project illustrated the extensive changes that occur in the endocrine pancreas during the neonatal time period, in terms of β-cell phenotype, anatomical location, and proliferative capacity.

While we did not generate significant numbers of β-cells as a potential donor source for use in transplant, we were able to identify a novel subset of progenitor cells in both the mouse and human pancreas, complementing evidence previously published which has not been replicated before now. We have contributed knowledge about these progenitor cells by showing that their predominant location is, in fact, outside of the islet, and their number significantly decrease with age. These findings may have implications in altering transplant criteria, and on a broader scale, illustrate a new parameter for study in pancreas maturation. Moreover, these results identify subpopulations of cells which may be responsible for adaptive growth during times of increased metabolic need such as pregnancy or diabetes.

6.2 Progenitor cell activation versus β-cell replication: the debate revisited

Much evidence has supported hypotheses favouring both progenitor cell differentiation and pre-existing β-cell replication as the mode for regeneration of endocrine cell mass during normal development, aging, and after injury. However, the breadth of convincing data for both arguments suggests that the timing of injury, as well as the manner and severity of damage play important roles in dictating the degree of endocrine regeneration, and which mechanism is responsible, i.e. pre-existing β-cell replication versus progenitor cell recruitment, activation, or cell trans-differentiation. This was illustrated by a study by Criscimanna and colleagues, which used models to destroy either "all" acinar and endocrine tissues (but not ductal cells) utilizing PdxCre;$R26^{DTR}$ mice, or the less-severe damage model created using ElaCre- ERT2;$R26^{DTR}$ mice, which selectively destroys only acinar tissue (8). The authors found that in the Pdx1-diptheria toxin model, 97% of all endocrine and acinar cells were destroyed, and both recovered by approximately 60% within 4 weeks, which was shown to be a result of the recapitulation of the endocrine developmental program. In contrast, knocking out the acinar compartment alone elicited trans-differentiation from ductal to acinar cells. That study is complemented by evidence from Thorel *et al*, which showed that under extreme β-cell loss, α-cells could trans-differentiate to become insulin-producing cells (9).

These data would indicate that under "normal" circumstances, i.e. aging, pregnancy, obesity, and even some damage models, β-cell (re)generation occurs through proliferation of existing cells exclusively. The trigger, or degree of damage, which elicits some other

mode of tissue regeneration, either from resident progenitor cell activation, or trans-differentiation from other tissue types, remains to be determined in both its scale and ability to regenerate functional endocrine mass. Indeed, nearly 2 decades ago this concept was purported (10); however, in the absence of lineage-tracing technology, and under the current and now-canonical model of exclusive β-cell self-duplication, it was dismissed.

Superimposed on this, of course, is the role of autoimmunity in diabetes; the loss of β-cell mass and attempts at regeneration, no matter whether β-cells are produced from a progenitor cell source or by self-duplication, being countered by T-cell-mediated destruction. This has been likened to a bathtub filling with water: new sources of β-cells, from replication or progenitor cell source, contribute new water to the tub from the faucet, while rates of cell death oppose this, and water flows out of the drain. In normal physiology, these two forces are roughly equal, and the net result is a maintenance of β-cell mass and/or function, which allows glucose homeostasis to be achieved. However, in the case of diabetes, the "drain" gets bigger (i.e., increased β-cell death), which far exceeds the potential of "new water" (insufficient β-cell regeneration and/or extremely slow β-cell proliferation), and hyperglycemia results (11). Furthermore, even when β-cell regeneration was shown to occur, anti-rejection drugs commonly used in the transplant setting mitigated the regenerative process (12). Whether the $Ins^{+}GLUT2^{-}$ cells are also targeted for destruction during T1D disease progression has yet to be ascertained, and the data presented here provides a novel arena for investigation.

6.3 Progenitor cells and β-cells exist within a spectrum of maturity

The concept that stem/progenitor cells may express mature markers, such as insulin, opens up the field to semantic arguments regarding what constitutes a stem cell; the present conclusion hinging on the premise that cell state can be assigned according to a binary code. Our findings suggest that a more appropriate assessment of cell maturity may include the concept of a continuum of cell "stem-ness" (or its corollary, maturity). As was noted by Murtaugh, it also contributes to the long-held debate over stem cell differentiation versus replication of pre-existing cell with regards to generation of postnatally-formed β-cells: it is both, in the same cell; or as Murtaugh states, "yes" [to both questions] (13). Furthermore, if progenitor cells can express insulin, substantial work by Melton (7) and Kushner (14), that β-cells are formed postnatally by self-duplication exclusively, are reconciled with the evidence of progenitor cell origin (5). In other words, the contribution from (the $Ins^{+}Glut2^{-}$) progenitor β-cells is masked because they are (mis) interpreted to be mature, insulin-expressing β-cells.

The concept of β-cell heterogeneity has recently emerged as a major contender in understanding the population of cells available for clinical use, either for retention of β-cells *in vivo* or for transplant procedures. It may be a philosophical argument, but is heterogeneity defined by cell maturity only? Is cell maturity defined exclusively by age? And, could β-cell anatomical location also dictate its function?

It is well known that mouse β-cells in the immediate postnatal period are immature, with poor GSIS and lower expression of Glut2 compared to adult β-cells (15–17). It was recently reported that neonatal β-cells require a glucose threshold for maturation, which

can be shown by gene expression changes in Urocortin3 (Ucn3) (18). This peptide hormone has been shown to be expressed in mature mouse β-cells after P7, and acts as a maturation marker (19). Similarly, human infant β-cells demonstrate some plasticity in insulin secretory machinery not seen in the adult state (20).

β-cells that reside outside of islets have long been thought to reflect newly generated, and hence immature, cells (10,21–25). However, none of these publications utilized lineage tracing, and hence none determined cell origin. The dogmatic publication by Dor *et al* then showed that there was no significant difference in lineage labelling index between β-cells found within and outside of islets during aging or after injury in the adult pancreas (7), implying that the anatomical location of β-cells was irrelevant to function or maturity. However, recent work has shown that, indeed, β-cells found in clusters outside of the islet demonstrate lower levels of Ucn3, especially in the young pancreas (18). Furthermore, β-cells outside of islets lack the exquisitely intimate contact with both the vascular network and innervation patterns as found in islets, as well as cell-cell contact with other β-cells for communication (26). We (**Chapter 4**) and others (27,28) have shown that age influences location of endocrine cell mass, concomitant with maturation. Taken together, it would appear that β-cell function, maturity, and location are intrinsically conflated.

For completeness this concept requires an investigation into the origin of the Glut2-negative β-cells, to test the assumption that “immature” equates to “newly generated” cells. The abundant evidence in the literature suggests that “all” (if not the majority) of new β-cells present during aging or after injury are derived from pre-existing β-cells. It is

presently unknown if the $Ins^{+}Glut2^{-}$ β-cells act as facultative progenitor cells *in vivo*. However, we do know that in the absence of pathologic metabolic challenge, the presence of multi-potential β-cells is extremely rare in the adult mouse and human pancreas. As posited in **Chapter 5**, the $Ins^{+}Glut2^{-}$ cells may represent a reserve pool of β-cells present in the postnatal pancreas, available for recruitment and/or utilization during remodelling of the endocrine compartment in early life. This reserve pool theory then implies that there is a finite number of $Ins^{+}Glut2^{-}$ β-cells, and perhaps, all β-cells, in the postnatal pancreas. Data from the Melton laboratory supports the idea of a set number of progenitor cells in the young, and a final β-cell number in the established pancreas (7,29). Our findings suggest that $Ins^{+}Glut2^{-}$ cells represent a part of this progenitor pool. Differential utilization of "true" stem cells within this population (the rare, multi-potential cells versus the majority of $Ins^{+}Glut2^{-}$ β-cells present in the early postnatal pancreas) as a means of changing the ultimate size of the β-cell pool remains to be examined. We can speculate that the majority of $Ins^{+}Glut2^{-}$ cells present in the young, mammalian pancreas normally mature directly into $Ins^{+}Glut2^{+}$ cells, and the multi-potential cells only undergo lineage expansion under pathological situations of β-cell loss. The signals required for activation of these stem cells in the adult context, and whether they can realistically contribute to β-cell regeneration to rectify hyperglycemia, is currently unknown. It was shown that these $Ins^{+}GLUT2^{-}$ cells normally ceased to be proliferative *in vivo* by adulthood (**Chapter 4**), but *in vitro* manipulation of β-cells was shown to select for a population of cells capable of differentiation towards proliferative "neurospheres", which after transplantation into diabetic mice was shown to be effective at combating glycemic excursions (5). We were also able to replicate these experiments from young mice, generating neurospheres from β-

cells present in neonatal islets and BCA (**Chapter 3**). Moreover, the evidence that a minority of β-cells and their progeny have been shown to change cell fate *in vivo*, by expressing proteins other than insulin after injury or a long chase (5,6) (**Chapter 3**), suggests that β-cells do demonstrate some plasticity *in vivo*. However, the minority cell presence or activity *in vivo* during diabetes progression or treatment suggests that there are strong mechanisms within the body to control growth, in addition to known mechanisms within β-cells themselves which limit proliferation, such as accumulation of $p16^{INK4a}$. Indeed, if the $Ins^{+}GLUT2^{-}$ cells are to have any meaningful role in human diabetes mitigation, they likely require manipulation *in vitro* before they can be re-introduced into a mammalian system.

Identification of these progenitor cells is therefore paramount, and the roadmap of what might define a β-specific progenitor cell is extensive. Putative β-cell progenitors have been proposed to express higher levels of early endocrine transcription factors such as *Pdx1*, *Ngn3* and *MafB* (5,30) and low/absent expression levels of genes indicative of mature gene function, including *Nkx6.1* and *Glut2* (6,31). Some β-cells have been shown to be capable of transitioning into mature β-cells during culture, distinct from those that maintained a progenitor phenotype regardless of *in vitro* conditions. As described by Szabat *et al*, "together, these findings suggest multiple levels of complexity underlying functional maturation in adult β-cells, and that maturation state in part underlies functional heterogeneity" (31).

Other markers have been proposed to represent maturity markers for β-cells, including Flattop (*Fltp*) (32), a planar cell polarity gene important for the ordered arrangement of cells in a plane of tissue. Fltp was recently shown to maintain the rosette-

like structure of mature β-cells around a blood vessel, and increased in expression up to 80% during postnatal β-cell growth. Furthermore, during pregnancy, proliferation increased in $Fltp^-$ β-cell progenitors more than 6-fold (33). Fltp expression was also shown to correlate to other markers of maturation, including Ucn3, Glut2, and transcription factors including Nkx6.1 and MafA (33).

Pax4 is a transcription factor with a well-defined role in β-cell maturation (34,35). Recent work has shown Pax4 to be present in a subpopulation of β-cells which are capable of expansion during pregnancy (36). Furthermore, evidence from the Melton laboratory has shown that certain transcription factors usually present in other [non-pancreatic] tissues representing the transition from fetal to adult programs were expressed in altered proportion during human stem cell differentiation to insulin-expressing β-cells, such as NFIB, PROX1, HHEX, and KLF9 (37).

Overall, these data suggests that β-cell progenitors may be identified by the combined presence or absence of multiple factors, as depicted in detail in F**igure 6.1**. We propose that Ins^+Glut2^- cells themselves exist within a spectrum of immaturity, and represent a reservoir of cells present during developmental, ranging from rare early β-cell progenitors (capable of multi-potential cell fate) to glucose non-responsive β-cells. Indeed, further sub-categorization of Ins^+Glut2^- cells will likely be necessary in the future. Moreover, phenotype identification of these progenitor cells preceding insulin expression would potentially yield a larger cell pool for manipulation.

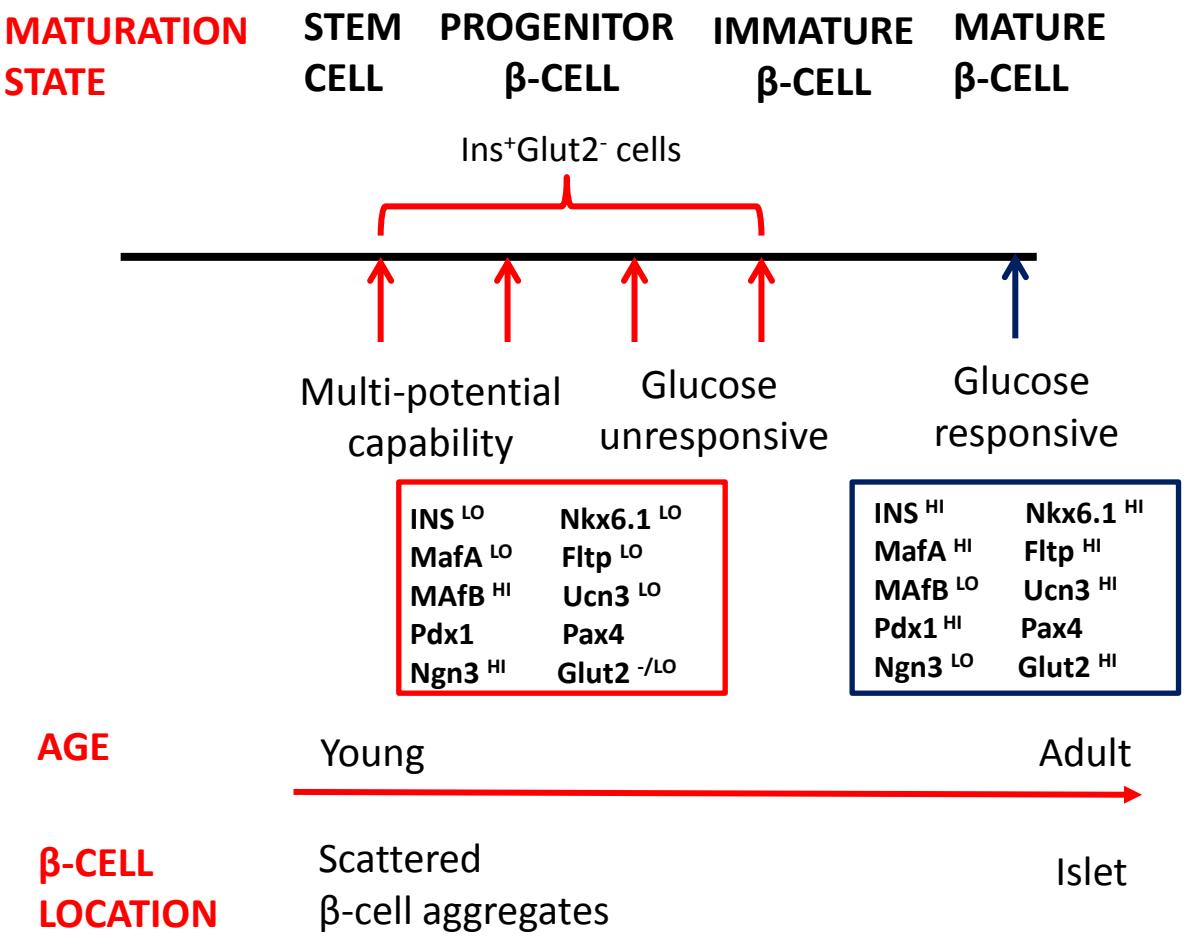

Figure 6.1. **Proposed model of Ins^{+}Glut2^{-} progenitor β-cells**.
β-cells exist within a spectrum of maturity, especially during early postnatal development. A subpopulation of β-cells can be identified by the lack of Glut2, and which have been identified by others as having low expression of Urocortin 3 (Ucn3), insulin, Flattop (Fltp), MafA, Nkx6.1, and increased expression of Pdx1 and Ngn3. Within this spectrum of maturity exist rare multi-potential cells; however, the majority represent immature β-cells which are not glucose responsive. Most Ins^{+}Glut2^{-} β-cells may be found outside of the islet, scattered throughout the pancreas in small clusters of cells. As the endocrine pancreas matures and ages, fewer Ins^{+}Glut2^{-} cells found and which coincides with a decrease in the proportion of small clusters, as more β- cells reside within islets. This also coincides with a decrease in endocrine regeneration potential.

6.4 Limitations and Uncertainties

6.4.1 Lineage tracing: a necessary evil?

Lineage tracing studies are qualified by the design of the genetic system, and involve multiple decisions including promoter selection and transgene inducibility, and are effected by pharmacological intervention. The implications of introducing another drug into a physiological system, however commonly used, should not be underestimated. Tamoxifen is widely used to cause transgene induction in a dose-dependent manner as described in **Chapter 5**. However, the primary application of this drug is for the prevention of estrogen-receptor- expressing breast cancer recurrence. In humans the standard dose is 20-40 mg/day (38), whereas the dosing regimen in transgenic mice is 3-5 doses of 0.1 mg/g body weight (i.e, ~3 mg/dose), and in some instances is increased to 4-8 mg/dose (7,39). In mice, this translates into drug exposure of several-fold higher dose in a much shorter timeframe than would be given to humans, and which has been shown to be poorly tolerated. While it was initially established, and widely assumed, that tamoxifen was only active in tissues for ~48 hours after administration (30), it was recently shown that the drug may persist, and hence continue to label cells, for weeks *in vivo*, particularly when used at high doses, and which may confound the interpretation of a pulse-chase experiment (39). While administration of tamoxifen via oral gavage instead of injection may mediate some of these discrepancies in absorption, physiological consequences of the drug may influence health outcomes independent of the disease model, such as the effect of tamoxifen on fertility (39,40). Due to a limited time frame between tamoxifen dosing and STZ exposure in our model, it cannot be conclusively determined that the results yielded, showing a doubling of

lineage-label over the first 4 weeks of life, were due to selective tagging of a highly proliferative subpopulation, or whether the tamoxifen continued to label β-cells in the normal developmental program for longer than was expected. Nonetheless, as this trend was not experimentally demonstrated after STZ exposure, this result points to the differential utilization of β-cells during regeneration, and implies the existence and involvement of a progenitor population (**Chapter 5**).

A similar universal challenge of lineage tracing experiments is that only a subset of cells are tagged, and after such careful design, we are left to rely on overall trends alone. As was shown in our inducible animal model in **Chapter 5**, ~30% of β-cells in islets were tagged with HPAP after tamoxifen administration, and fewer in BCA. Considering that only ~1% of β-cells in islets lack Glut2 at P7, and which decreases with age, our assessment of lineage-tagged $Ins^{+}Glut2^{-}$ cell proportion at the initiation of the experiment was <0.3% in islets, and <3% in BCA, making definitive conclusions challenging. Many groups have shown positive reporter presence in a small population of cells than was expected after lineage-tracing, but which was consequently dismissed as an artifact of Cre transgene "leakiness" (41,42), negating the impact of details which can drastically alter interpretation. The assessment of a rare population of cells using a system that is even minimally imperfect creates an opportunity for criticism; however, it also highlights that our reliance on lineage tracing technology has both reformed, and perhaps paradoxically restricted our knowledge of β-cell biology. The mounting, and seemingly contradictory results provided by multiple groups trying to answer the same question does lead one to ponder if there is in fact more than one correct answer. The bold conclusion by the Melton laboratory that β-cells are formed

exclusively through self-duplication and not stem-cell differentiation may be too restrictive in the face of years of research showing alternate phenomena. As was testified by Bonner-Weir and Sharma in reference to this controversy, there exists "... the difficulty of definitively proving that something does not happen" (43).

In a similar vein, limitations in lineage tracing technology restricted us from following $Ins^{+}Glut2^{-}$ cells → $Ins^{+}Glut2^{+}$ cells directly as there is no specific marker available for the absence of the glucose transporter. We also do not have the technology to differentially label β-cells present within BCA versus islets, which could be used to determine their hypothesized maturation and coalescence. It would be useful if multiple transgenic lines could be combined to label both insulin and Glut2 with separate reporters, however as shown in **Chapter 5** and above, pharmacological effects and a short window for transgene induction in young mice would make this system difficult to appropriately study.

As discussed in **Chapter 3**, the use of the cytoplasmic protein HPAP as a reporter in our system required us to employ an alternate cell marker for use in fluorescent cytometric sorting procedures. It was previously demonstrated by the Grompe laboratory that membrane-bound, non-endocrine, non-hormonal proteins efficiently labelled islet and acinar cell subpopulations, such as Glycoprotein M6a as a proxy marker for β-cells (44). Within the pancreas, it can be confidently ascertained that $Gpm6a^{+}$ cells further defined by positive expression of Glut2 appropriately selects for the mature β-cell. However, using a neural marker and the absence of Glut2 as the selection criteria for a progenitor β-cell population does allow for some degree of uncertainty. Despite insulin immunostaining of recovered, FAC-sorted fractions and *ex vivo* tissue sections using the Gpm6a antibody, there

exists the theoretical possibility that resident neurons could be collected along with β-cells. Furthermore, while the culture of $Ins^{+}Glut2^{-}$ cells in neural-differentiating conditions generated neurospheres, and which expressed HPAP, it is theoretically possible that neurons use insulin as a neurotransmitter (45), and could therefore also retain the reporter protein, suggesting that this result is not wholly conclusive, however statistically unlikely this is. Moreover, this has been further complicated by recent evidence from the van der Kooy laboratory which showed that there are in fact two populations of progenitor cells within the pancreas: the majority derived from the Pdx1 lineage and which produce the insulin-expressing cells as described, and a rarer population which is derived from Wnt1 neural crest lineage (46).

6.4.2 Animal models as proxy for human disease

Besides the limitations of interpreting data obtained through lineage tracing, we must be cautious in extrapolating data provided by studies on rodents directly to the human context. Knowledge about human β-cell cell biology and proliferation has been obtained largely through static, post-mortem examination, and which has recently been questioned as artificially low due to warm ischemia time which influences antibody immunostaining (47). Relatively few studies have been able to lineage trace human β-cells *ex vivo* (48,49), and none *in vivo*, due to obvious ethical reasons. Data from **Chapter 4** showed a surprisingly high degree of correlation between human and mouse in the progression of $Ins^{+}GLUT2^{-}$ cells during endocrine pancreas development. However, the numerous species differences known, with differential utilization of primary glucose transporter subtype, islet macro-

organization with regards to cell-cell contact, vascular arrangement, basement membrane, and innervation patterns (50–56), timing of endocrine pancreas maturation, widely variable genetics, and lifestyle factors among others, render paralleling these situations directly as unsuitable. Furthermore, animal models of diabetes do not directly educate diabetes disease progression in humans. While it is commonplace to effect hyperglycemia by pharmacological methods, and much can, and has been learned by the damage and regeneration models used in mice, STZ-mediated hyperglycemia is not a direct substitute for the complex physiological changes occurring in patients with type 1 diabetes, such as continued β-cell damage and lack of C-peptide exposure in the human context. Indeed, promising results from animal studies have generally failed in human clinical trial (especially with regards to autoimmune mitigation), forcing frank reassessment on the appropriateness of basing diabetes therapy on data gleaned from a 30g rodent. This is, however, nominally reconciled with the advent of studies involving humanized mice (57,58), as well as a strong push within the islet biology community to include experiments with human tissue to ensure that appropriate conclusions are being drawn. It is therefore critically important, and reassuring, that the results by Smukler *et al* (5) and by us in **Chapter 4** did indeed illustrate that this population of progenitor cells is present and active within the human pancreas, in addition to its presence in mice.

6.5 Future Studies

To complete the studies in **Chapter 3**, quantitative PCR experiments are currently underway to examine the genetic differences between subsets of P7 RIPCre;Z/AP$^{+/+}$β-cells,

flow cytometrically-sorted by the expression of Glut2. Similar to data by Smukler *et al* (5), we will assess the relative proportion of β-cell-specific genes (*Ins*, *Glut2*, *Ngn3*, *Pdx1*, *Nkx6.1*).

The model which we have established to stratify β-cells according to function can be applied to study multiple related endocrine pathologies. We have defined a time-course for these $Ins^{+}GLUT2^{-}$ cells, a primary location, and that the actions of STZ are (indirectly) influenced by their presence, at least in mice. To ascertain conclusively if there are differences in β-cells based on location, experiments will be undertaken to separate these cells. Islet isolation will be performed as previously described; however, the ~98% of pancreas-cell content remaining will then be retained and examined. These two compartments of β-cells – intra- islet vs extra-islet – would be independently separated into single cell suspension and prepared for flow cytometric sorting for insulin, or its appropriate cell surface proxy marker, such as GPm6a, as shown in **Chapter 3** (44), or by transgenic β-cell lineage marker, such as green fluorescent protein present in MIP-GFP mice (5). And, as shown by us in **Chapter 4**, these experiments should be undertaken using young (<21 days) and old (>6 months) mice to characterize the multi-potential population, and if it concurrently changes with age.

The obvious next step is to interrogate the human pancreas, islets and BCA alike, to assess whether the $Ins^{+}GLUT2^{-}$ cells also demonstrate a multi-potential lineage capacity, specifically over different ages. Furthermore, it must be determined whether the added labour and expense of collecting extra-islet β-cells in addition to intact islets yields improvements after transplant. With the introduction of new cell-surface markers for

human β-cells allowing for their isolation by flow cytometry (59), this capability should soon be within reach.

The multitude of physiological situations requiring enhanced metabolic substrates, and the situations in which they fail (i.e., diabetes subtypes) could all be examined for the role of $Ins^{+}GLUT2^{-}$ cells during their progression. As we have successfully identified these cells in sections of human pancreas, tissue samples from newly diagnosed versus established type 1 diabetes pancreata could be examined, including those around adolescence, as speculated in **Chapter 4**.

Investigation of the role of $Ins^{+}Glut2^{-}$ cells during pregnancy is currently underway, using RIPCreER;Z/AP mice. β-cells are tagged prior to pregnancy with HPAP, and proliferation assessed by EdU and Ki67. Pancreata are collected at key points prior to parturition, with the intent of determining if compensatory β-cell mass increases involve these putative progenitor cells. We also anticipate a correlation of $Ins^{+}Glut2^{-}$ cells with $Ucn3^{-}$ (18,19), $Fltp^{-}$ (33) and $Pax4^{+}$ (36) cells. Examination of human pancreata during pregnancy and postpartum will also be ideally performed, although the availability of these samples is limited.

Our study illustrating the progressive decline in $Ins^{+}GLUT2^{-}$ cells with advancing age was accomplished using lean human individuals alone (**Chapter 4**); examining this population in overweight humans, with and without diabetes present could also identify (pre)clinical parameters for study. Both of these conditions can be mirrored using appropriate animal models, with the use of non-obese diabetic (NOD) mice to track the role of immune attack in type 1 diabetes (60), the role of insulin resistance without obesity

using β-cell-specific insulin-receptor knock-out mice (IRKO) (61), and the use of C57Bl6 (62) or liver specific insulin-receptor knockout (LIRKO) mice (63) fed a high fat diet to induce glucose intolerance and type 2 diabetes with obesity present.

6.6 Conclusions

The work presented here has contributed to the body of knowledge regarding pancreatic β-cells in three primary ways: first, that a subset of β-cells demonstrates lineage fate plasticity, which can be stratified into categories of maturity and function based on the expression of Glut2 in both human and mouse. This has allowed the identification and retrieval of these cells relatively simply, while integrating succinctly into the current paradigm of β-cell biology. Second, that this β-cell subtype is temporally defined, with fewer $Ins^{+}Glut2^{-}$ cells present in mouse and human pancreas with advancing age, which identifies a window for clinical intervention. And third, that these progenitor β-cells are predominantly found outside of mature islets, which provides an anatomical location wherein research efforts can be focussed, and has been heretofore untapped.

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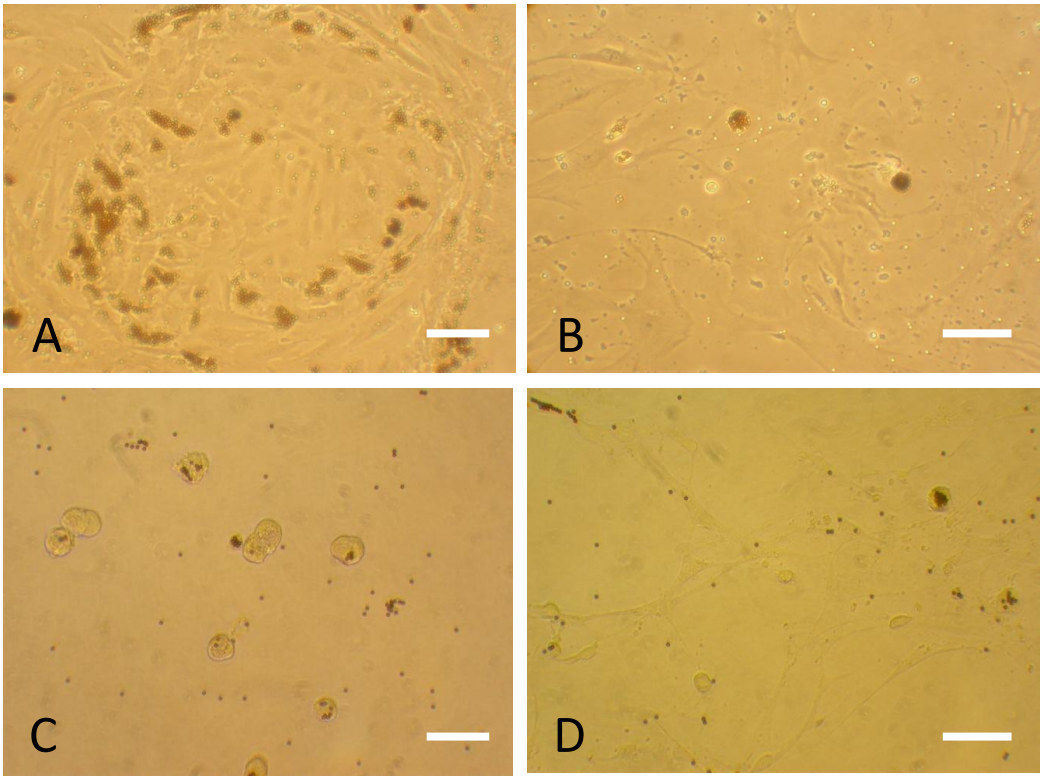

Figure S2.1. Pancreatic endothelial cells. Freshly isolated mouse endothelial cells (ECs) grown on fibronectin-coated dishes with EBM-2 media (A), and which could be passaged up to 3 times (B). Alternatively, ECs were harvested and mixed in Matrigel (C). After 7 days, ECs had formed tubular structures (D). Scale bars represent 50 µm, *n* = 4.

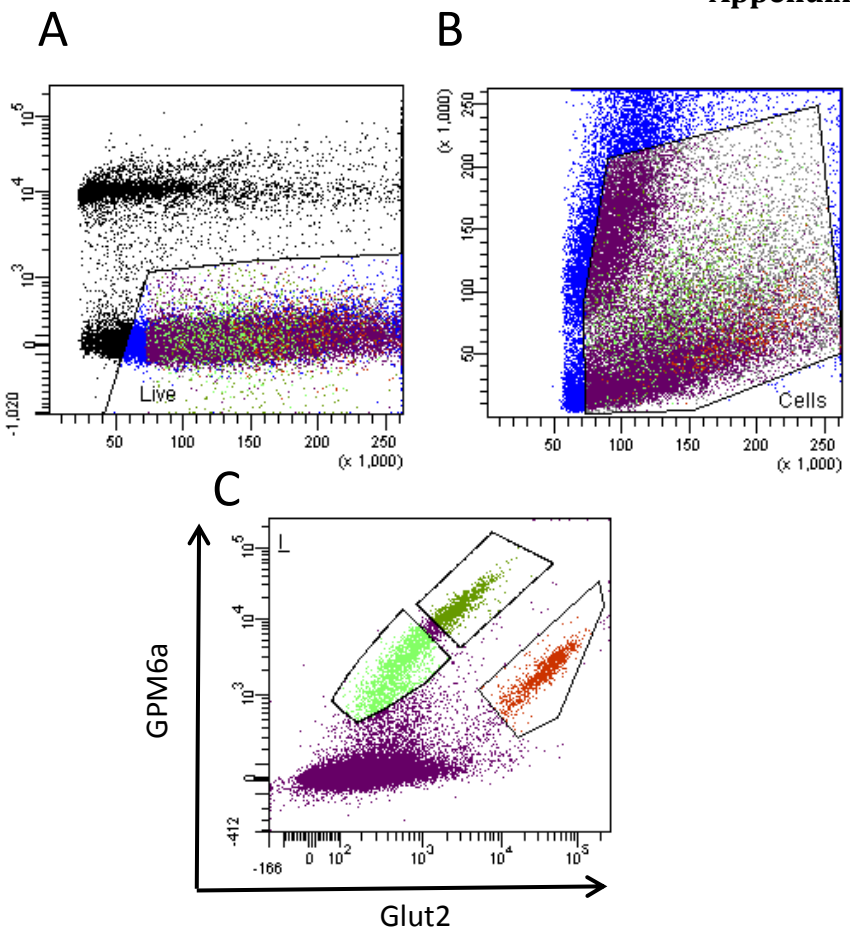

Figure S3.1. Strategy for gating neonatal pancreas cells used for flow cytometric cell sorting by GPM6a and Glut2. Dissociated pancreatic cells were selected by the 'Live' gate based on 7-AAD exclusion (A). Forward scatter (FSC) and side scatter (SSC) voltages were adjusted to clearly visualize cells as indicated by the 'Cells' gate (B). Doublets were excluded based on consecutive gates on FSC-Height vs FSC-Width and SSC-Height *vs* SSC-Width plots (not shown). Viable GPM6A (insulin)-expressing cells (B, C) were selected based on differential Glut2 expression (C, red and green) by comparison to cells lacking GPM6a expression (C, purple). Gates were set based on fluorescence minus one controls and cluster gating. The FACSAria III cell sorter is equipped with a 30 mW Coherent solid state 405 nm violet laser, a 20 mW Coherent Sapphire solid state 488 nm blue laser, a 50 mW Coherent Compass solid-state diode-pumped 561 nm yellow-green laser, and a 17 mW JDS Uniphase Helium Neon 633 nm red laser. The blue laser trigon was configured to detect AlexaFluor 488 from detector B (530/30 bandpass and 502 longpass filter). The yellow-green laser octagon was configured to detect AlexaFluor 594 from detector D (610/20 bandpass and 600 longpass filter), and 7-AAD from detector C (670/14 bandpass and 630 longpass filter). Cells were sorted at 4°C using a 100 µm nozzle at 20 psi and at a maximum of 6,000 events/second.

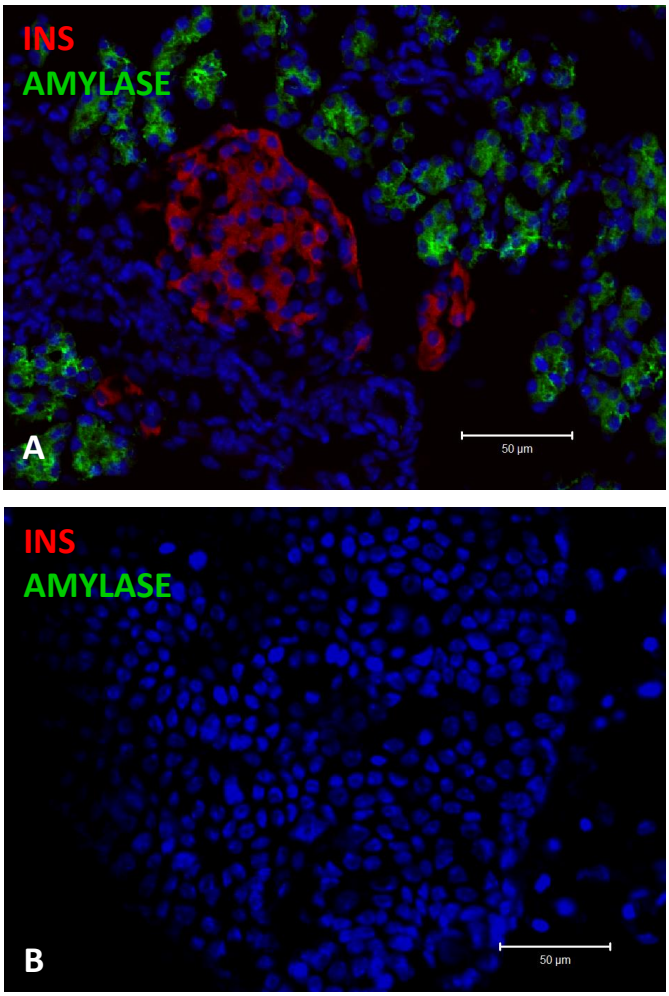

Figure S3.2. Neither insulin nor amylase are expressed after dedifferentiation of neonatal mouse islets to ductal epithelium. Pancreatic beta cells within P7 islets immunostained for insulin (A, red), and were negative for amylase (A, green), which immunostained the acinar compartment. After isolation and culture of islets for 7 d towards a ductal epithelial phenotype, insulin expression was lost (B, red), as was amylase expression (B, green). Scale bar represents 50 µm, *n* = 3.

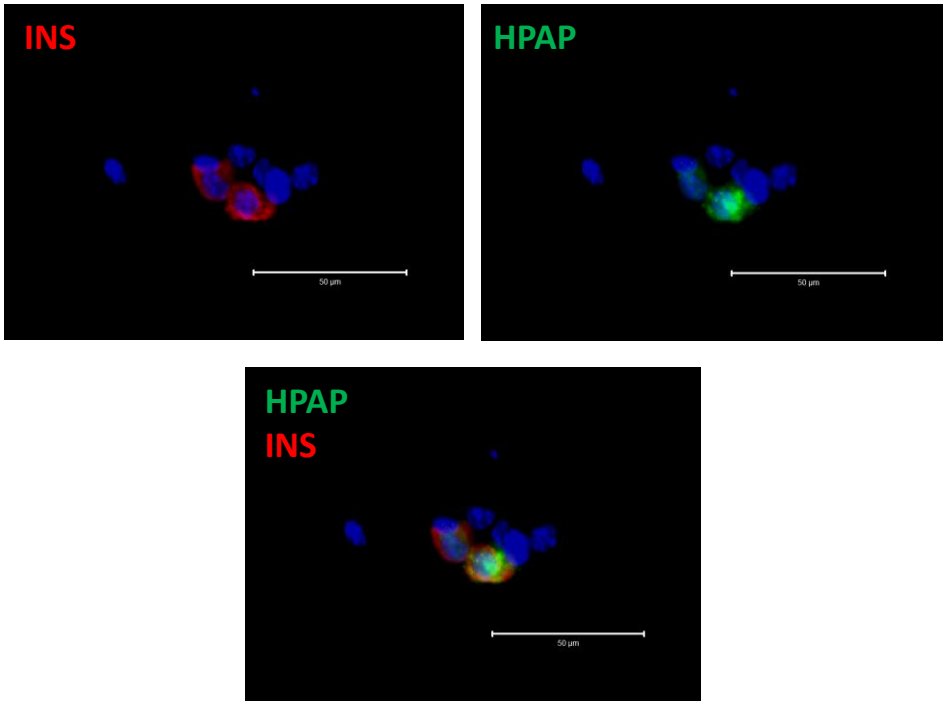

Figure S3.3. HPAP faithfully tracks the β-cell during dedifferentiation *in vitro*.
RIPCre;Z/AP$^{+/+}$ mouse islet stained for insulin (red) and HPAP (green) after culturing for 3 days in dedifferentiation conditions. Note that insulin immunostains only 3/8 cells present, with HPAP immunostaining 2/3 β-cells. Scale bar denotes 50 µm, *n* = 3.

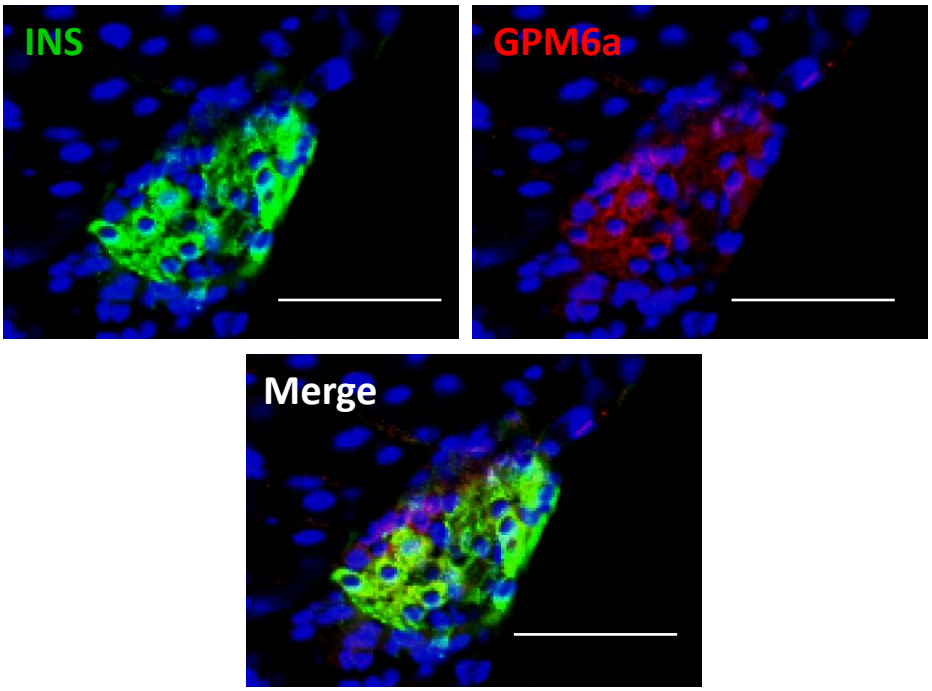

Figure S3.4. Insulin-expressing β-cells immunostained by GPM6a.
Beta cells were immunostained for insulin (green) and the cell surface marker GPM6a (red) within an islet. Scale bar denotes 50 µm, *n* = 3.

Supplemental Methods: Genotyping

At maturity, one RIPCre$^{+/+}$ and one LacZ/HPAP$^{+/-}$ mouse of the opposite sex were caged together for mating. Genotyping of resultant P5 mouse pups was accomplished by ear/tail snips. The DNA extraction buffer consisted of 5 µL 0.5 M NaCl, 2.5 µL each 1 M Tris pH 8.0 and 20 mg/ml proteinase K (Roche Ltd, Mississauga ON), 0.1 µL 0.5 M EDTA, 0.5 µL 10% SDS, and 39.2 µL DNase-free , RNase-free H_20, to a final volume of 50 µL per tissue sample. Samples were heated at 56°C for >3 h, vortexed, then centrifuged at 600 x G for 15 min at 4°C. Samples were stored at -20°C, and 0.5 µL of DNA within the supernatant was used in downstream PCR reactions.

The PCR reaction master mix contained 5 µL each 10x buffer and $MgSO_4$ (UBI Life Sciences Inc, Saskatoon, SK), 2 µL 10 mM dNTPs (Invitrogen, Carlsbad, CA), 1 µL each 20 mM forward and reverse primers (Sigma-Aldrich, St Louis MO), and 1 U (0.2 µL) Taq (UBI), to a final volume of 50 µL in DNase-free, RNase-free water. The following primer sequences for the transgenes of interest were used: RIPCre (FW – *gcggtctggcagtaaaaactatc* and RV – *gtgaaacagcattgctgtcactt*, 100 bp), Z/HPAP (FW- *ccgcttcccatatgtggctctgtcc;* RV- *gcatgagctcagtgcggttccacac*, 548bp), and the internal control (FW- *caaatgttgcttgtctggtg*; RV- *gtcagtcgagtgcacagttt*, 200 bp). All primer sequences were available from Jackson Laboratories. PCR reactions were performed using an initial denaturation at 94 C for 3 min, followed by 35 cycles of denaturation (30 sec at 94°C), primer annealing (1 min at 59°C), and transcript extension (1 min at 72°C). cDNA was run on a 2% agarose gel as described in **Chapter 2**.

Supplemental Material

S5.1. Identification of LacZ-expressing tissues

To confirm the presence of β-galactosidase, sections from LacZ/AP mouse pancreas were subjected to the X-gal reaction (1) containing X-gal reagent (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) (Sigma Chemical, St Louis, MO), potassium ferricyanide, and potassium ferrocyanide. Cells expressing β-galactosidase demonstrated an indigo-blue precipitate in RIPCreER;Z/AP$^{+/+}$ mouse pancreas sections without tamoxifen administration within the islet (**Figure S5.1A,** red circle). Conversely, RIPCreER;Z/AP$^{+/+}$ mouse pancreas sections stained for X-gal after tamoxifen induction did not contain the blue precipitate (**Figure S5.1B**), and neither did sections from RIPCreER;Z/AP$^{-/-}$ mouse pancreas (**Figure S5.1C**).

S5.2 Identification of HPAP-expressing tissues using NBT/BCIP

HPAP^{+} cells were identified using multiple methods: (A) the alkaline phosphatase substrate chromogen Nitro-Blue Tetrazolium Chloride/ 5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt (NBT/BCIP, Sigma) which produces a purple-blue precipitate in alkaline phosphatase-expressing cells; (B) a fluorescent substrate using the Vector Red Alkaline Phosphatase Substrate Kit (Vector Labs, Burlingame, CA), which works similarly to NBT/BCIP but by adding a red fluorescent tag; or (C) immunohistochemical staining of HPAP^{+} cells using a Mouse-on-Mouse (MOM) kit (Vector Labs) with a mouse anti-human HPAP antibody (Sigma), or a rabbit anti-human HPAP antibody (Abcam, Cambridge,

UK). Blocking of endogenous pancreatic alkaline phosphatase, but not HPAP, was accomplished by pre-treatment with levamisole (2) (Dako, Glostrup Denmark), or by incubating slides in 70°C PBS for 20 min for the chromogenic assays.

By NBT/BCIP staining, $HPAP^{+}$ cells within islets could be visualized in P7 RIPCreER;$Z/AP^{+/+}$ sections as indicated by the presence of purple-blue precipitate after tamoxifen administration (**Figure S5.2A**); islets from RIPCreER;$Z/AP^{-/-}$ mice did not stain (**Figure S5.2B**), and neither did RIPCreER;$Z/AP^{+/+}$ mouse pancreas sections not induced by tamoxifen (not shown). Pancreas sections not pre-treated with levamisole/heating in PBS to block endogenous alkaline phosphatase displayed substantial background staining, especially within ducts (**Figure S5.2C**).

S5.3 Dose response to tamoxifen in RIPCreER;$Z/AP^{+/+}$ pancreas and specificity of HPAP to the β-cell

RIPCreER;$Z/AP^{+/+}$ pancreas sections from P7 pups were stained for insulin (green) and Vector Red alkaline phosphatase substrate (red) demonstrated no positive areas of staining for AP when tamoxifen (TM) was not administered (**Figure S5.3A**). After induction with TM, the HPAP protein clearly and faithfully tracked insulin in the β-cell, showing a graded increase in AP presence after one (**Figure S5.3B**) and two injections of TM (**Figure S5.3C**). However, there was considerable variability in staining frequency between islets after 2 doses of tamoxifen when stained using either chromogenic method (between 10-90%), likely due to the enzymatic nature of the NBT/BCIP and Vector Red substrate reactions, and not directly to HPAP-expressing cells. More than 2 injections of TM were not

tolerated by the pups (not shown). Moreover, any pup injected with tamoxifen were not viable breeders as adults, indicating a negative effect on fertility. None of the non-insulin^{+} cells at P7 positive for AP (red) co-stained for glucagon (**Figure S5.3D**, green). Pancreas sections not pre-treated with levamisole or heated to 70°C in PBS show endogenous alkaline phosphatase presence, primarily around ducts (**Figure S5.3E**, red).

Due to the heterogeneity of Vector Red staining within islets, we utilized anti-HPAP antibodies for quantification, although the enzyme-based methods were still used to quickly and easily assess transgene induction presence.

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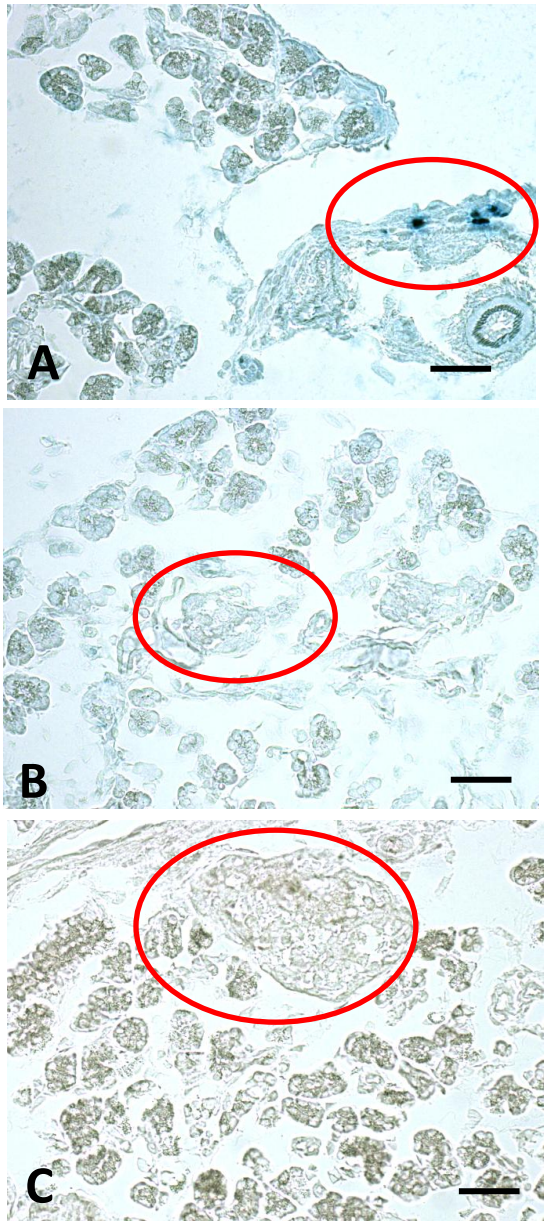

Figure S5.1. X-gal staining for β-galactosidase presence in transgenic (LacZ/HPAP) mouse pancreas.
RIPCreER;Z/AP$^{+/+}$ mouse pancreas without tamoxifen injection (A). Blue precipitate inside the islet (red circle) indicates areas of positive beta-galactosidase enzyme, correlating with lacZ content. RIPCreER;Z/AP$^{+/+}$ mouse pancreas after tamoxifen induction (B) was absent for blue precipitate, as were sections from RIPCreER;Z/AP$^{-/-}$ mouse pancreas (C). Cells were counterstained with Carazzi' s haematoxylin. Scale bars denote 50 µm, $n > 3$.

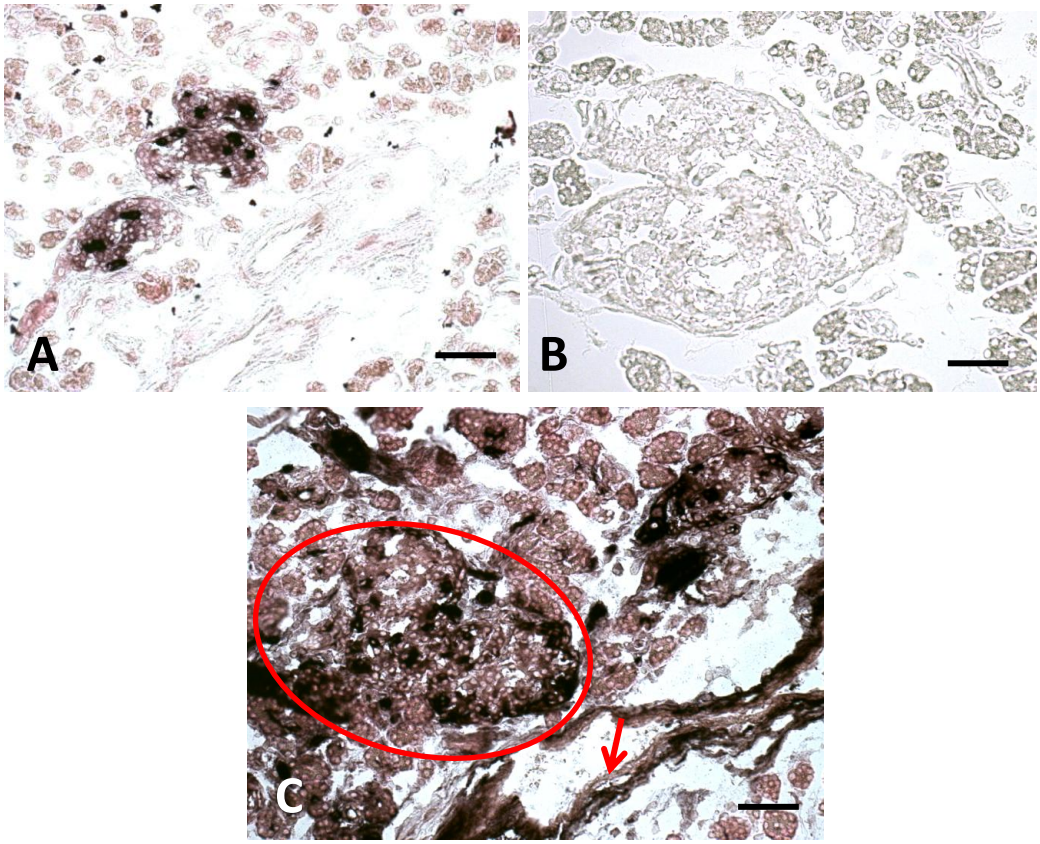

Figure S5.2. NBT/BCIP staining of alkaline phosphatase-expressing pancreatic tissue.
RIPCreER;Z/AP$^{+/+}$ islet stained for AP indicated by purple-blue precipitate (A). RIPCreER;Z/AP$^{-/-}$ pancreas section pretreated with levamisole, indicating a lack of blue precipitate (B). Pancreas section neither pre-treated with levamisole nor 70°C PBS; positive staining for AP is seen within the islet (red circle), however there is substantial background staining for endogenous AP, especially within ducts (C, arrow). Cells were counterstained with Carazzi's haematoxylin. Scale bars denote 50 µm, $n > 3$.

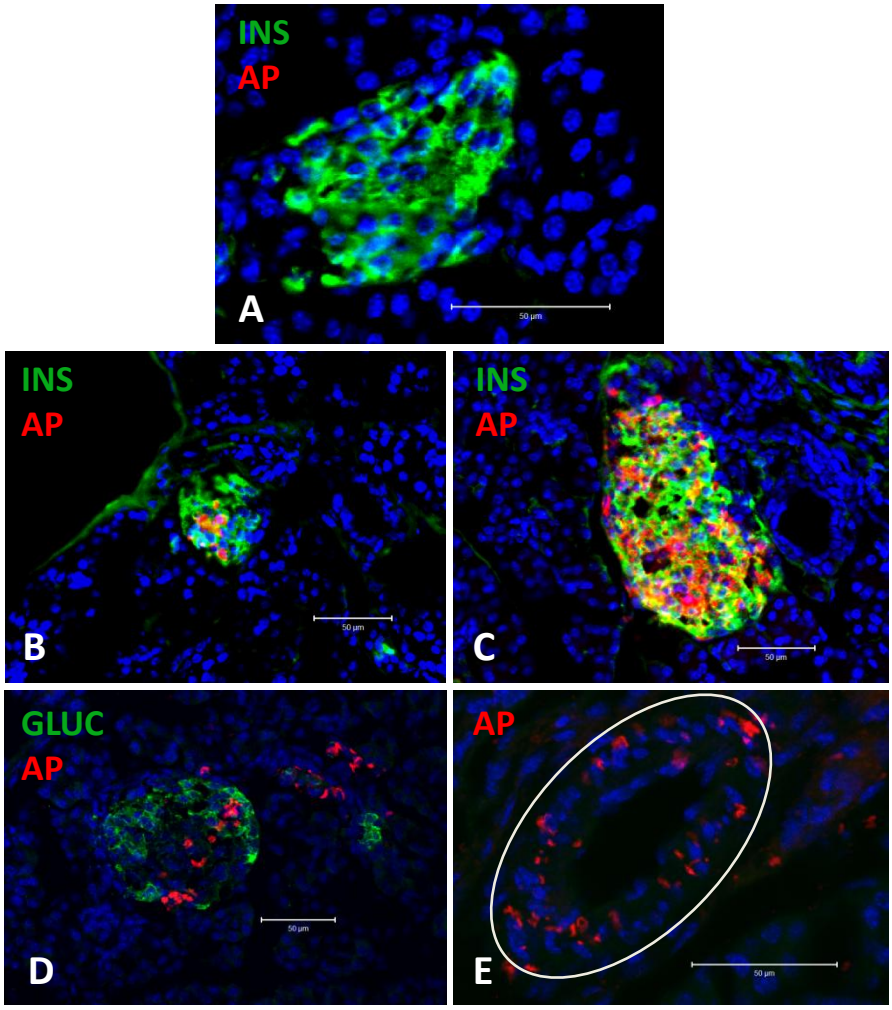

Figure S5.3. **Dose response to tamoxifen in RIPCreER;Z/AP$^{+/+}$ pancreas, and specificity of HPAP to the β-cell.**
P7 RIPCreER;Z/AP$^{+/+}$ pancreas sections stained for insulin (green) and Vector Red alkaline phosphatase substrate (red) shows no staining for AP when tamoxifen was not administered (A), minimal staining for AP after one dose of TM (B), and a moderate proportion of AP staining in insulin-expressing cells (C) after 2 injections of TM. Alpha (α)- cells immunostained for glucagon (green) and AP (red) clearly occupy different cells within the islet (D). Pancreas section not blocked for endogenous AP expression shows AP staining (red) around ducts (E, white circle). Size bar represents 50 µm, $n > 3$.

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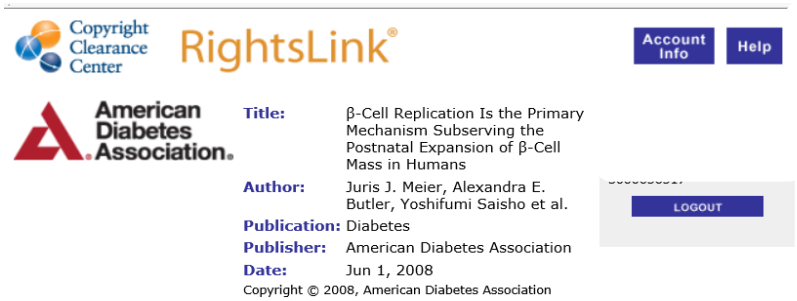

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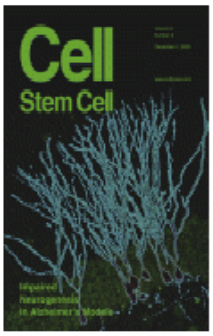

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   Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Kinchlea, Will D
on behalf of the Animal Use Subcommittee

LAWSON
HEALTH RESEARCH INSTITUTE

LAWSON FINAL APPROVAL NOTICE

LAWSON APPROVAL NUMBER: R-12-501

PROJECT TITLE: Contribution of beta-cell progenitor population to pancreatic beta-cell plasticity.

PRINCIPAL INVESTIGATOR: Dr. David Hill

LAWSON APPROVAL DATE: April 10, 2014

Health Sciences REB#: 103167

Please be advised that the above project was reviewed by the Clinical Research Impact Committee and Lawson Administration and the project:

Was Approved

Please provide your Lawson Approval Number (R#) to the appropriate contact(s) in supporting departments (eg. Lab Services, Diagnostic Imaging, etc.) to inform them that your study is starting. The Lawson Approval Number must be provided each time services are requested.

Dr. David Hill
V.P. Research
Lawson Health Research Institute

All future correspondence concerning this study should include the Lawson Approval Number and should be directed to Sherry Paiva, Research Administration Officer, Lawson Approval, Lawson Health Research Institute, 750 Baseline Road, East, Suite 300.

cc: Administration

CURRICULUM VITAE
CHRISTINE A. BEAMISH

EDUCATION

Bachelor of Medical Sciences, Biochemistry (2005)
University of Western Ontario, London, Ontario.

Doctor of Philosophy, Physiology (2014)
University of Western Ontario, Lawson Health Research Institute

AWARDS

Joseph Gilbert Leadership Award (2013)

Children's Health Research Institute's Trainee Travel Award (2013, 2012)

2nd prize Oral Presentation, Diabetes Research Day. London ON (2012)

2nd prize Oral Presentation, Lawson Research Day. London ON (2009, 2012)

Nominated, University of Western Ontario Graduate Teaching Award (2009)

EXPERIENCE

Graduate research thesis (September 2005 - present)
University of Western Ontario, London ON

Research assistant, Mechanisms of preeclampsia (May 2010 – June 2011)
St Joseph Health Care, University of Western Ontario, London ON

Teaching Assistant (2008-11)
Dept of Physiology, University of Western Ontario, London ON

BMSc. Research Thesis (Sept 2004 – April 2005)
University of Western Ontario Children's Health Research Institute, London ON

PUBLICATIONS

Journal Articles

Cellular Mechanisms Underlying Failed Beta Cell Plasticity in Offspring of Protein Restricted Pregnant Mice. AR Cox, CA Beamish, DJ Carter, EJ Arany, DJ Hill. *Exp Biol Med (Maywood).* 2013 Oct; 238(10):1147-59.

Insulin^{+}GLUT2^{-} Cells isolated from Neonatal Mouse Pancreas exhibit Lineage Plasticity and are enriched within Extra-Islet Endocrine Cell Aggregates. CA Beamish, BJ Strutt, Sofia Mehta, EJ Arany, DJ Hill. *Submitted to AJP Endo Metab, 2015.*

Book Chapters

Mechanisms of plasticity of pancreatic β-cell mass. *Trends in Endocrinology and Metabolism, Cell Press.* DJ Hill, CA Beamish, and TG Hill. August 2014.

INVITED TALKS

Tracking cells in the pancreas, from duct to beta cell... and back again? *Endocrinology Grand Rounds*, Schulich School of Medicine, Western University, Canada, March 2011.

Lineage tracing of insulin+ cells during differentiation. CA Beamish, BJ Strutt, EJ Arany, DJ Hill. *"Talks on Fridays"* Seminar Series, London ON, March 18 2011.

CONFERENCE PROCEEDINGS

Oral Presentations

Ontogeny of Ins^{+}GLUT2^{-} β-cell Progenitors in the Human and Mouse Pancreas. CA Beamish, BJ Strutt, S Chakrabarti, M Hara, and DJ Hill. *Lawson Research Day*. London ON, March 18, 2014

Extra-Islet Ins^{+}GLUT2^{-} Progenitor Cells Contribute to β-cell Plasticity in the Neonatal Mouse Pancreas. CA Beamish, BJ Strutt, EJ Arany, DJ Hill. *73rd Scientific Sessions, American Diabetes Association,* Chicago IL, June 23 2013. Selected for a Guided Audio Poster Tour.

Contribution of Ins^{+}GLUT2^{-} progenitor cells to endocrine pancreas regeneration after streptozotocin ablation in the neonatal mouse. CA Beamish, BJ Strutt, and DJ Hill. *European Association for the Study of Diabetes*, Barcelona, Spain, September 26, 2013. (Declined due to conflict).

Rare Transition of Beta Cells to Duct Cells: Beta Cell Dedifferentiation or Activation of Insulin+ Precursors? CA Beamish, BJ Strutt, EJ Arany, DJ Hill. *Keystone Symposia on Islet Biology*. Monterey, CA. March 27, 2012.

Differentiation of Beta Cells to Duct Cells: Beta Cell Dedifferentiation or Activation of Insulin+ Precursors? CA Beamish, BJ Strutt, EJ Arany, DJ Hill. *Lawson Research Day*. London ON, March 22, 2012

Neonatal Mouse Beta Cells can be Differentiated *In Vitro*. CA Beamish, BJ Strutt, EJ Arany, DJ Hill. *Lawson Research Day.* London, ON. March 19 2009

Poster Presentations

Contribution of $Ins^{+}GLUT2^{-}$ Cells to Beta Cell Regeneration after STZ Ablation in the Neonatal Mouse. CA Beamish, BJ Strutt, S Mehta, and DJ Hill. *74th Scientific Sessions, American Diabetes Association*, San Francisco CA, June 15 2014.

Ontogeny of $Ins^{+}GLUT2^{-}$ β-cell Progenitors in the Human and Mouse Pancreas. CA Beamish, BJ Strutt, Sofia Mehta, Y Tong, S Chakrabarti, M Millis, P Witkowski, M Hara, and DJ Hill. *Keystone Symposia on Islet Biology*. Keystone, CO. April 7, 2014.

Lineage Tracing of Beta Cells during Dedifferentiation to Duct Epithelium: Involvement of Insulin+ Progenitor Cells. CA Beamish, BJ Strutt, EJ Arany, DJ Hill. *Annual Meeting for the Endocrine Society.* Houston, TX. June 22-25, 2012.

Rare Transition of Beta Cells to Duct Cells: Beta Cell Dedifferentiation or Activation of Insulin+ Precursors? CA Beamish, BJ Strutt, EJ Arany, DJ Hill.*Keystone Symposia on Islet Biology*. Monterey, CA. March 27, 2012.

Tracking Mouse Beta Cell Differentiation *In Vitro*. CA Beamish, BJ Strutt, EJ Arany, DJ Hill. *Keystone Symposia on Islet Biology*. Whistler, BC. April 12-17, 2010.

Neonatal Mouse Beta Cells Can Be Differentiated *In Vitro*. CA Beamish, BJ Strutt, EJ Arany, DJ Hill.*International Diabetes Federation's 20th World Diabetes Congress*. Montreal, QC. October 19, 2009. Selected for a poster discussion group.

Islet Differentiation *In Vitro*: A Potential Source of Cells for Transplant? CA Beamish, BJ Strutt, EJ Arany, DJ Hill. *Keystone Symposia on Islet Biology*. Snowbird, Utah. April 7-12, 2008.

Imaging of Redifferentiated Islet-like Clusters using a Novel Immunocytochemical Method. CA Beamish, BJ Strutt, EJ Arany, DJ Hill. *67th Scientific Sessions, American Diabetes Association*. Chicago, IL. June 22-26, 2007.

Neonatal Mouse Beta Cell Dedifferentiation *In Vitro*. CA Beamish, BJ Strutt, EJ Arany, DJ Hill. *Annual Meeting of the Endocrine Society*. Toronto, ON. June 2-5, 2007.

Murine Islet Differentiation *In Vitro*. CA Beamish, BJ Strutt, EJ Arany, DJ Hill. *Annual Meeting for the Canadian Diabetes Association*. Toronto, ON. October 21, 2006.