Morphogenetic Plasticity of Adult Human Islets of Langerhans in the Context of the Healthy and Diabetic Pancreas

by

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Abstract

As the limited utility of islet transplantation becomes established, interest turns to other means of restoring β-cell mass in diabetes. Specifically, reports suggest that the endogenous pancreas may be stimulated to regenerate β-cells, thereby restoring normoglycemia. However, the source of this regeneration is debated, with the islet proper proposed as a source, via replication or neogenesis. The mechanism is uncertain, given *in vitro* models of islet plasticity whereby islets can be induced to dedifferentiate, proliferate, and subsequently redifferentiate into functional endocrine tissue. We sought to examine the cells and signals involved in islet plasticity.

Isolation stimulated the expression of TNFα in islets, whereas TNFα inhibition reduced apoptosis and improved β-cell function. Conversely, insulin secreted by islets improved islet survival. Thus, isolation induces the expression of both pro- and anti-apoptotic signals.

Cultured islets produced EGF ligands that induced islet dedifferentiation. This effect was countered by TGFβ expression, which inhibited endocrine dedifferentiation. Thus it appears that within the context of this culture model, the major determinants of the differentiated state of the islet are signals provided by the islet itself.

Cell tracking experiments were performed to identify the cells involved in islet dedifferentiation. Cultures of dedifferentiated islets were comprised primarily of two cell types: a cytokeratin$^+$ epithelial population derived from α-, δ- and PP-cells, and a nestin$^+$ fibroblast-like population derived from β-cells. While the origins of islet redifferentiation were not ascertained, the process appears to require the presence of β-cell-derived nestin$^+$ cells.

Islets from control and type 2 diabetic sources were compared, with islets from diabetic sources overexpressing nestin and vimentin, markers of β-cell dedifferentiation. Moreover, while cultures derived from diabetic donor tissue were equally capable of dedifferentiating, these cultures did not undergo any islet redifferentiation, suggesting a possible defect in β-cell mass dynamics in type 2 diabetes.

These results provide evidence to suggest that islet plasticity may be a normal contributor to β-cell mass dynamics, and that a defect in such a process may be involved in the pathophysiology of type 2 diabetes. Moreover, identification of the cells and signals responsible for the control of islet plasticity may provide a novel target for the development of subsequent therapies.

Résumé

Alors que l'on découvre les limites du succès de la transplantation d'ilots, l'intérêt se porte sur d'autres moyens de restaurer la masse de cellules β chez le diabétique. Plus spécifiquement, certaines études suggèrent que le pancréas endogène peut être stimulé afin de régénérer des cellules β et rétablir une glycémie normale. Toutefois, la source de cette régénération est débattue, les ilots eux-mêmes étant proposés comme en étant la source, via réplication ou néogénese. Si le mécanisme de cette régénération reste incertain, des modèles *in vitro* montrent la capacité des ilots à se dédifférencier, proliférer et se redifférencier à nouveaux en ilots fonctionnels. Nous avons examiné les cellules et signaux impliqués dans la plasticité des ilots.

L'isolation stimule l'expression de TNFα dans les ilots, alors qu'il a été montré que l'inhibition de TNFα réduit l'apoptose et améliore la fonction de la cellule β. Réciproquement, l'insuline secrétée par les ilots améliore la survie des ilots. Ainsi, l'isolation induit l'expression à la fois de signaux pro- et anti-apoptotiques.

Les ilots en culture produisent des ligands EGF qui induisent leur dédifférenciation. Cet effet est contrecarré par l'expression de TGFβ qui lui inhibe la dédifferenciation. Il apparaît donc que dans les contraintes de ce modèle, les déterminants majeurs de l'état différencié des ilots sont des signaux produits par l'ilot lui-même.

Nous avons conduit des expériences de traçage cellulaire pour identifier les cellules impliquées dans la dédifférenciation des ilots. Nous avons montré que les cultures d'ilots dédifférenciés comprenaient principalement deux types cellulaires : une population de cellules épithéliales cytokératine$^+$ dérivée des cellules α, δ et PP, et une population de cellules fibroblastiques nestine$^+$ dérivée des cellules β. Alors que l'origine cellulaire de la redifférenciation en ilot endocrine n'a pas été déterminée, le processus semble nécessiter la présence de cellules nestine$^+$ dérivées de cellules β.

La plasticité des ilots provenant d'individus normaux et de diabétiques de type 2 ont été comparées. Les ilots de diabétiques surexpriment les marqueurs de dédifférenciation nestine et vimentine. De plus, alors que les cultures dérivées

d’ilots de donneurs diabétiques sont capable de se dédifférencier de manière équivalente aux ilots de donneurs contrôles, une fois dédifférenciés leur cultures ne sont pas capables de se redifférencier en ilots, ce qui suggère un défaut potentiel de la dynamique des cellules βchez le diabétique de type 2.

Ces résultats suggèrent que la plasticité des ilots pourrait jouer un rôle important dans le dynamique de la masse des cellules β, et qu’un defaut dans ce processus pourrait être impliqué dans la physiopathologie du diabète de type 2. De plus, l’identification des types cellulaires et signaux impliqués dans la plasticité de l’ilot pourrait permettre de découvrir de nouvelles cibles pour le développement de futures thérapies.

For Deborah

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Abbreviations

ADP	adenosine diphosphate
AKT	v-akt murine thymoma oncogene homologue
ARNT	aryl hydrocarbon receptor nuclear translocator
ATP	adenosine triphosphate
BAX	Bcl-2-associated X protein
bHLH	basic helix-loop-helix
BMI	body mass index
BrdU	5-bromo-2'-deoxyuridine
BSA	body surface area
BTC	betacellulin
cAMP	3',5'-cyclic adenosine monophosphate
cDNA	complementary DNA
CK	cytokeratin
CM	control medium
CMV	cytomegalovirus
CR5	cumate-inducible promoter
cTA	CR5 transactivator
DLS	duct-like structure
DM	differentiation medium
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
DN	dominant-negative
DNA	deoxyribonucleic acid
DPP	dipeptidyl peptidase
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	EGF receptor
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
FDA	fluorescein diacetate
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GCG	glucagon
GFP	green fluorescent protein
GLP	glucagon-like peptide
GLP1R	GLP-1 receptor
GLUT	glucose transporter
GSIS	glucose-stimulated insulin secretion
HbA_{1c}	glycosylated hemoglobin
HBSS	Hank's buffered salt solution
HCM	heat-inactivated conditioned medium
HES	hairy and enhancer of split
HNF	hepatocyte nuclear factor

^{3}H-TdR	^{3}H-thymidine
IAPP	islet amyloid polypeptide
IBMX	3-isobutyl-1-methylxanthine
ICM	islet-conditioned medium
IE	islet equivalent
IFN	interferon
IGF	insulin-like growth factor
IGFR	IGF receptor
IHH	Indian hedgehog
IL	interleukin
ILS	islet-like structure
INGAP	islet neogenesis-associated protein
INS	insulin
IR	insulin receptor
IRS	insulin receptor substrate
JNK	c-jun NH_2-terminal kinase
LIM	Lin11-Isl1-Mec3
LPS	lipopolysaccharide
MAPK	mitogen-activated protein kinase
MODY	maturity-onset diabetes of the young
MOI	multiplicity of infection
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NeuroD/BETA2	neurogenic differentiation / β-cell E-box transactivator-2
NGN	neurogenin
NICD	Notch intracellular domain
NKX	NK-related homeobox
NOD	non-obese diabetic
OPO	organ procurement organization
PARP	poly(ADP-ribose) polymerase
PAX	paired box gene
PBS	phosphate-buffered saline
PC	prohormone convertase
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDK	phosphoinositide-dependent kinase
PDX	pancreatoduodenal homeobox gene
PH	pleckstrin homology
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PP	pancreatic polypeptide
PPAR	peroxisome proliferator-activated receptor
qRT-PCR	quantitative real-time PCR
RDP58	H-*d*-Arg-*d*-Nle-*d*-Nle-*d*-Nle-*d*-Arg-*d*-Nle-*d*-Nle-*d*-Nle-Gly-*d*-Tyr-NH_2
Reg	regenerating gene/protein
RNA	ribonucleic acid

RT-PCR	reverse transcription-PCR
SHH	Sonic hedgehog
SMAD	mothers against decapentaplegic homolog
SST	somatostatin
STZ	streptozotocin
SUR	sulfonylurea receptor
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TCN	triciribine
TGF	transforming growth factor
TGFβR	TGFβ receptor
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase-mediated X-dUTP nick end labelling.
UW	University of Wisconsin

Contributions of Authors

Clinical islet isolations to provide source tissue were carried out by the candidate, along with Despina Agapitos, Béatrice Assouline, Emily Austin, Jason Blaichman, Mauro Castellarin, Sharon Clark, Jamal Daoud, Mila D'Cunha, Jieping Ding, Deborah Driver, Ali Hazrati, Mimi He, Jordanna Kapeluto, Erika Leung, Julia Makhlin, Xinfang Li, Mark Lipsett, Mandana Moosavi, Norina Nordin, Soon-Hyang Park, Maria Petropavlovskaia, Lawrence Rosenberg, Ryan Scott and Sandeep Vanamala.

Mark Lipsett prepared the artwork depicted in Figure 1.5. All experiments were performed by the candidate with the following exceptions. The experiments depicted in Figures 3.2B, 3.3, 3.4 and 3.5 were performed by Shuqing Liu. The experiments depicted in Figures 4.1A&C, 4.3, 4.5 and 4.6 were performed by Reid Aikin. The adenoviral vectors described in Chapter 7 were designed and prepared by Amélie Pilotte. The experiments depicted in Figures 8.2C and 9.2B were performed by Emily Austin. The experiments depicted in Figures 8.2B, 9.1A&B, 9.2C, 9.3 and 9.4 were performed in collaboration with Jordanna Kapeluto and Mandana Moosavi. All other co-authors contributed to manuscript preparation. Lawrence Rosenberg provided guidance and supervision for all work carried out and the preparation of the thesis.

Preface

The aim of this thesis was to elucidate the morphogenetic plasticity potential of adult human islets in the context of the healthy and diabetic pancreas, using an established *in vitro* model of human islet plasticity. This thesis is composed of 9 chapters. The first chapter provides a general introduction to pancreatic and islet development, the *in vitro* model to be used, as well as the current knowledge of pancreatic and islet plasticity and how these phenomena relate to the healthy and diseased pancreas. Chapter 2 examines the efficiency of the clinical islet isolation program at McGill University, and is published in *Transplantation* (85: 950-955, 2008). Chapters 3 and 4 explore the response of the islet to the isolation procedure in the form of induced signals leading to either islet death or survival. Chapter 3 examines the induction of TNFα, leading to post-isolation cell death, and is published in *Transplantation* (82: 813-818, 2006), while chapter 4 elucidates the protective role of autocrine insulin, and is published in *Diabetologia* (49: 2900-2909, 2006). Next, chapters 5 and 6 examine the autocrine response of the islet to the aforementioned *in vitro* model of islet plasticity. Once again, opposing signals are induced, with chapter 5 examining the induction of EGF, leading to islet dedifferentiation. This work has been submitted for publication. Chapter 6 describes the induction of TGFβ as a signal to maintain the endocrine phenotype and function of the islet, and is published in *Molecular Endocrinology* (21: 1467-1477, 2007). Chapter 7 presents an assessment of the cellular origins of the islet plasticity that occurs in this model. This work is published in *Laboratory Investigation* (88: 761-772, 2008). Chapter 8 examines whether the plasticity of human islets is affected by the diabetic environment, and has been submitted for publication. Finally, chapter 9 contains a discussion of the results presented, and attempts to frame these results within the current understanding of islet plasticity.

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1 Review of the Literature

1.1 Pancreatic Anatomy

The pancreas is an organ of the digestive system, found in the retroperitoneal space posterior to the stomach. The tail of the pancreas is found close to the spleen, while the head of the organ is connected directly to the proximal duodenum by way of the ampulla of Vater (Fig. 1.1A).

The adult pancreatic parenchyma consists of three principal cell types: the exocrine, endocrine, and ductal cells (Fig. 1.1B). These cells encompass one organ with two distinct functions; the exocrine pancreas secretes digestive enzymes carried through the ductal network to the gastrointestinal tract, while the endocrine pancreas produces the hormones responsible for glucose homeostasis.

Briefly, the acinar cells secrete digestive enzymes into the ductal network, which produces bicarbonate and other components of pancreatic juice. The pancreatic juice is then released, in a controlled manner, into the duodenum via the ampulla of Vater. Acinar cells secrete enzymes responsible for the digestion of proteins (trypsin, chymotrypsin, carboxypeptidase), carbohydrates (amylase), lipids (lipase) and other nutrients. Most of these enzymes are secreted as inactive zymogens, which are activated by tryptic cleavage. Trypsin is also secreted as a zymogen (trypsinogen) that is cleaved and activated by duodenal enterokinase. In this way, digestive activity is delayed until after these enzymes have reached the duodenum.[1]

Digestive enzyme secretion is under hormonal control, including such gut-derived peptides as secretin, gastrin and cholecystokinin, as well as islet hormones. In this way, enzyme secretion responds to the fed state.[1]

1.2 Islet Anatomy

The endocrine cells of the pancreas are typically grouped into organised clusters known as the islets of Langerhans, although individual cells or small clusters are also observed.

1.2.1 Islet Cell Types

With a diameter ranging from 40 to ≥400μm, the adult human pancreatic islet is a collection of a few to several thousand cells. While 75% of islets are less than 160μm in diameter, these only account for 15% of the total endocrine volume, while islets with diameters of greater than 250μm account for only 15% of islets by number, but 60% by volume.[2] There are four principal endocrine cell types found in the human pancreatic islet: the α-, β-, δ- and PP-cells produce glucagon, insulin, somatostatin and pancreatic polypeptide (PP), respectively. In addition there exist smaller numbers of other cell types, including the ghrelin-secreting ε- and D_1-cells, serotonergic enterochromaffin (EC)- and the polyhormonal small cells, about which less is known.[2-5]

1.2.1.1 β-Cells

β-Cells are the most common cells of the islet. Under normal conditions, these cells secrete insulin in response to the body's needs. Insulin is synthesised and packaged into immature secretory granules, which then mature as proinsulin is cleaved by prohormone convertases (PC), producing insulin, consisting of an A-chain (21 amino acids) and a B-chain (30 amino acids) connected by two sets of disulfide bonds, and C-peptide, a byproduct that is co-secreted with mature insulin. Insulin release is glucose-responsive: in addition to constitutive basal secretion,[6] insulin secretion spikes post-prandially in response to increased circulating glucose levels.

1.2.1.1.1 Insulin Secretion

Briefly, glucose enters the β-cell via the glucose transporter-2 (GLUT2), whereby it is rapidly phosphorylated by glucokinase, producing glucose-6-phosphate. Glucose-6-phosphate is a substrate for the glycolytic reactions that produce two moles of pyruvate and a net conversion of eight moles of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) for every mole of glucose catabolised. As a result, the ATP:ADP ratio within the β-cell shifts in favour of ATP. Under normal

β-cell ATP:ADP ratios, ATP-sensitive K^+ channels are constitutively open, allowing K^+ outflow to set and maintain the resting potential of the β-cell. The glycolytic shift in intracellular ATP levels induces a conformational change at the level of the K^+ channel, inhibiting K^+ conductance across the membrane, and inducing depolarisation of the β-cell, yielding an action potential. This action potential travels along the cellular membrane via induced changes in voltage-gated ion channels. When the action potential reaches L-type voltage-sensitive Ca^{2+} channels, it causes them to open, allowing an influx of Ca^{2+} into the depolarised cell. This rapid influx of Ca^{2+} initiates insulin exocytosis. Increased intracellular Ca^{2+} causes degranulation by acting at the level of the vesicular fusion proteins as well as calmodium, which contain calcium-binding motifs. In this manner, glucose is directly responsible for the secretion of insulin.[7-9] However, insulin secretion can also be potentiated by the administration of insulin secretagogues, including 3-isobutyl-1-methylxanthine (IBMX), KCl and arginine.

Gap junctions, allowing for electrochemical coupling, connect β-cells within an islet and as such β-cells may act as a syncitium.[10-13] Of interest, however, is the presence of intra-islet variability with regards to glucose-stimulated insulin secretion (GSIS); not all β-cells respond equally to a given stimulus.[14-16] This fact suggests the notion of individual β-cell recruitment in response to increased glucose loads, and lends support to the notion of sub-populations of β-cells within an islet.

As indicated above, insulin is also secreted by a constitutive pathway, with release at a constant rate irrespective of local glucose levels.[6]

During the processing of proinsulin into mature insulin, C-peptide is produced in equimolar amounts. While C-peptide had been considered a biologically inert byproduct of insulin biosynthesis, evidence now suggests that this peptide may also have biological activity, possibly inducing arterial muscle and nociceptive neuron repair.[17, 18]

The β-cell also produces islet amyloid polypeptide (IAPP),[19-21] a 37 amino acid peptide whose function is poorly understood. Both increased age and type 2

diabetes mellitus (T2DM) are associated with a buildup of intra-islet IAPP, which is related to defective β-cell secretory function.[22-25] IAPP is found in immature insulin granules in a pro-form, which is much more conducive to plaque formation than the mature molecule that is produced by PC-mediated cleavage. However, pro-IAPP, and the resultant plaques, are common in T2DM, a condition associated with increased release of unprocessed proinsulin,[22, 26] possibly due to impaired PC function.[24] Intra-islet IAPP plaques are β-cell-toxic, and have been proposed as contributors to the pathophysiology of T2DM.[24]

1.2.1.1.2 Insulin Action

Insulin acts at peripheral tissues, primarily muscle, liver and adipose tissue, to induce glucose uptake and storage via glycogen synthesis, as well as fatty acid synthesis and esterification. Conversely, insulin also acts to downregulate catabolic processes including gluconeogenesis, glycogenolysis, proteolysis and lipolysis. In this way, circulating insulin levels act to maintain extracellular glucose levels.

Briefly, the insulin receptor (IR) is a tetrameric complex comprising two extracellular insulin-binding α-subunits and two intracellular tyrosine kinase β-subunits. Insulin binding induces transphosphorylation of the two intracellular subunits, activating the kinase domains. The IR kinase domains then phosphorylate so-called docking molecules, namely the IR substrates (IRS). These docking molecules bind to, and activate, other molecules involved in various signal transduction pathways. In this way, insulin rapidly elicits varied intracellular effects, including increased glucose uptake via increased GLUT4 shuttling to the cell membrane. Meanwhile, other insulin signalling pathways are directing intracellular glucose towards storage pathways, and inhibiting catabolic pathways, typically via changes in target protein phosphorylation, induced by various kinases and phosphatases. Finally, other insulin signalling pathways are affecting gene expression of proteins involved in these processes, eliciting longer-term effects.[27, 28]

One of the principle target organs of insulin activity is the liver. This organ is exposed to significantly higher insulin concentrations than other organs, however, as insulin is secreted into the hepatic portal vein, and first-pass insulin metabolism at the liver leads to reduced systemic insulin concentrations.

1.2.1.2 α-Cells

α-Cells are the second most frequent cell type in the adult islet, and produce a variety of hormones via post-transcriptional modification of preproglucagon. Tissue-specific processing of preproglucagon yields glucagon, glucagon-like peptide-1 (GLP-1) and other C-terminally extended glucagon derivatives in the islet, while enteroendocrine cells produce glicentin, GLP-1 and GLP-2 from the same starting material.[29] α-Cell secretory products co-localise to the same secretory granules, but as the granules mature, the level of glicentin decreases while that of glucagon increases,[30] suggesting glicentin is a precursor to other glucagon-related products.

Glucagon, the 29 amino acid peptide hormone, is released by the α-cell in response to decreased blood glucose[31] and amino acid levels[32] - typically found in the fasting state - as well as nervous stimulation,[33] and acts to increase blood glucose levels by inducing glycogenolysis as well as hepatic gluconeogenesis and other effects.[31, 34, 35] Glucagon also acts to counter glycolysis and other glucose use and storage mechanisms, effectively countering the effects of insulin. Thus, insulin and glucagon, with opposing effects, act to maintain blood glucose levels within a tightly controlled range.

1.2.1.3 δ-Cells

Less frequent than β- or α-cells, the δ-cells produce somatostatin. This multi-functional peptide is an inhibitor of insulin, glucagon and growth hormone secretion, as well as that of other polypeptide hormones. The circular 14 and 28 amino acid sequences function as both hormone and neurotransmitter, acting in an endocrine manner when released into the circulation, and in a paracrine manner when released locally, both by δ-cells and neurons. δ-Cells have a

somewhat dendritic appearance; they exhibit unipolar elongation of their cytoplasm, which extend to other cells as well as capillaries, and which are thought to be the site of somatostatin release.[2]

1.2.1.4 PP-Cells

The least common of the four principal islet cell types, the PP-cell, secretes PP, a 36 amino acid hormone whose function remains poorly understood.[36] Reports indicate that PP induces satiety and reduced food intake,[37] possibly secondary to delayed gastric emptying.[38]

1.2.2 Architecture

It was long held that islet architecture is conserved in mammals in that β-cells are found predominantly in the core of the islet, with a mantle composed of the other endocrine cell types (Fig. 1.1B).[2] Recent evidence, however, suggests that human and non-human primate islets are marked by a more random distribution of cell types throughout the islet, as compared to the β-cell core that is more prevalent in rodent islets.[39]

The relative proportion of endocrine cell types changes during maturation. At birth, the β:α:δ:PP cell ratio is 45:22:32:1 in humans, although with maturation comes an increase in the relative proportion of β-cells and a decrease in the frequency of δ-cells. By adulthood, the final ratio is estimated at 68:20:10:2,[40] although recent studies suggest these frequencies are quite variable in humans.[39] Relative frequencies also vary throughout the pancreas itself; islets in the tail of the organ typically contain more α- and less PP-cells, while islets in the head have decreased α- and β-cell numbers, increased PP-cell numbers and less well-defined borders.[40] Not surprisingly, data suggests islets in the head of the pancreas release less insulin in response to a glucose stimulus. Further heterogeneity comes from the fact that an adult human pancreas contains approximately 10^6 islets with the majority found within the tail of the organ.[41] Finally, larger islets (>250μm), which account for a minority of islets by number

but a majority by mass, are found predominantly in proximity to large ducts and blood vessels.[2, 42]

1.2.3 Vasculature

While comprising less than 2% of the pancreas by volume, islets receive approximately 20% of pancreatic blood flow.[43] Arterioles travel directly to the islet core before opening into a highly fenestrated capillary bed. The capillary network then passes through the islet mantle before joining into post-capillary venules. In larger islets, these venules coalesce within the boundaries of the islet, while in smaller islets the capillaries persist into the acinar tissue, although the proportion of acinar tissue that is vascularised in this fashion is limited.[44]

Rodent studies suggest that the endocrine cells are arranged in such a way as to maximise vascular exposure. Furthermore, β-cells appear to display a polarity with regards to the vasculature in that the secretory granules are directed towards the capillaries so as to maximise systemic release.[2] Within the rodent islet, the orientation of blood flow gives rise to a specific order of vascular exposure – β-to-α-to-δ-cell – which may relate to cell-cell interactions.[45, 46] However, due to differences in architecture between primate and non-primate islets, this orientation may not be preserved in human islets,[39] given that the origin of this ordered exposure may be based primarily on location and not cell type.[670]

Not only do the islet hormones act in an endocrine manner, but these hormones also act in a juxtacrine fashion.[2, 47] For example, somatostatin downregulates both insulin[48] and glucagon release,[49] while insulin diminishes somatostatin[50] and glucagon release.[51] Finally, studies using infused hormones suggest that glucagon induces insulin and somatostatin secretion.[46]

The islet vasculature also plays a role in dictating β-cell maturation and differentiation, with endothelial cells mediating these effects through extracellular matrix (ECM) interactions[671-674] as well as paracrine signals.[675]

1.2.4 Innervation

Islets are richly innervated, receiving afferent innervation from cholinergic, adrenergic and peptidergic neurons.[52] Pre-ganglionic parasympathetic nerves enter the pancreas, along with blood vessels, via the gland's intrinsic ganglia. These nerves, along with the post-ganglionic sympathetic adrenergic fibers from the celiac plexus, then innervate the islets. While parasympathetic cholinergic nerves run to the β-cell core, and vagal stimulation promotes insulin release, adrenergic innervation is found primarily at the mantle, with sympathetic stimulation causing glucagon release and inhibiting insulin release.[36]

1.3 Pancreatic Development

The pancreas derives from the epithelium of the primitive foregut via separate ventral and dorsal protrusions. While these evaginations become evident by gestational week six, pancreatic commitment is established earlier in development. During week seven, the ventral pancreatic bud rotates and fuses with the dorsal bud, resulting in the formation of the fetal pancreas. The ventral bud comes to form the head of the organ, connected to the duodenum by the duct of Wirsung, while the dorsal bud eventually comes to form the pancreatic body and tail, emptying into the duodenum via the duct of Santorini.[36, 53, 54]

While the pancreatic parenchyma derives from endodermal origins, epithelial-mesenchymal interactions are key for proper pancreatic development.[55] It is well established that appropriate mesenchymal signalling is required for pancreatic organogenesis, though the exact mediators are only now beginning to be elucidated.[56-58] Repression of Sonic hedgehog (SHH) and Indian hedgehog (IHH) is required in the portions of the primitive foregut that come to form the pancreas.[59, 60] The proximity of the notochord to the dorsal pancreatic bud prompted the suggestion that notochord-derived factors may be responsible.[53, 61] However, the ventral bud does not contact the notochord but is induced nonetheless. Factors proposed as responsible for repression of SHH and IHH signalling include fibroblast growth factor-2 (FGF2) and activin-β.[62, 63]

Briefly, pancreatic organogenesis begins via the branching morphogenesis of the pancreatic buds. These structures proliferate and branch, giving rise to a primitive ductal network from whence targeted cells undergo specification and differentiation. Terminal ductules are postulated to give rise to acini, which come to form a majority of the organ. Specified cells undergo endocrine commitment, and migrate away from the ductal network, clustering to form the islets of Langerhans. It appears that a definitive ductal phenotype is a default phenotype for cells not otherwise committed.

1.3.1 Growth Factor Regulation

Epithelial-mesenchymal interactions are responsible for specifying pancreatic lineage early in pancreatic organogenesis. Certain mesenchyme-derived growth factors have been identified as key to this process, although little is known about the exact mechanisms involved.

1.3.1.1 FGF

While pancreatic specification and development is dependent on epithelial-mesenchymal interactions,[55] the effect of mesenchyme can be partially mimicked by FGF2, possibly via its repression of SHH.[64] *In vitro* studies demonstrate that FGF2 induces proliferation of the pancreatic epithelium,[65] and FGFR2b signalling induces exocrine differentiation.[66] Given that FGF signalling has been implicated in branching morphogenesis in *Drosophila Melanogaster*,[67] it is not surprising that mice overexpressing dominant-negative (DN)-FGFR2b[68] or having FGF10 mutations[69] have a grossly underdeveloped pancreas. Interestingly, FGF10 appears to be upstream of key signalling pathways involved in maintaining the pancreatic progenitor cell population, namely the Notch signalling pathway.[70] Finally, FGF signalling also appears to be required for maintenance of adult β-cell function via expression of GLUT2 and PC1/3.[71]

1.3.1.2 EGF

Epidermal growth factor (EGF) signalling appears to play a variety of roles in pancreatic growth and differentiation, with these roles being difficult to delineate due to the redundancy of receptors and ligands.[72] Early in pancreatic development, EGF ligands have the effect of inducing the proliferation of the pancreatic epithelium, while limiting endocrine differentiation.[73] After specification of endocrine progenitors, however, EGF ligands contribute to individual cell lineages within the endocrine component, with betacellulin (BTC) stimulating β-cell development, while neuregulin-4 appears crucial to the normal development of δ-cells.[74] EGF receptor $(EGFR)^{-/-}$ mice have disrupted pancreatic growth, but pancreatic function appears to be maintained, suggesting that EGF is more important in expanding the pancreatic epithelium prior to differentiation than in the induction of differentiation.[75]

Persistent EGF signalling may also be important to the maintenance of adult islet phenotype, as pancreas-specific expression of a DN-EGFR results in decreased β-cell proliferation and β-cell function, along with increased β-cell apoptosis.[76]

1.3.1.3 TGFβ / Activin / BMP

The family of transforming growth factor-β (TGFβ) ligands and receptors is a large and varied one, with ligands sometimes having opposite effects within the same system. Activin-β is likely involved in the SHH repression required for pancreatic specification,[62, 77] while also being a regulator of branching morphogenesis[78, 79] and islet development.[80] TGFβ family members also have variable effects on specification of individual pancreatic cell fate; BMP signalling appears to maintain epithelial duct-like cells,[81] while activin and TGFβ may favour the formation of endocrine cell types.[82, 83] In fact, expression of dominant-negative-TGFβ receptor-II (DN-TGFβRII)[84] or follistatin, an endogenous inhibitor of activin signalling,[85] result in increased exocrine differentiation, while TGFβ treatment of pancreatic explants induces a reversal in relative frequencies to favour endocrine cell types over acinar and ductal.[86] Finally, as with other

mesenchyme-derived growth factors, TGFβ signalling is also important to maintenance of adult islet function, as inhibited activin,[80] BMP[87] or TGFβ[88] signalling results in decreased islet function and phenotype. This distinction is noteworthy, as it appears the majority of effects during development are inductive, while signals to adult tissue promote maintenance.

1.4 Islet Development

Adult pancreatic cell types derive from the proliferation, branching and subsequent differentiation of the primitive pancreatic ductal epithelium. Specified endocrine precursors appear during the course of pancreatic development. These precursors proliferate and subsequently give rise to immature endocrine cells, which mature as they migrate into the surrounding mesenchyme, grouping with other mature endocrine cells into islets of Langerhans.

1.4.1 Transcriptional Regulation

Because of the common derivation of pancreatic cell types from a relatively homogeneous epithelial layer, the process of islet differentiation and maturation requires the coordinated expression of a variety of transcription factors, both spatially and temporally (Fig. 1.2). Gene knockout experiments have allowed the determination of the order of expression as well as the relative requirement of each of the following transcription factors. Of note, many of the transcription factors crucial to islet development are also implicated in neuronal development.

1.4.1.1 PDX1

Pancreatoduodenal homeobox gene-1 (PDX1) is a homeobox transcription factor that is the earliest marker of pancreatic specification. Adult pancreatic cell types derive from $PDX1^+/p48^+$ co-expressing precursor cells,[89-91] and targeted or spontaneous mutations of the *pdx1* gene result in pancreatic agenesis,[90, 92-94] although the associated mesenchyme appears unaffected.[90] PDX1 expression is not required for budding from the foregut;[90, 93] this process may be regulated by Hlxb9 or other, unidentified, factors.[95] PDX1 is required, however, for subsequent

steps in pancreatic and islet development, possibly regulating the response to mesenchymal factors.[90, 96] In fact, it appears that a lack of SHH and the presence of PDX1 are prerequisites to pancreatic development.

While ubiquitously expressed early in pancreatic development, PDX1 is later restricted to adult β-cells,[97] and plays a role in promoting and maintaining the β-cell phenotype, as evidenced by direct effects on insulin, glucokinase, GLUT2 and IAPP transcription.[98, 99] While homozygous mutations in *pdx1* result in pancreatic agenesis in humans,[94] a heterozygous *pdx1* mutation is responsible for maturity-onset diabetes of the young-4 (MODY4; Table 1.1).[100]

Common pancreatic progenitors express PDX1,[89, 90] though subsequent endocrine progenitors begin to express a more specific subset of transcription factors, including neurogenin-3 (NGN3), neurogenic differentiation / β-cell E-box transactivator-2 (NeuroD/BETA2), paired box gene-4 (PAX4), NK-related homeobox-2.2 (NKX2.2) and NKX6.1.[56, 57] Conversely, exocrine progenitors continue to express PDX1 and p48,[101] which is largely responsible for exocrine specification.[102] It would appear that the ductal phenotype is the default pathway for cells that remain uncommitted to either an endocrine or an exocrine phenotype.[56, 57]

1.4.1.2 ISL1

ISL1 is a Lin11-Isl1-Mec3 (LIM) homeodomain transcription factor expressed in the dorsal mesenchyme early in pancreatic development.[103] Targeted deletion of the *isl1* gene results in a lack of dorsal pancreatic mesenchyme and impaired differentiation of islet cells,[103] suggesting an important role in endocrine cell differentiation. ISL1 has also been established as an activator of glucagon,[104, 105] somatostatin[106] and IAPP[107] transcription. ISL1 is expressed in differentiated endocrine cells as these cells appear, and its expression is maintained in all adult endocrine cells of the islet.

1.4.1.3 NGN3

As evidenced by the scattered differentiation of endocrine cells, followed by subsequent migration into the mesenchyme and formation of islets, endocrine cells of the pancreas undergo lateral specification mediated by Notch signalling.[108] This phenomenon was studied in neuronal development prior to its application to endocrine pancreatic development.[109, 110]

The notion of lateral specification implies that the phenotypic specification of one cell will result in its cell surface expression of ligands that bind receptors on adjacent cells, maintaining these neighbouring cells in an undifferentiated state. In this way, during pancreatic development, expression of the basic helix-loop-helix (bHLH) transcription factor NGN3 specifies endocrine fate. NGN3 expression in turn induces the cell surface expression of Notch ligands. These ligands bind Notch receptors on adjacent cells, stimulating γ-secretase-mediated release of the Notch intracellular domain (NICD), in turn activating such transcription factors as RBP-Jκ, which activate expression of the hairy and enhancer of split (HES) family of transcription factors. These Hes proteins then block expression of target genes, including ngn.[111, 112]

Thus, elevated Notch signalling maintains cells in a proliferative progenitor state via repression of *ngn3*, whereas decreased Notch signalling derepresses *ngn3* and allows endocrine differentiation.[111, 112] Developmental NGN3 expression is short-lived,[108] and while expression is observed in the adult pancreas, these $NGN3^+$ cells are putative islet precursors.[113] Initial activation of *ngn3* expression is controlled by several transcription factors, among them hepatocyte nuclear factor-1α (HNF1α), HNF3β and HNF6.[114]

Mice overexpressing either NGN3 or inhibitors of Notch signalling experience pancreatic hypoplasia due to the precocious differentiation of endocrine cells, resulting in a limited pool of pancreatic progenitors.[115-118] Conversely, $NGN3^{-/-}$ mice are characterised by a pancreas that is grossly normal, but with an absolute lack of endocrine cells,[119] indicating the importance of NGN3 in endocrine cell differentiation.

1.4.1.4 NeuroD/BETA2

One of the immediate downstream targets of NGN3 is NeuroD/BETA2,[120] a bHLH transcription factor expressed in developing endocrine cells as these cells appear. NeuroD is also expressed in adult islet cells, and is a transactivator of insulin gene expression,[121] synergistically with PDX1,[122] and may also be a transactivator of the pdx1 gene.[123] NeuroD$^{-/-}$ mice are characterised by a reduction in the number of all four principal endocrine cells, with a failure to form mature islets.[124] In fact, mutations in the neuroD gene define MODY6 (Table 1.1).[125]

1.4.1.5 PAX4

Expression of PAX4, a paired-box homeodomain transcription factor, is found in endocrine progenitor cells during development, followed by restriction to developing β-cells.[126, 127] Examination of the *pax4* promoter region suggests that its expression may be immediately downstream of PDX1 and/or NeuroD.[128] PAX4$^{-/-}$ mice are deficient in β- and δ-cells, with an increased number of α-cells.[126] It appears that PAX4 requires coordinated NKX2.2 expression in order to mediate β-cell specification.[127, 129]

1.4.1.6 NKX2.2

NKX2.2, a member of the NK family of homeobox transcription factors, is expressed late in common pancreatic progenitors, and is maintained in endocrine progenitors, until finally being restricted to adult α-, β- and PP-cells.[56, 57] While NKX2.2$^{-/-}$ mice lack β-cells and have diminished frequencies of α- and PP-cells, NKX2.2 is crucial for insulin expression, if not terminal differentiation of β-cells, as immature cells expressing other β-cell markers, but not insulin, can be found in these animals.[130]

1.4.1.7 NKX6.1

With expression limited to the endocrine progenitor, followed by restriction to the differentiating β-cell, NKX6.1 appears to act downstream of NKX2.2.[131]

Moreover, targeted deletion of *nkx6.1* leads to a phenotype characterised by a shortage of mature β-cells, without affecting other endocrine cell types.[131]

1.4.1.8 PAX6

PAX6 expression is observed in endocrine cells as these cells appear, and is maintained in adult endocrine cells. ISL1$^{-/-}$ mice lack PAX6 expression, suggesting that pax6 is downstream of ISL1.[56] Given that PAX6 binds to an element common to the insulin, glucagon and somatostatin promoters,[132] it is not surprising that targeted deletion of the pax6 gene results in a decreased frequency of endocrine cells, especially α-cells, and reduced hormone expression levels.[133]

1.4.2 Lineage Inter-Relationships

Though all endocrine pancreatic cell lineages derive from a common endocrine progenitor,[57] specification of individual endocrine cell fate occurs fairly quickly, with some cells maintaining a more common ancestry than others. Early evidence suggested the existence of glucagon (GCG)$^{+}$/insulin (INS)$^{+}$ and/or GCG^{+}/PP^{+} precursor cells.[676, 677] Targeted cell ablation studies have since indicated that α-cells do not derive from insulin^{+} progenitors, and vice versa,[134] while NGN3^{+}/PP^{+} cells beget both β- and PP-cells, either directly or indirectly.[113] Moreover, targeted ablation of PP-cells leads to a paucity of β-, δ- and PP-cells, but not α-cells, suggesting either a lineage or paracrine relationship between these cells during development.[134] Thus, it would appear that α-cells develop from a precursor cell that branches before the formation of a common β-/δ-/PP-cell precursor.

While the lineage relationships of pancreatic endocrine cells are not yet fully understood, what is clear is the complexity of the transcriptional control and genetic expression profiles that are required to arrive at an adult islet.[678] It also remains to be seen whether these common lineages can be harnessed for regenerative purposes in pathological settings.

1.5 Pancreatic Pathology

Pancreatic development indicates that all adult pancreatic lineages derive from a common pancreatic precursor, from whence specification and differentiation occur. Pancreatitis and pancreatic cancer are marked by the appearance of a primitive phenotype that is similar to that of pancreatic precursors.

The role of dedifferentiation is not as certain in the pathogenesis of diabetes mellitus.[135, 136] Moreover, the persistence of pancreatic precursors in the adult organ, or lack thereof, is an area of intense debate and research.

1.5.1 Pancreatitis

While the etiologies of acute and chronic pancreatitis differ somewhat, the diseases are commonly characterised by inflammation of the organ, associated with increased circulating levels of pancreatic digestive enzymes, as well as autodigestion associated with ectopic activation of acinar-secreted digestive enzymes.

1.5.1.1 Acute Pancreatitis

Acute pancreatitis is secondary to gallstones in almost half of all cases,[137] with other common causes including alcoholism,[138] hyperlipidemia[139] and hyperthyroidism.[140] This disease is characterised by a sudden onset with patients presenting with elevated circulating and urinary digestive enzyme levels, as well as severe abdominal pain.[141] While acute pancreatitis generally tends to resolve untreated, infection rapidly leads to pancreatic abscesses and/or pseudocysts, with associated increases in morbidity and mortality.[142] Common therapy includes antibiotic prophylaxis, while octreotide, a long-acting somatostatin analogue, is under study for its ability to limit pancreatic enzyme secretion.[143, 144]

1.5.1.2 Chronic Pancreatitis

Chronic pancreatitis typically presents as recurring acute pancreatitic attacks, leading some to suggest that acute pancreatitis can lead to chronic pancreatitis.[138] Alcoholism is associated with almost 80% of cases of chronic

pancreatitis,[145-150] with 5-20% of heavy drinkers suffering from this disease.[151, 152] While chronic pancreatitis is marked by combined exocrine and endocrine insufficiency caused by calcification and fibrosis of the organ, pancreatic insufficiency is not usually observed until almost ten years after onset of the disease.[138, 150, 153] In fact, though chronic pancreatitis is associated with a one-third increase in mortality rate,[154] this increased risk is due mainly to the co-morbidities that are also likely causes of the chronic pancreatitis.[155]

While the exact induction mechanisms are not yet fully elucidated, a picture is beginning to emerge of the steps that precipitate the fibrosis responsible for the symptomology of chronic pancreatitis.[156] Briefly, damage to the organ induces the release of cytokines, namely TGFβ and platelet-derived growth factor (PDGF),[157, 158] that in turn activate fibroblast-like cells resident within the pancreas. These cells, termed pancreatic stellate cells, proliferate and become active in response to the aforementioned cytokines.[159, 160] Activated stellate cells begin to deposit ECM proteins that in turn modify the ultrastrucure of the organ,[161] resulting in pancreatic fibrosis and eventual exocrine and endocrine insufficiency, as the pancreatic parenchyma is replaced by fibrous tissue. Pancreatic duct obstruction is also possible under these circumstances.

Currently, the most recommended curative treatment of chronic pancreatitis is surgical resection, with patients sometimes requiring subsequent digestive enzyme and insulin supplementation.[162]

1.5.2 Pancreatic Cancer

The most common cancer of the pancreas, pancreatic adenocarcinoma has an ever-increasing incidence and prevalence,[163] as well as a five-year survival rate of no more than 5%.[164] With few symptoms, these adenocarcinomas are usually detected late, further worsening a patient's prognosis.

So little is known about the initiators of pancreatic adenocarcinoma that even the cell of origin remains highly debated.[165] While the expression profile and morphology of pancreatic adenocarcinoma suggest a duct cell origin, there exists significant data to suggest that cells of the islet[166-170] or acinus[171-173] could also

serve as the cell of origin. Furthermore, pancreatic development suggests that the ductal phenotype may in fact simply represent a more proto-differentiated, proliferative cell type.[90, 96] Thus, research has focused on the molecular initiators and characteristics of the disease.

To this end, common mutations in tumor suppressors have been identified, including p53, p16/CDKN2A and mothers against decapentaplegic homolog-4 (SMAD4).[174, 175] In fact, SMAD4 was identified based on the high mutation rate in pancreatic cancer, [176] though mutations in various other TGFβ signalling molecules are also common in pancreatic adenocarcinoma.[177, 178] The K-ras oncogene is also commonly mutated in pancreatic cancer,[179] typically very early in the process of carcinogenesis, which suggests a causal effect.[180] Pancreatic cancers also tend to overexpress growth factor receptors.[181, 182] Thus, while the best treatment option available is surgical resection ± chemotherapy, novel adjuvant therapies have focused on the apparent growth factor-dependence of these tumors.[180]

In addition to pancreatic adenocarcinoma, other cancers of the pancreas include insulinomas, glucagonomas and gastrinomas, although these hormone-secreting tumors are much less common.

1.5.3 Diabetes Mellitus

Far and away the most common pancreatic disease is diabetes mellitus. Diabetes afflicts approximately eight percent of Canadians, with a prevalence and incidence that continue to rise.[183, 184] Half of sufferers will develop secondary complications, including cardiovascular disease, nephropathy, neuropathy and retinopathy, that contribute to over $\$10^9$ in yearly health-related costs.[184]

While the etiology and treatments options differ between type 1 diabetes mellitus (T1DM) and T2DM, both are characterised by a relative deficit in functional β-cell mass such that the body's insulin requirements are not met. As a result, blood glucose levels are not tightly controlled, leading to both hypo- and hyperglycemic episodes.

Hypoglycemia is the result of insufficient circulating glucose to meet the body's needs, usually due to excessive doses of insulin or certain oral antihyperglycemics. In healthy individuals, the α-cells respond by secreting glucagon, which stimulates glycogenolysis and gluconeogenesis.[31, 34, 35] However, this response may be attenuated in individuals suffering from diabetes. As a result, organ function is impaired, including that of the brain, which eventually leads to loss of consciousness. While treatable with administration of simple sugars, hypoglycemia can be fatal if untreated.

Hyperglycemia is more common in diabetic patients undergoing treatment, and in fact defines T2DM, [183] although it is also common in T1DM. In hyperglycemia, circulating glucose levels are above normal physiologic levels, although cells may be glucose-starved if circulating insulin levels are insufficient to induce glucose uptake. Diabetes is marked by chronic hyperglycemia, typically measured by the level of glycosylated hemoglobin (HbA_{1c}). It is this persistent hyperglycemia that leads to the secondary complications of diabetes; although the exact mechanisms are not clearly understood, diabetic hyperglycemia is associated with the development of neuropathy, nephropathy and retinopathy, while intensive glycemic control can delay, if not reverse, the progression of these complications.[185, 186]

While the classical symptoms of the onset of diabetes – polyuria, polydipsia and weight loss – are common to T1DM and T2DM, the age of onset differs significantly, with T1DM occurring much younger in life, mainly due to the pathophysiology of the diseases. [184]

1.5.3.1 Type 1 Diabetes Mellitus

While the causal factor(s) remain to be elucidated, T1DM is characterised by the virtual ablation of β-cell mass due to auto-immune attack.[187] Clinical onset of the disease is associated with a residual β-cell mass comprising approximately 20% that of age-matched non-diabetic individuals.[188] It is at this point that the remaining β-cell mass is incapable of maintaining normoglycemia, and persistent hyperglycemia occurs.

Recent case reports of elderly individuals with T1DM indicate chronic β-cell apoptosis,[189, 190] suggesting that attempts at β-cell regeneration may persist throughout the duration of the disease.

Thus, T1DM is marked by significant, persistent β-cell death, irrespective of compensatory β-cell regenerative efforts. As indicated above, however, β-cell formation may be ongoing, but at a rate that is easily eclipsed by the predominant rate of β-cell death. Moreover, given the paucity of β-cells, as well as the short lifespan of any newly forming β-cells, β-cell replication likely plays little, if any role. As such, the current state is such that β-cell mass is almost non-existent in T1DM individuals, and therefore exogenous insulin is required to control circulating glucose levels.

1.5.3.2 Type 2 Diabetes Mellitus

T2DM accounts for more than 90% of diabetes worldwide, and is characterised by insulin resistance in peripheral tissues combined with deficient β-cell GSIS.[191, 192] Pathogenesis of the disease involves both genetic[193] and environmental factors, and is frequently associated with obesity.[194-197]

Until recently, there existed significant controversy as to the role of β-cell mass dynamics in the pathophysiology of T2DM.[188, 198-205] However, a recent report provided strong evidence for a β-cell mass deficit in T2DM. In fact, while insulin resistance in peripheral tissues is certainly an element of the pathophysiology of the disease, the hyperglycemia observed in T2DM may be the result of defective β-cell mass dynamics, given the observed step-wise decline in β-cell mass from normoglycemia to impaired fasting glucose to overt diabetes.[22]

Further confounding the pathology of T2DM is the metabolic state of the patient. Studies have demonstrated that β-cell mass is increased in obese individuals without diabetes,[22, 188, 206] presumably via a compensatory response to insulin resistance.[207, 208] Thus, a β-cell mass regulatory mechanism does exist. However, reports have also indicated that exposure to elevated glucose and free

fatty acid levels are directly toxic to β-cells,[209, 210] leading some to propose a role for glucolipotoxocity in the pathogenesis of T2DM.[211-213]

Recent evidence indicates that T2DM is marked by a relative β-cell mass deficit, regardless of metabolic state.[22] While not necessarily discounting obesity-related hyperglycemia and hyperlipidemia from the pathogenesis of T2DM, this observation does suggest a more general deficit in functional β-cell mass common to T2DM.[136, 214] Furthermore, the mechanism of this β-cell mass deficit appear to be common between lean and obese T2DM; a marked increase in the β-cell apoptotic index.[22] Interestingly, however, compensatory mechanisms seem to respond to the diabetic state; obese T2DM patients have increased rates of β-cell neogenesis, while lean T2DM patients have increased rates of β-cell replication. In both cases, however, the rate of β-cell apoptosis outpaces these regenerative efforts.

Of note, while morphologic studies of β-cell mass provide an absolute measure of insulin-producing tissue, these studies do not indicate the functional capacity of such tissue. In fact, correlations between β-cell mass and glucose tolerance are not direct,[215] suggesting that sufficient insulin-expressing tissue does not necessarily guarantee normoglycemia. Thus, insulin resistance at peripheral tissues,[191, 195, 216] as well as possible β-cell dysfunction,[135, 136, 191] contribute to the pathophysiology underlying T2DM.

1.5.3.3 Treatment Options

Two predominant forms of therapy exist for the treatment of diabetes; those that seek to enhance the efficiency and/or function of the remaining β-cells mass, and those that serve to replace the lacking β-cell mass. Evidently, therapeutics designed to optimise the effect of residual β-cell mass have little or no clinical relevance to patients suffering from T1DM, while mechanisms to replace β-cell mass are the standard of care in T1DM, but are becoming increasingly important in the management of T2DM.[183]

1.5.3.3.1 Oral Anti-Hyperglycemics

As indicated, T2DM is often marked by β-cell dysfunction[135, 136, 191] combined with insulin resistance at peripheral tissues.[191, 195, 216] As a result, therapeutics aimed at improving GSIS or insulin sensitivity may prove effective at improving hyperglycemia (Table 1.2). Only those therapeutics that act to improve insulin resistance may be of relevance to T1DM.

1.5.3.3.1.1 α-Glucosidase Inhibitors

Although not technically insulin secretagogues or insulin sensitisers, α-glucosidase inhibitors previously represented a mainstay of anti-diabetic therapy. These compounds are designed to remain in the lumen of the gastrointestinal tract, where they bind to, and inhibit, the α-glucosidase enzyme that digests complex carbohydrates prior to their absorption. As competitive inhibitors, these compounds do not completely inhibit glucose uptake, but rather dull the spike in glycemia that normally occurs post-prandially. Given that β-cell functional stress will be greatest with the increased insulin requirements of a post-prandial glucose load, limiting the peak of such a load allows partially functioning β-cells to work within functional limits. However, due to unpleasant side effects, namely the flatulence associated with excessive glucose in the gut feeding the local flora, these medications have since seen limited utility.[217]

1.5.3.3.1.2 Sulfonylureas

One of the earliest classes of insulin secretagogues are the sulfonylureas.[218] These medications act by binding to the aptly named sulfonylurea receptor (SUR) on β-cells. This cell membrane protein is a component of the ATP-sensitive K^+ channels that are responsible for transducing the glucose-stimulated electrochemical signal that mediates insulin release.[219] Thus, sulfonylurea binding maintains SUR in a closed conformation, potentiating the effect of a glucose load, and thereby increasing insulin secretion in response to a given glucose challenge. However, sulfonylurea activity is not entirely glucose-

dependent, and as a result hypoglycemic events are a possibility with this class of medication.[220]

1.5.3.3.1.3 Meglitinides

Meglitinides function in a way that is similar to sulfonylureas; that is these drugs act to inhibit K^+ channels at the β-cell, potentiating GSIS.[221] As such, the same risk of hypoglycemia is associated with these medications.

1.5.3.3.1.4 Biguanides

Metformin is by far the most common biguanide, and is in fact the first-line recommended treatment for T2DM.[183] While the exact mechanism of action is not known, it appears that biguanides elicit a primary activity not at the β-cell, but rather at the liver, inhibiting gluconeogenesis.[222] Increased insulin sensitivity is also reported for this category of medications.[223] Due to a mechanism of action that targets insulin sensitivity, as opposed to insulin secretion, hypoglycemia is not reported in patients using biguanides.

1.5.3.3.1.5 Thiazolidinediones

Thiazolidinediones are selective agonists of peroxisome proliferator-activated receptor-γ (PPARγ), a nuclear receptor family that controls transcription of a variety of genes in a number of tissues.[224] Thiazolidinediones regulate the transcription of genes controlling insulin resistance, leading to increased glucose uptake and storage at adipose, skeletal muscle and hepatic tissue. The net result is, again, an increase in insulin sensitivity, without risk of hypoglycemia.[225] There are reports, however, of hepatotoxicity associated to this medication, thus liver enzyme levels are typically monitored, and these are not recommended first-line medications.[226]

1.5.3.3.1.6 GLP-1

Established biological activities of GLP-1 include increased insulin biosynthesis and GSIS, decreased glucagon secretion, decreased appetite and delayed

gastric emptying, all of which result in decreased blood glucose levels.[227] As a result, long-acting GLP-1 analogues,[228] as well as inhibitors of dipeptidyl peptidase-4 (DPP4), the enzyme responsible for GLP-1 degradation,[229] are now available for the management of T2DM.

While previous clinical practice guidelines have indicated the optimal course of pharmacotherapy for the management of diabetes,[183] the advent of GLP-1 and other recent therapeutics suggest a re-examination of such guidelines is inevitable.

1.5.3.3.2 Insulin

The aforementioned oral anti-hyperglycemics certainly have a role to play in the management of diabetes, along with diet and exercise. Secondary complications, however, cannot be prevented without tight blood glucose monitoring that appears to require the administration of exogenous insulin.[185, 230] Moreover, insulin secretagogues and sensitisers are of limited use to patients suffering from T1DM. Thus, administration of exogenous insulin has remained the standard of care for the treatment of T1DM since its discovery.[231, 232] Insulin therapy is also becoming increasing commonplace in the treatment of T2DM.

While originally porcine or bovine in origin, the insulin that is used clinically is now a recombinant form of the human protein.[233, 234] As a result, manipulation of the primary sequence has allowed the modification of the pharmacokinetics of insulin, such that long- and short-acting formulations exist.[235] Moreover, osmotic pumps can be worn whereby a constant basal insulin infusion rate can be set, with user-determined bolus administration after meals.[236] The next generation insulin pumps will seek to integrate continuous glucose monitoring with insulin infusion via an automated algorithm, essentially creating the first artificial pancreas.[237]

1.5.3.3.3 Islet Replacement

Despite the advent of numerous insulin formulations to optimise glycemic control, the fact remains that exogenous insulin replacement does not succeed in

providing the meticulous control provided by native islets. As a result, recent research has focused on the replacement of functional β-cell mass. As of the present, donor organs represent the only viable source of functional human islets.

1.5.3.3.3.1 Pancreas Transplantation

Pancreas transplantation was pioneered in the 1960s, and has since seen a decrease in the morbidity and mortality associated with the procedure.[238] As a result, solid organ pancreas transplantation represents the only available means of providing long-term glycemic control that prevents, and even reverses, diabetic complications.[239]

The procedure consists of grafting the donor organ, either alone, simultaneous with, or after, a kidney transplant. While the native pancreas is left untouched, the donor organ is typically placed in the lower abdomen, with vascular anastomoses, and the main pancreatic duct anastomosed unto the recipient's bladder.

However, the risks and side effects of chronic immunosuppression are such that pancreas transplantation usually only represents a viable treatment option to patients suffering from extremely brittle diabetes, or receiving immunosuppression in conjunction with another graft. Moreover, a shortage of donor organs means that pancreas transplantation cannot serve as a major curative therapy for diabetes.

1.5.3.3.3.2 Islet Transplantation

With other factors considered equal, overt diabetes is only observed when there is a ≥50% reduction in β-cell mass relative to matched controls.[188] Given this observation, it was hypothesised that the isolation of islets from a donor pancreas could provide sufficient tissue to reverse diabetes in more than one recipient, improving the 1:1 donor:recipient ratio existing for solid organ transplantation. Moreover, it was also hypothesised that islets may be less immunogenic than other pancreatic components, meaning that islet grafts would

require less immunosuppression than whole-organ grafts. Thus, recent clinical research has focused on transplanting islets as a means of replacing or expanding β-cell mass.

In this technique, the islets of Langerhans are isolated from the surrounding acinar and ductal tissue such that a relatively pure preparation of islets can be infused into hepatic portal vein, where the islets will embolize into the capillary beds within the liver. Thus, the donor islets reside intravascularly.

Early results seemed to suggest that islet transplantation might in fact represent a viable alternative to whole organ pancreas transplantation.[240] However, the fact remains that the attrition that occurs throughout the processes of islet isolation and transplantation actually worsens the donor:recipient ratio.[240-242] Moreover, it appears that islets infused into the portal vein, the established graft site, have a much shorter half-life than those transplanted as part of the whole organ, with the average duration of graft function reaching only 15 months in islet recipients,[243] as compared to ten years in pancreas recipients.[239] Nevertheless, work continues to improve the technique,[244] with one recent positive side effect being the study of *ex vivo* islet expansion as a means of expanding the pool of transplantable tissue obtained per donor.[245]

Of note, however, is the fact that while transplantation-based techniques are useful at replacing a deficit in β-cell mass, these treatment options do not address the issue of insulin resistance.

1.6 β-Cell Mass Balance

Pancreatic β-cell mass is not a static entity, but rather is in constant flux, and as such can adapt to the prevailing physiologic needs.[246, 247] For example, insulin resistance associated with pregnancy[248] or obesity[22, 188, 206] leads to as much as a doubling of β-cell mass. Conversely, a deficit in β-cell mass is associated with both T1DM and T2DM.[22, 189]

Changes in β-cell mass over time reflect the net effect of diametrically opposed pathways; those that serve to increase β-cell mass, and those that serve to decrease β-cell mass. Possible β-cell mass expansion pathways include

β-cell hypertrophy and replication, as well as the formation of new β-cells from non-β-cell sources - neogenesis. Each of these factors has an equivalent opposing force; β-cell atrophy and death, via apoptosis or necrosis, as well as β-cell dedifferentiation, all serve to decrease β-cell mass. However, studies suggest that the three main mechanisms at play in controlling β-cell mass dynamics are β-cell replication, neogenesis and death.[22, 249, 250]

Based on these observations, Finegood *et al*.[246] proposed a simple mass-balance equation to follow the prevailing trends in β-cell mass:

$$d(\beta\text{-cell mass})/dt = rate_{\beta\text{-cell replication}} + rate_{\beta\text{-cell neogenesis}} - rate_{\beta\text{-cell death}}$$

While animal studies using 5-bromo-2'-deoxyuridine (BrdU) or ^{3}H-thymidine (^{3}H-TdR) incorporation have allowed for an estimation of the rate of β-cell replication,[251, 252] such direct methods are not applicable to humans, and as such, the absolute individual contributions of these mechanisms cannot be determined; only the net sum can be estimated based on changes in overall β-cell mass.

1.6.1 Mechanisms of β-Cell Mass Dynamics

Pancreatic β-cell mass is under tight regulation in the normal individual, with the mechanisms of β-cell mass dynamics varying between species and according to the prevailing physiologic condition.

For example, neonatal rats undergo β-cell mass remodelling characterised by minimal β-cell replication, but elevated β-cell neogenic and apoptotic rates,[246, 253] while β-cell mass involution in the post-partum rat occurs via β-cell apoptosis and atrophy, coupled with decreased β-cell replication.[248] In mice, obesity induces a compensatory increase in neogenesis and, more dramatically, in β-cell replication, to increase β-cell mass as much as ten-fold.[249] Similar increases in β-cell replication are observed in models of insulin resistance.[208] Mouse models of T2DM are characterised by increased β-cell apoptosis,[249] whereas

normoglycemic mice respond to induced β-cell mass expansion via decreased β-cell replication and increased β-cell apoptosis.[250]

As indicated earlier, human T1DM and T2DM are marked by a deficit in functional β-cell mass. While patients suffering from T1DM have a virtual lack of β-cell mass,[188] chronic β-cell apoptosis in individuals with T1DM suggests β-cell neogenesis is also ongoing.[189, 190] Recent evidence also indicates that increased β-cell apoptosis also leads to a deficit in β-cell mass in T2DM.[22] This increased β-cell apoptosis is observed in both lean and obese cases of T2DM, while obese T2DM individuals do show evidence of increased neogenesis and lean T2DM individuals have increased β-cell proliferation indices, suggesting compensatory mechanisms are intact, but outpaced by β-cell apoptosis.[22] Thus, these findings suggest that regenerative mechanisms do exist within the human pancreas. The exact nature, and potential utility, of these mechanisms remains to be determined.

1.6.2 Islet Regeneration

While the mechanisms responsible for depleting β-cell mass are well defined, and are predominated by β-cell death,[22, 246] either in the form of apoptosis or necrosis, the mechanisms of increasing β-cell mass are much more controversial.[254, 255] The two principal mechanisms of increasing β-cell mass are neogenesis, the formation of β-cells from non-β-cell precursors,[256-260] and replication, the formation of β-cells by the division of pre-existing β-cells. While the formal definitions of these two mechanisms are mutually exclusive, the working definitions are less clearly distinguished, and as such, in this thesis, the term islet regeneration has been used to describe the expansion and/or replenishment of β-cells, irrespective of exact mechanism.[261, 262]

1.6.2.1 Replication

While the adult β-cell turnover rate is low,[263] *in vivo* and *in vitro* studies have determined that β-cells proliferate in response to physiologic concentrations of

relevant growth factors.[264-271] However, *in vitro* β-cell proliferation is associated with loss of endocrine phenotype and expression profile,[272, 273] suggesting a balance between differentiated and proliferative states, as is common to most cell types. Recent evidence suggests that β-cell proliferation may function as a compensatory mechanism in T2DM,[22, 249] although proliferating β-cells are also more susceptible to apoptosis.[274, 275] A recent transgenic animal study indicated that β-cell replication was the main mode of β-cell mass maintenance in the adult mouse, as well as the prevailing mechanism of β-cell regeneration in the partial pancreatectomy model of pancreatic regeneration.[276] Thus, β-cell replication is a real, semi-quantifiable component of β-cell mass dynamics, both in the normal and diseased pancreas.

1.6.2.2 Neogenesis

Although β-cell replication and neogenesis are dynamic processes, the availability of detectable markers of cellular proliferation[251, 252] renders the quantification of cell replication much more straightforward than the measurement of neogenesis. Recent advances in lineage tracing techniques have facilitated the process, but questions regarding the cell(s) of origin and inter-species differences mean that neogenesis is still a polarising subject.

Neogenesis implies that β-cells form from non-β-cell precursors,[257-259] though the source and location of such precursors is not specified. Thus, the term neogenesis could be applied to the formation of β-cells from intra- or extra-pancreatic stem cells, as well as the direct or indirect transdifferentiation of other adult cell types.

1.6.2.2.1 Stem Cell-Derived

Recent publications suggest that the definition of a stem cell has been relaxed somewhat as compared to the original,[277] which required the ability to self-renew and give rise to differentiated cells from multiple lineages.[278]

Current stem cell research focuses on bone marrow-derived cells, with contradictory results existing regarding the role, if any, of these cells in

pancreatic regeneration.[279-282] The revised definition of stem cells includes tissue-specific stem cells that may reside within an organ, in a proto-differentiated state, and with a multipotency that is limited to the cell types of the organ in question. The existence and identification of such a pancreatic stem cell is also questionable.[276, 283-285] While one group has indicated that cells with such multipotency do appear to exist within the adult rodent pancreas, isolation and characterisation are rendered difficult by the lack of an appropriate cellular marker.[284] Thus, based on available evidence, it appears more likely that islet neogenesis occurs via the transdifferentiation of other adult pancreatic cell types, which may act as facultative stem cells.

1.6.2.2.2 Duct-Derived

Ample evidence exists to suggest that adult pancreatic ducts can give rise to newly forming islets. The incidence of small clusters of β-cells in close proximity to pancreatic ducts, not associated with other endocrine cells, has long been held as an indicator of duct-associated islet neogenesis,[256-260] given that this is the mechanism by which islets form during development.[54]

Numerous studies have characterised duct-associated islet neogenesis, based mainly on morphological and/or immunohistochemical evaluation.[259, 260, 286-292] However, with static techniques being used to quantify a dynamic process, studies of this kind cannot be considered definitive. Moreover, while double labelling of cells suggests a transition between cell types,[288, 290, 291] no directionality is implied. To clarify this issue, one study used the pulse-chase administration of an indelible cell marker in the partial pancreatic duct obstruction model of islet regeneration. Researchers detected ^{3}H-TdR labelling, first in ducts and later in islets, indicating that islets were derived from ductal cells that had previously incorporated the label.[293]

In an effort to further confirm the existence of duct-derived islet neogenesis, groups have developed *in vitro* culture models whereby islet regeneration from cultured ductal tissue has been reported,[294, 295] although the true ductal nature of the starting tissue has since been called into question.[296]

Finally, further complicating the issue is the lack of a definitive marker of precursor cells resident within the duct. Cytokeratins (CK) have been proposed as a marker of islet progenitors,[287] although such a marker is relatively non-specific. Other groups have proposed ductal expression of PDX1,[297-299] NGN3,[91, 300] vimentin[301] or nestin,[302] although the research community has not reached a consensus.[303, 304]

1.6.2.2.3 Acinar-Derived

Although adult pancreatic acinar tissue was long thought not to participate in islet regeneration, recent studies have begun to describe situations of acinar-derived islet neogenesis, through either direct or indirect mechanisms.

As mentioned earlier, acinar-to-ductal dysplasia has been proposed as a mechanism of pancreatic carcinogenesis.[171-173] Tubular complexes, that is entire lobes of pancreatic acinar tissue that transdifferentiate into a series of tubular duct-like structures (DLS), are frequently observed in chronic pancreatitis and pancreatic adenocarcinoma.[305-307] Recent studies now suggest that tubular complexes may also act as sites of islet neogenesis,[308-311] although again directionality is not implicit.[312] That is, the question remains as to whether a duct is giving rise to an islet, or vice versa. Other groups have also reported *in vivo* islet regeneration from acinar-derived ductal structures.[313]

To confirm the ability of adult acinar to pass through a duct-like cellular intermediate en route to islet neogenesis, several groups have developed *in vitro* culture models.[314-317] There is evidence that cultured acini give rise to DLS, with lineage tracing studies confirming acinar origin.[318] This laboratory has since reported that adult human acinar-derived duct cultures can actually be induced to regenerate islet-like structures (ILS);[319] findings that confirm previous observations.[320, 321] Acinar-derived islet regeneration may be of therapeutic interest, as acinar tissue retains the potential to regenerate islets in animal models of T1DM.[322]

While most reports focus on acinar-derived regeneration passing through a duct-like intermediate, limited evidence exists to indicate a direct acinar-to-endocrine differentiation pathway in islet neogenesis.[309, 323-326]

1.6.2.2.4 Islet-Derived

Seemingly contradictory, β-cell neogenesis can also occur from islet cells, provided these cells are not adult β-cells. However, this notion is rendered all the more complicated by two additional caveats.

First, β-cell neogenesis only implies the formation of β-cells, meaning that adult α-, δ- and PP-cells, along with any resident precursor, could serve as the cell of origin. This is not the same case for islet neogenesis, which implies the formation of a fully functional islet, including the differentiation of all four endocrine cell types, and as such involves a precursor that is not of adult endocrine origin.

Secondly, the definition of a β-cell is not hard and fast, and may in fact vary depending on the study design. For example, a functional study would likely define an adult β-cell based on a composite phenotype including measures of function and gene expression. A lineage tracing study, however, might only define a β-cell based on the presence of an active insulin promoter.[276] Thus, the study of islet-derived precursors and β-cell neogenesis requires careful attention.

In vivo studies regarding islet-derived regeneration are difficult due to the presence of pre-existing β-cells. While duct- or acinar-derived islet neogenesis would be observed as a novel source of islet tissue, intra-islet neogenesis could easily be misconstrued as β-cell proliferation given the resultant increase in β-cell number. As a result, *in vivo* work on intra-islet progenitors has focused on the regeneration of islets in animal models of β-cell depletion, typically employing streptozotocin (STZ) as a β-cell-specific toxin.[327-329] However, the obvious concern arises as to the destruction of putative precursor cells by STZ.

Researchers have identified several putative *in vivo* intra-islet progenitors, with marker profiles that include PDX^{+}/somatostatin $(SST)^{+}$[327, 329] and INS^{+}/SST^{+}.[328] These phenotypes are significant given the common

developmental ancestry of β- and δ-cells.[134] $GCG^+/GLUT2^+$ cells have also been described.[328] Petropavlovskaia and Rosenberg also described a heterogeneous population of small cells in adult human islets, with characteristics that suggest regenerative potential.[5] Putative $nestin^+$ intra-islet progenitors have also been described in the mouse.[330] Finally, a population of $PDX^+/NGN3^+/nestin^{+/-}$ cells has been described, with these cells apparently residing in both ducts and islets in the mouse.[284, 285] Thus, while it is clear that much work has gone into identifying intra-islet progenitors *in vivo*, a definitive cell type with an established marker profile does not yet exist.

Several *in vitro* models have also been developed to study the incidence of islet-derived neogenesis; these will be discussed later in the context of the islet response to isolation and culture.

1.6.2.3 Regenerative Factors

A variety of animal models have been developed to study the mechanisms of islet regeneration, and identify the factors involved. These models include partial pancreatic duct obstruction[258, 331] or ligation,[288, 332] partial pancreatectomy,[259, 333] chronic and acute glucose infusion,[309, 334, 335] STZ administration[327] and transgenic models,[286, 336, 337] all of which are associated with an endogenous pancreatic regenerative response. As such, researchers have screened the regenerating pancreas to identify factors that may regulate the response, as well as general changes in gene expression.[338, 339]

Several novel therapeutics have been developed based on these studies, all of which are currently in clinical trials as novel therapies of diabetes.

1.6.2.3.1 Gastrin and EGF

Studies of the pancreatic regenerative response to pancreatic duct ligation[288] noted the upregulation of gastrin and TGFα, a member of the EGF family of ligands.[332] Subsequent studies indicated that alone, these factors acted mainly as duct cell mitogens,[340-342] whereas co-administration or generation of double transgenic animals led to a significant increase in β-cell mass.[286, 343, 344] It now

appears that the role of EGF ligands may be to generate metaplastic DLS,[307, 318] which then form islets in response to gastrin.[310]

In vitro studies suggest that human tissue can be expanded and differentiated in the same way.[295, 319, 345] There is also pathologic evidence for such an islet regenerative effect of gastrin, as Zollinger-Ellison syndrome, hypergastrinemia secondary to a gastrinoma, is associated with increased islet neogenesis and replication.[346, 347]

Based on the above studies, combination therapy with gastrin and EGF analogues is currently in clinical trials,[348] although due to the established carcinogenicity of EGF, more recent trials focus on gastrin and GLP-1 co-administration.[349]

1.6.2.3.2 GLP-1

As indicated earlier, GLP-1 is a product of alternative post-translational modification of preproglucagon.[29] While α-cells produce and secrete glucagon, enteroendocrine L-cells of the intestine produce GLP-1.[350] Due to their incretin and other effects,[227] long-acting GLP-1 analogues[228] and DPP4 inhibitors[229] are already available for the treatment of T2DM. Additionally, however, GLP-1 has been implicated in islet regeneration,[267, 283, 292, 351-355] leading to its study as a regenerative factor.

Data regarding the regenerative role of GLP-1 and related agonists are controversial. There is strong evidence to suggest that GLP-1 receptor (GLP1R) activation can protect β-cells from apoptosis.[356-359] There is equally convincing evidence of a β-cell mitogenic effect of GLP-1.[267, 352, 356, 360] However, while there are reports of GLP-1-mediated islet neogenesis,[267, 283, 292, 353, 361] recent statements by researchers examining the *in vivo* effects of GLP-1 call into question a neogenic response.[362-366] Nevertheless, GLP-1 certainly has a role to play in the evolving treatment of diabetes.

1.6.2.3.3 Reg Proteins

Given that subtotal pancreatectomy combined with poly(ADP-ribose) polymerase (PARP) inhibitor treatment promotes islet regeneration,[367] researchers screening a complementary deoxyribonucleic acid (cDNA) library generated from the regenerating islets identified a novel protein, termed regenerating protein (Reg, now known as RegI).[368] It was later determined that while some Reg proteins are expressed in regenerating islets,[369] expression is primarily at acinar tissue.[370]

Reg proteins have been postulated to play a role in inhibition of pancreatic stone formation,[371] bacterial aggregation,[372] regulation of inflammation[373, 374] and cellular adhesion to ECM components,[375] among others. Most interestingly, however, *in vivo* studies suggest that administration of RegI may be sufficient to induce pancreatic regeneration in both surgically-induced[265] and genetic[376-379] models of diabetes. Moreover, RegI-overexpressing mice have increased islet proliferation, and crossing unto a non-obese diabetic (NOD) background delays the onset of diabetes and increases β-cell mass.[380] RegI$^{-/-}$ animals are not overtly diseased, although islet proliferation is reduced.[380] Reg proteins are mitogenic to both islet and ductal cell lines, as well as isolated islets and primary duct cultures,[381-383] and may also have anti-apoptotic effects.[374, 384]

While the exact Reg mechanism of action and signalling pathways are still unknown, it is noteworthy that Reg expression or activity has been associated with other putative regenerative factors. For example, administration of a GLP-1 analogue upregulates pancreatic Reg expression prior to observation of islet regeneration,[339] while the effects of gastrin may be mediated by Reg expression.[385, 386]

Thus, while results are promising, Reg proteins, with the exception of one, have not elicited much clinical interest.

1.6.2.3.4 INGAP

The Reg proteins can be divided into three general families; RegI, RegIII and Reg IV.[387] The RegIII subfamily is characterised by the presence of a five amino acid insert, which would likely be found on an outside turn in the three-

dimensional structure of the protein, based on available models.[388] Islet neogenesis-associated protein (INGAP) is the only RegIII protein identified in hamsters, although two family members exist in humans, and four exist in mice, including one - RegIIIδ - that is highly homologous to INGAP.[389]

INGAP was discovered in a surgical model of partial pancreatic duct obstruction associated with increased β-cell mass and what appeared to be foci of islet neogenesis.[258] Initial work identified a crude pancreatic extract containing biological activity,[390] while subsequent genetic work identified INGAP as the main biologically active compound.[381] Based on three-dimensional modelling and sequence comparisons, a synthetic 15 amino acid fragment was designed, which includes the aforementioned five amino acid insert. This fragment ($INGAP^{104-118}$), termed INGAP peptide, was shown to induce the proliferation of primary ductal cultures and ductal cell lines.[381]

While identified initially in partially pancreatic duct-obstructed hamsters, INGAP was subsequently found to be upregulated in other models of islet regeneration, including sucrose administration in hamster drinking water,[391] sucrose administration to pregnant hamsters,[392] chronically glucose-infused rats and diabetes-prone BioBreeding rats.[345]

Administration of INGAP peptide increases β-cell mass sufficiently to reverse STZ-induced diabetes in mice.[326] While INGAP peptide did not reverse diabetes in chronically diabetic NOD mice, co-administration with immunosuppression improved survival and indices of diabetes severity.[345] Studies on isolated rat islets indicate that acute INGAP peptide treatment can increase both basal and stimulated insulin secretion from isolated rat islets.[393]

Interestingly, while administration of INGAP peptide to normoglycemic hamsters,[326] mice[250] or monkeys[345] does induce islet neogenesis and β-cell mass expansion, this increase appears to be transient, at least in healthy animals. Based on this observation, it has been proposed that the mechanisms of β-cell mass dynamics are actively regulating β-cell mass, even in the context of induced islet neogenesis.[250]

While no direct human homologue appears to exist for INGAP, studies using *in vitro* cultures of human islet- or acinar-derived tissue indicate that the islet regenerative potential of INGAP peptide also extends to human tissue.[319, 394] Clinical trials have also yielded promising results, showing an increase in arginine-stimulated C-peptide levels in T1DM patients,[395] and a decrease in HbA_{1c} in T2DM patients.[396]

1.7 Isolated Islets

As indicated earlier, clinical research has focused on transplanting islets as a means of replacing or expanding β-cell mass.[240] The justification for such a methodology was the hypothesised reduced immunogenicity of islets as compared to the solid organ, as well as an improved donor:recipient ratio based on the apparent need for only 50% of β-cell mass to maintain normoglycemia.[188] While certainly a valuable option, islet transplantation no longer appears to have the potential therapeutic impact it was once hoped.[240-242] Recent work has focused, therefore, on the response of islets to isolation and culture, with a view to improving islet viability in the post-isolation pre-transplantation period.

Another rapidly developing area of research, however, is the study of the proliferative and/or regenerative potential of isolated human islets. This work concentrates on increasing functional β-cell mass via proliferation of isolated islets, either as differentiated adult islets, or via dedifferentiated proliferative intermediate cells that can then be redifferentiated to an adult islet phenotype.

1.7.1 Islet Isolation

In order to study the response of the islet to the isolation procedure, a basic understanding of the process is required (Fig. 1.3).

The goal of an islet isolation procedure is the preparation of highly purified, functional, intact islets. Because islets are dispersed heterogeneously throughout the pancreas, the first step is the enzymatic digestion of the pancreas into individual acini and islets.[397] Next, differences in density between islets and other pancreatic tissues are exploited to isolate the islet fraction.[398]

Pancreata for clinical islet isolation are received through local organ procurement agencies (OPO), and are procured following established protocols.[399] As such, the organ has been flushed *in situ* with cold organ preservation solution, removed *en bloc* along with the spleen and a stapled loop of duodenum, and shipped on ice in a sterile container containing organ preservation solution. Upon reception, ideally within eight hours of aortic cross-clamping, the pancreas is dissected under sterile conditions to remove the spleen and duodenum, taking care to close the pancreatic duct at the site of its entrance into the duodenum, as well as any extraneous tissue, including fat.

Next, according to established protocols,[400] the pancreas is dissected transversely to visualize the main pancreatic duct. The duct is cut and fitted with two cannulas; one towards the tail of the organ and the other towards the head. In this way, a specially blended enzymatic solution can be delivered preferentially to the islets, which sit in close proximity to the ductal network.[397, 401] The enzymatic blend is infused cold to prevent precocious digestion of the organ. After infusion, the pancreas is cut into pieces and placed in a digestion chamber that is fitted with stainless steel marbles for mechanical dissociation of the organ and mesh filters to prevent the escape of large, undigested pieces of pancreas. This digestion chamber is part of a closed circulating system that allows sampling of the digestate, as well as temperature control.

The system is heated to activate the enzymatic blend of collagenases and neutral proteases, and shaken to allow mechanical dissociation of the organ. After islets are observed to be free of attached tissues, as determined by real-time sampling and visualization using dithizone, the system is cooled, to inactivate the enzyme blend, and opened, to allow collection of the digestate. The total pancreatic digestate is pooled and washed, and allowed to recover in cold organ preservation solution before the second step of the isolation procedure. Proper tissue recovery is an essential step in the isolation procedure, as the actual isolation step relies on density gradient centrifugation, and the various insults incumbent to the first steps of the isolation may have disrupted cellular densities.

The pancreatic digestate is applied to a polysuccrose density gradient of 1.077 to 1.100g/mL, established in a COBE 2991 cell processor.[398] After allowing tissues to migrate to their respective densities within the gradient, the entire volume is collected in fractions, and samples taken to determine the location and purity of the remaining islets. These fractions are pooled and washed before counting, again using established protocols. Briefly, due to the variability in islet diameters, counts are established in islet equivalents (IE), that is, normalized to the number of islets with a diameter of 150μm.

All told, the islet isolation procedure takes eight hours from reception of the organ until the production of a final sample of purified islets. Final purity ranges from <30% to >90%, and islet function may be equally variable. Islets may or may not be cultured prior to transplantation, which is accomplished by percutaneous transhepatic portal embolization, using unit gravity.

1.7.2 Response to Isolation

In light of the variety of stresses and insults that islets face during the course of isolation, it is not surprising that cultures of isolated islets are marked by high levels of cell death.[241, 242] Some of these insults are unfortunate secondary effects of the isolation procedure; mechanical, sheer, osmotic and hypoxic stress, for example, while some insults are a direct result of the isolation procedure, namely loss of trophic support, cell-cell and cell-ECM interactions. The result is both apoptosis and necrosis. Moreover, it appears that when an islet cell is committed to cell death, inhibition of one of these cell death pathways is not sufficient to prevent cell death.[402]

1.7.2.1 Apoptosis

Apoptosis is an active cell death pathway in that it is an energy-dependent process that uses genetically programmed pathways to target a cell for controlled destruction.[403] General apoptotic signalling involves the activation of caspases, cysteine proteases that function as both the initiators and effectors of the apoptotic pathway.[404] Another common step in apoptotic cell death is

mitochondrial permeabilisation and release of cytochrome C,[405] typically controlled by the interplay of pro- and anti-apoptotic members of the Bcl-2 family of proteins.[406, 407] The end result of pro-apoptotic pathways is the controlled digestion of DNA and blebbing of cells such that intracellular contents are not released, and there is minimal inflammatory reaction at the site.[408]

Most molecular targets of apoptosis are characterised by the presence of endogenous inhibitors, indicating that apoptosis is marked by a succession of checkpoints along the path towards cell death. Thus, pro-apoptotic signals activate apoptotic pathways, and pro-survival signals inhibit apoptotic pathways, with the sum of these signals determining the fate of the cell. Hence, while some isolation-associated stresses activate pro-apoptotic signals, others might represent removal of constitutive pro-survival signals.[409]

The first step in the islet isolation procedure, the enzymatic digestion of the pancreas,[400] removes the trophic interactions between islets and the surrounding ECM.[410-412] Anoikis, that is, apoptosis caused by disrupted cell-ECM interactions, functions as a defence mechanism to prevent carcinogenesis and metastasis,[413] but may act to target islets for apoptosis during isolation.[411, 412, 414] Likewise, cell-cell interactions within the islet are also very important,[45, 46] and while islet isolation is not meant to disrupt these connections, this event surely occurs.[412]

Proximity to non-endocrine pancreatic tissue is also important in maintaining functional islets, as evidenced by co-culture studies and observation of expression of islet trophic factors in duct and acinar tissue.[370, 415, 416] Thus, purification of islets from non-endocrine tissue may also remove necessary trophic factors.[409] Growth factor dependence is another preventative mechanism aimed at limiting carcinogenicity.[417]

As described earlier, islets are normally highly vascularised structures,[43] and as such are highly susceptible to hypoxic stress.[418] However, with more than eight hours of cold ischemia prior to isolation, followed by isolation, culture and up to two more weeks at the graft site prior to vascularisation, hypoxia is also a likely cause of significant levels of islet apoptosis.[419]

While the above represent mainly apoptotic pathways induced by a loss of survival signals, there are also apoptotic mechanisms at play that are activated in the context of islet isolation. These mechanisms are focused on pro-inflammatory cytokines, namely interleukin-1β (IL1β), interferon-γ (IFNγ) and tumor necrosis factor-α (TNFα), which are induced throughout islet isolation, culture and transplantation,[420-424] and induce islet apoptosis.[425] Possible inducers include endotoxin found in various isolation reagents,[423, 426, 427] as well as a host response at the graft site.[420, 421]

1.7.2.2 Necrosis

While necrosis is less energy-dependent and less regulated than apoptosis, this mechanism of cell death does not function in isolation, but appears to represent the other extreme of a spectrum of cell death mechanisms.[428] In fact, inhibition of apoptotic signalling simply causes islets to undergo necrosis,[402] suggesting the possibility of programmed mechanisms of necrosis.[429] Effectors are less well defined, but include PARP, which is involved in DNA damage repair.[430]

Incidents of islet necrosis induced by isolation are less common, but evidence exists to suggest that necrosis may be induced by mechanical, sheer and osmotic stresses associated with the isolation procedure, as well as targeted necrosis of cells that are too energy-depleted to undergo apoptosis.[402]

Thus, the islet response to isolation is beginning to be elucidated. It is unclear, however, how active a role the islet plays in this response, or whether it is simply a passive reaction to the addition or removal of specific stimuli.

1.7.3 Response to Culture

Following isolation, islet culture prior to transplantation has become common, given the therapeutic window that exists for interventions that improve islet phenotype or function.[244, 431] Moreover, research indicates that the majority of islet cell death occurs early in the culture period, meaning islets cultured for several days express lower levels of pro-inflammatory cytokines.[432, 433]

However, culture for up to one week also provides an additional opportunity for islets to undergo apoptosis, become contaminated, or respond adversely to a microenvironment that provides little, if any, trophic support via growth factors and ECM. This negative response, alluded to earlier, is the loss of phenotypic stability associated with loss of these maintenance signals.[434, 435]

1.7.3.1 Extracellular Matrix

As indicated above, a highly specialised ECM surrounds islets within the pancreas, whereas islet isolation typically removes or severely damages this supportive environment.[411, 412] While one of the default pathways in response to a loss of the support appears to be induction of cell death,[411, 412, 414] another common response is cellular differentiation.[436, 437]

The ECM, in addition to serving as a cellular scaffold, also regulates differentiation.[434, 438-441] Thus, co-culture with ECM proteins or embedding in various matrices has been tested as a means of normalising islet phenotype, function and proliferation.[411, 412, 414, 415, 442-445] However, it does appear that a duct epithelial phenotype is the default pathway during pancreatic development,[446] and may also be the default state to which islets revert if proper ECM support is not provided. Thus, isolated islets readily dedifferentiate in culture,[245, 296, 447-450] even if some degree of ECM support is provided.[394, 451-456]

1.7.3.2 Growth Factors

Lack of growth factor signalling can activate pro-apoptotic pathways. However, growth factors, and ECM, do not function alone, but rather work in concert to provide a very specific microenvironment, with disruptions possibly causing not only cell death but also changes in cellular differentiation.[457, 458] Thus, while EGF appears to be involved in islet cell differentiation in the *in vivo* context,[74] *in vitro* administration of EGF appears to favour islet dedifferentiation.[245, 307, 318, 455]

These data suggest that the specific microenvironment provided to islets *in vivo* is crucial to maintaining proper endocrine phenotype and function. As such, partial replacement of this microenvironment does not appear to prevent islet

dedifferentiation. While early studies were aimed at inhibiting this dedifferentiation, recent reports have attempted to capitalise on dedifferentiation into proliferative cellular phenotype, with subsequent redifferentiation, as a means of expanding β-cell mass.[245, 296, 394, 449, 450] Thus, recent research focuses on the potential positive side effects of islet plasticity.

1.8 Models of Islet Plasticity

There exists a wealth of knowledge that indicates that islet cells dedifferentiate when placed in an *in vitro* environment.[245, 296, 447-452] Moreover, differentiated state and proliferative capacity seem to be mutually exclusive, with attempts at inducing β-cell proliferation resulting in loss of endocrine phenotype.[442, 459, 460] To this end, two models of islet plasticity have been developed whereby islets are cultured under specific conditions that induce islet dedifferentiation, followed by proliferation and subsequent redifferentiation into functional ILS.

1.8.1 Monolayer

When plated, cells migrate out from adult human islets, eventually forming a monolayer.[245, 296, 447-450] These cells are proliferative and amenable to passaging. Studies have identified two principal phenotypes associated with the islet-derived monolayer cultures: a nestin$^+$/vimentin$^+$ mesenchymal-type fibroblast-like cell, and an E-cadherin$^+$/CK19$^+$ epithelial-type cell.[394, 448, 459, 461] When subjected to specific induction conditions, namely serum deprivation, these islet-derived monolayers give rise to clusters of cells that begin to express islet-like markers.[245, 296, 449, 450] While this culture process does not produce fully functional ILS, transcription of key β-cell markers, expression of multiple endocrine hormones, GSIS and *in vivo* graft function have been reported.[245]

Preliminary results of immunofluorescence[245, 450] and pulse-chase[448] experiments have suggested that both populations of putative precursor cells are of islet endocrine cell origin. The exact cellular origin of these cells, as well as the origin of the regenerating ILS, remains to be determined, however.

More definitive approaches have been reported recently, in which *in vivo* lineage tracing techniques were applied to identify the specific cellular origins of the precursor cells in mouse islets.[462-465] For example, Chase *et al*. isolated islets from mice engineered to permanently express green fluorescent protein (GFP) in cells having had active *insulin* or *pdx1* promoters.[463] Thus, either β-cells or cells from all pancreatic lineages (having all derived from a common $PDX1^+$ precursor in development[466]) were indelibly marked with GFP expression. Applying the same culture conditions reported, Chase *et al*. reported few, if any, GFP^+ precursor cells, suggesting a non-pancreatic origin for these cells.

However, minimal regeneration has been reported from any mouse precursor cell cultures,[462-465] suggesting an innate difference between mouse and human islet-derived cells. This distinction is supported by the observation that the primary β-cell mass expansion mechanism *in vivo* in mice appears to be β-cell replication, while β-cell neogenesis may to be the predominant mechanism in humans. [22, 249] In fact, a most recent study now proposes that β-cell dedifferentiation is the main contributor to human islet monolayer cultures.[467]

1.8.2 Collagen Embedding

We have previously reported that embedding human islets in either type 1 collagen or Matrigel, and culturing in medium containing EGF and cholera toxin, which increases intracellular 3',5'-cyclic adenosine monophosphate (cAMP) levels, induces a phenotypic switch from adult islet into proliferative DLS (Fig. 1.4).[451, 452] DLS formation does not occur when islets are cultured in suspension with soluble laminin or fibronectin, in agarose (in the absence of matrix proteins), or in the absence of elevated cAMP levels,[454] suggesting that a combination of environmental and intracellular events are required for islet dedifferentiation to occur.

Assessment of cell death indicates an early wave of β-cell apoptosis associated with caspase and c-jun NH_2-terminal kinase (JNK) activity, followed by DLS cell proliferation, associated with increased extracellular signal-regulated kinase (ERK) and p38 signalling.[452, 454, 455] Moreover, electron microscopy and

immunofluorescence suggest that cells of the DLS form via transdifferentiation of mantle islet cells.[394]

DLS cells are highly proliferative, and DLS are somewhat amenable to passaging, given appropriate mechanical disruption.[394] While certainly not homogenous, cells of the DLS are predominantly CK^+, although increased nestin expression is also reported.[394]

Again, exposure to specific culture conditions causes DLS to undergo islet regeneration, with foci of ILS budding from the structure. These ILS appear to be more adult-like in phenotype than other reported ILS, given the transcription of several key markers of β-cell function, the presence of cells expressing each of the four endocrine hormones, and insulin content and GSIS that appear to match that of freshly isolated islets.[394] *In vivo* function has not yet been reported, however.

The factor first reported to induce islet regeneration from DLS was INGAP peptide,[394] although we have since identified other factors with similar abilities.[345]

We have proposed four possible mechanisms by which DLS could form from islets (Fig. 1.5). In the first case, transdifferentiating endocrine cells would come to form the DLS in its entirety, including whatever cell(s) retain the potential to regenerate islets. In the second possibility, some intra-islet stem/progenitor cell is activated by the *ex vivo* culture conditions, and comes to form the DLS in its entirety. Presumably, endocrine cells would undergo apoptosis in this model. Thirdly, some combination of endocrine cell transdifferentiation and stem/progenitor cell-activation cause the resultant DLS to be a heterogeneous collection of endocrine- and stem/progenitor-derived cells. The subsequent source of islet regeneration could then be either of these two cell types. Finally, transdifferentiation of endocrine cells forms the DLS, but a resident stem/progenitor remains associated with the DLS, and is only activated in response to the regenerative stimulus. Based on available data, the most likely scenario includes some degree of endocrine cell transdifferentiation, although the extent of its contribution to DLS formation, and islet regeneration, remains to be determined.[394]

It is difficult to determine to what extent cells produced via monolayer culture resemble those from the DLS. However, cellular marker expression, as well as regenerative potential, suggests that these two culture platforms likely represent alternative methods of arriving at highly similar, if not identical, populations of putative human islet precursor cells.

In any event, the cellular origins of cells of the DLS are not fully elucidated. Likewise, the contribution, if any, of these cells to the conversion process, in the form of intra- or extracellular signalling, is also unclear. Next, the relative roles of specific cell types in the subsequent reconversion has not yet been addressed, especially in light of the controversy surrounding the origins of these cells. Finally, given that the end goal of any such studies is their eventual application towards a therapy for diabetes, the extent to which these mechanisms are active, both in the native pancreas, and in the context of disease is an issue that has yet to be addressed.

Table 1.1 MODY Forms

Name	Mutated Gene	Reference
MODY1	HNF4α	[468]
MODY2	glucokinase	[469]
MODY3	HNF1α	[470]
MODY4	PDX1	[100]
MODY5	HNF1β	[471]
MODY6	NeuroD	[125]

Table 1.2 Medications Available in Québec for the Treatment of Diabetes

Class	Compound
α-Glucosidase Inhibitor	Acarbose
Biguanide	Metformin
Meglitinide	Repaglinide
Sulfonylurea	Chlorpropamide Gliclazide Glimepiride Glyburide Tolbutamide
Thiazolidinedione	Pioglitazone Rosiglitazone
Insulin	

Source: Régie de l'assurance maladie du Québec[472]

Figure 1.1 Human Pancreas and Islets of Langerhans
A. The pancreas is a flesh-colored organ found between the duodenum and the spleen, posterior to the stomach, in the retroperitoneum.
B. The pancreatic parenchyma consists of acini of exocrine cells, with lumens collecting into a ductal network (brown) that drains into the duodenum. Dispersed throughout the organ are islets of Langerhans, containing β-cell (red), α-cells (blue), δ-cells (green) and PP-cells (yellow).

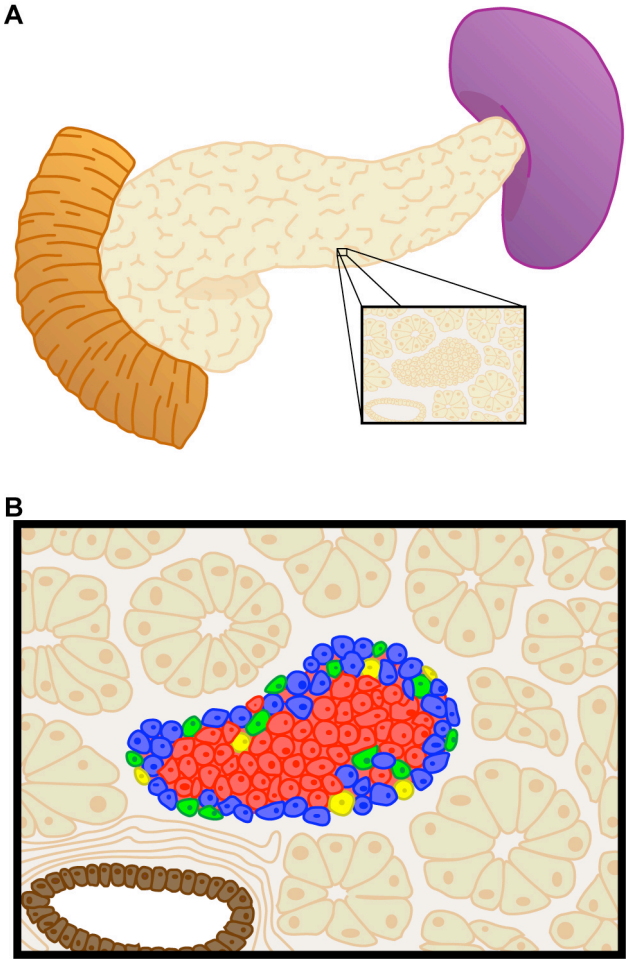

A
B

Figure 1.2 Transcriptional Control of Islet Development
Adult pancreatic cell types derive from a common $PDX1^+$ progenitor. Sequential transcription factor expression limits cell lineages until adult pancreatic phenotypes are achieved. However, knowledge gaps still exist as to the transcriptional control of development of certain cell types. Adapted from Habener *et al*.[473]

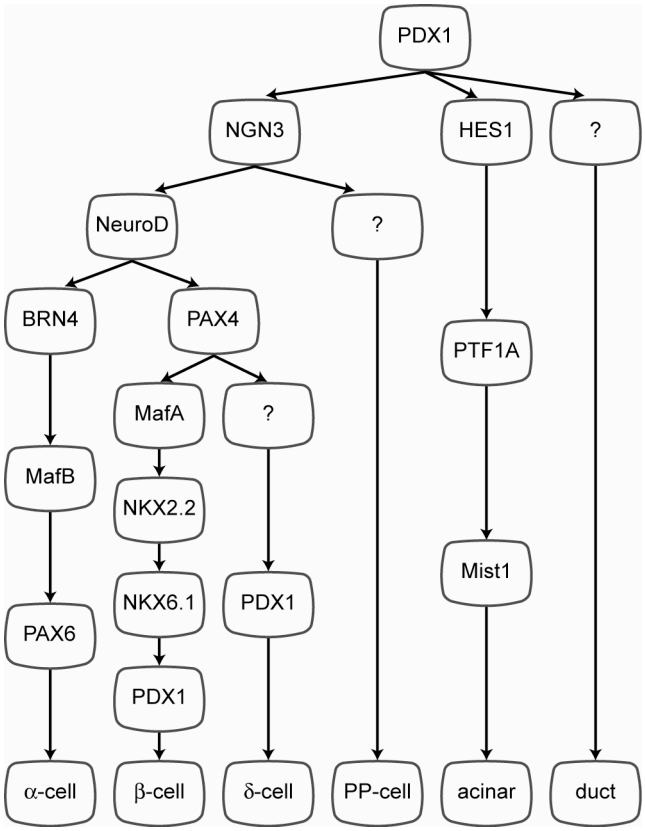

PDX1
NGN3
HES1
?
NeuroD
?
BRN4
PAX4
PTF1A
MafA
?
MafB
NKX2.2
Mist1
NKX6.1
PDX1
PAX6
PDX1
α-cell
β-cell
δ-cell
PP-cell
acinar
duct

Figure 1.3 Clinical Islet Isolation
A. The pancreas is received with the duodenum and spleen attached, and surrounded by fat and connective tissue. These extraneous tissues must be removed before proceeding with the isolation procedure.
B. The dissected pancreas weighs approximately 90g. The next step is to identify and isolate the main pancreatic duct at the neck of the pancreas to allow its cannulation.
C. Once cannulas are inserted into the main pancreatic duct, a chilled solution containing digestive enzymes can be delivered throughout the organ via the pancreatic ductal network.
D. After perfusion, the pancreas is cut into pieces and placed in a sterile chamber with stainless steel marbles. The recirculating fluid is heated to 37°C to activate the digestive enzymes, and the chamber is shaken to mechanically disrupt the organ.
E. The tissue pellet collected from the digestion of a human pancreas is approximately 30mL.
F. By the end of the digestion process, only fat and connective tissue remain in the digestion chamber.
G. After a recovery period, the digestate is applied to a continuous density gradient in a cell processor. Islets separate from acinar tissue based on the difference in specific gravity of the tissues.
H. A successful clinical islet isolation yields 250,000IE of at least 70% purity, as assessed in real time using dithizone, a Zn^{2+} chelator that stains islets red.

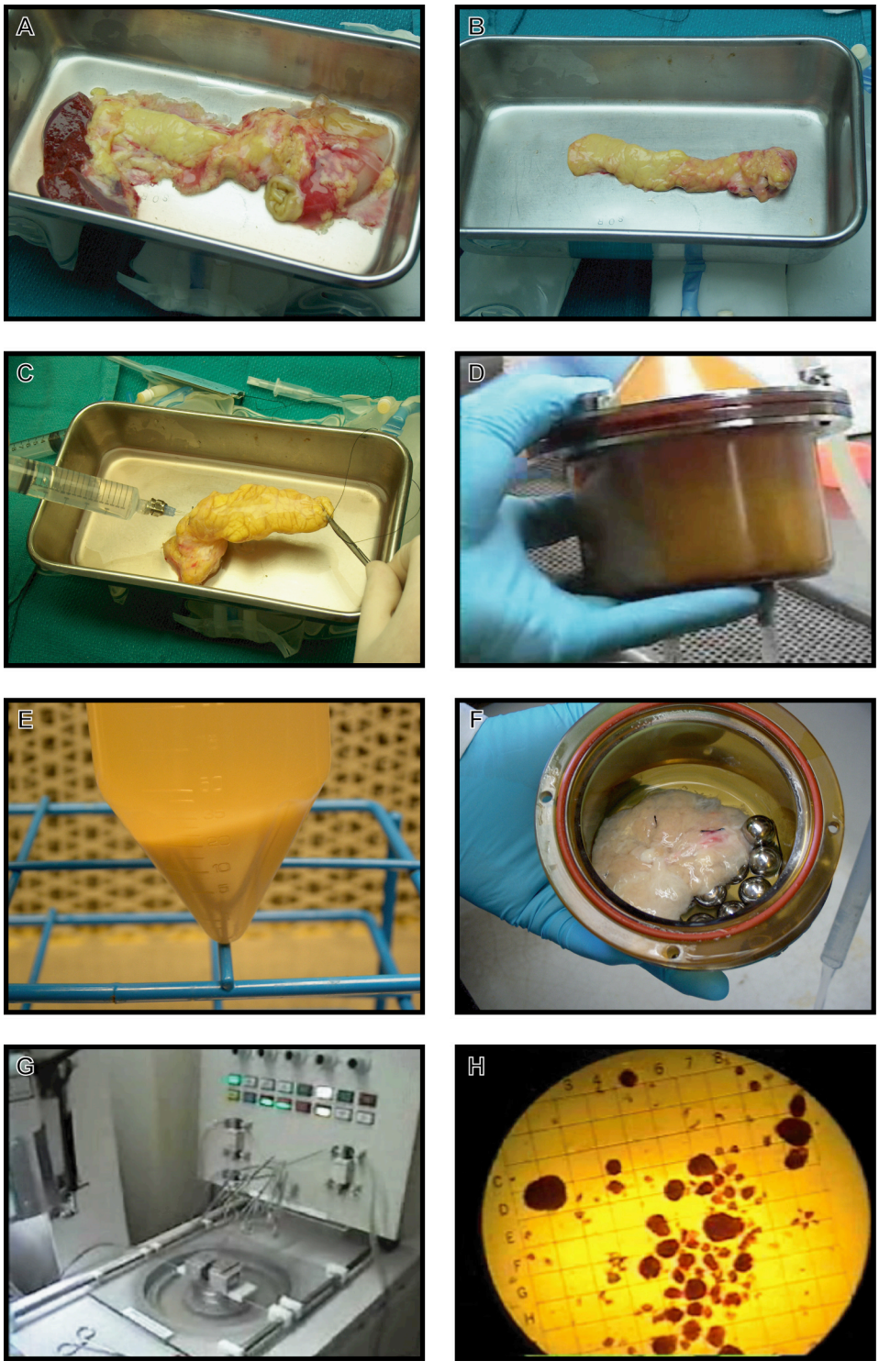

A
B
C
D
E
F
G
H

Figure 1.4 *In Vitro* Model of Islet Plasticity
Inverted and immunofluorescence microscopy of the conversion from adult islet (**A**) comprised of insulin$^+$, glucagon$^+$, somatostatin$^+$ or PP$^+$ endocrine cells (**D**), through a transitional structure with both islet and duct character (**B**), including expression of both endocrine and epithelial cell markers (**E**), to a hollow, cystic structure (**C**) that contains a heterogeneous population of CK$^+$ duct epithelial cells (**F**). Adapted from Hanley *et al*.[474]

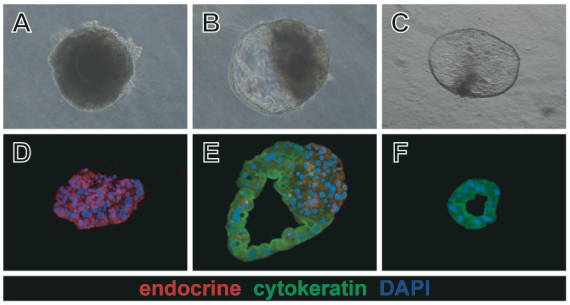
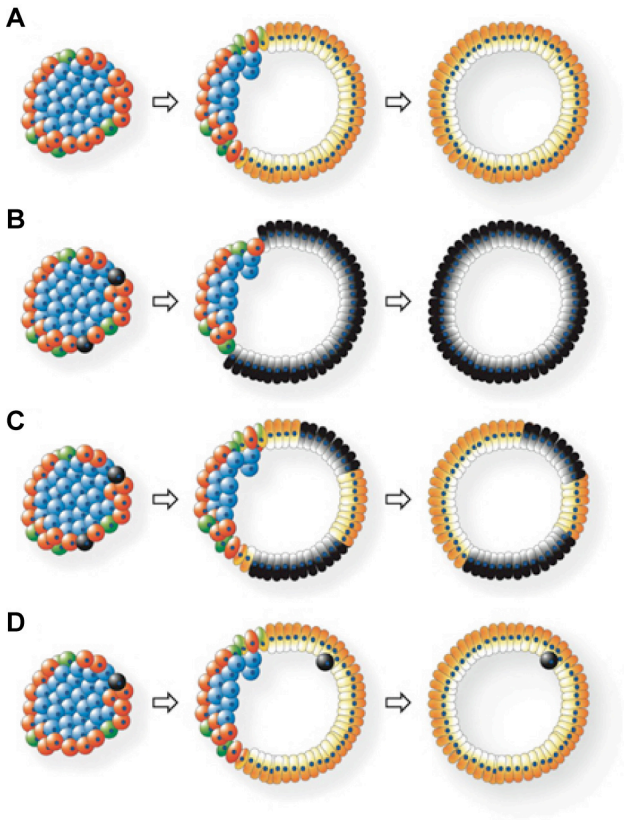

A
B
C
D
E
F
endocrine cytokeratin DAPI

Figure 1.5 Proposed Mechanisms for the Conversion of Islets to DLS
A. Transdifferentiation of endocrine cells to cells of the DLS, along with apoptosis of the cells at the core of the islet.
B. Activation of an intra-islet progenitor cell, which proliferates and comes to form the cells of the DLS, while the endocrine cells undergo apoptosis.
C. The cells of the DLS are formed from a combination of endocrine cell transdifferentiation and progenitor cell activation.
D. The cells of the DLS are formed by the transdifferentiation of endocrine cells, while an intra-islet progenitor cell remains associated. Adapted from Jamal *et al.*[394]


A
B
C
D

Rationale

The prevalence of diabetes mellitus is reaching epidemic proportions in the developed world, and its incidence continues to rise.[183, 184] While diabetes is also frequently characterised by insulin resistance, β-cell dysfunction is at the core of both T1DM and T2DM.[187, 191, 192] As a result, islet-based therapies are being increasingly considered as options for this disease, especially in light of reports the secondary complications of the disease can be prevented by the intensive glucose control that only islets can provide.[185, 230]

Current therapies have focused on the transplantation of human islets from cadaveric donors, either as part of a solid organ pancreas transplant,[238] or via the engraftment of isolated islets.[240] However, the shortage of donor organs relegates these therapies to proof-of-principle more than popular cure. Moreover, transplantation-based therapies require immunosuppression.

Recent research has therefore focused on identifying new sources of islets, including sources within the endogenous pancreas. One of the proposed sources is the islet itself. There exists significant literature describing the capacity of islets to dedifferentiate,[245, 296, 447-452] and recent evidence suggests that redifferentiation is also possible.[245, 296, 394, 449, 450] However, the contributions of these phenomena to diabetes, both with respect to the pathophysiology of the disease, as well as the potential therapeutic benefits of such processes, remain to be determined.

Hypothesis

Based on the available literature, we hypothesize that islet cell differentiation represents a reaction to the local microenvironment, including matrix and growth factor support. Moreover, the islet plays an autocrine role in determining its response to its local microenvironment via the production of specific factors.

Islet cell dedifferentiation is a contributing factor to the pathophysiology of T2DM, whereas *in vitro* islet cell dedifferentiation, proliferation and subsequent redifferentiation represents one means of expanding the number of functional islet cells derived from a single donor organ.

Objectives

- Establish the quality of isolated human islets used in the following studies, as compared to those produced by other clinical islet isolation programs, and identify factors predicting their successful isolation.
- Identify the secreted factors and signalling mechanisms by which isolated human islets determine their own survival following islet isolation.
- Identify the secreted factors and signalling mechanisms by which cultured human islets determine their own differentiated state following collagen embedding and culture.
- Identify the cellular lineages of the major phenotypes of precursor-type cells produced by the collagen embedding and culture of isolated human islets.
- Identify the effect of T2DM on the differentiation and redifferentiation capacity of isolated human islets.

2 Islet Isolations

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2.1 Abstract

Recent advances in the fields of islet transplantation and *in vitro* islet cell expansion place a renewed emphasis on the optimization of islet isolation from cadaveric human donor organs. We retrospectively analyzed 171 islet isolations to identify variables that predict islet yield and isolation success. Cadaveric human donor pancreata were procured and processed according to established protocols. Donor-, procurement- and isolation-related variables were analyzed for correlation with islet yield and isolation success (≥250,000IE). Univariate analysis suggested correlations between islet yield and donor age ($p<0.005$), body surface area ($p<0.005$), duration of enzymatic digestion ($p<0.001$), and pancreatic β-cell volume ($p<0.05$). Donor sex ($p<0.01$), procurement team ($p<0.05$) and peri-digestion serine protease inhibition ($p<0.05$) affected islet yield, while enzyme lot ($p<0.01$) and pancreatic fatty infiltration ($p<0.05$) influenced isolation success. By logistic regression, donor sex and age, and duration of enzymatic digestion could predict a successful isolation with 72% accuracy. The use of Liberase CI improved islet yield ($p<0.05$) in young donors (≤25 years). While donor-related variables are useful in predicting islet yield, these are likely surrogates for pancreatic β-cell volume. Enzyme lot, and the associated duration of enzymatic digestion ($p<0.05$), appears to be key determinants of isolation success.

2.2 Introduction

Diabetes is a disease of insufficient β-cell mass. In T1DM, this deficiency is nearly absolute,[188] with poorly understood auto-immune mechanisms targeting pancreatic islets for destruction.[187] Conversely, T2DM is marked by a decreased functional β-cell mass,[22] characterized by a combination of β-cell dysfunction and insulin resistance.[191] Consequently, therapies aimed at restoring β-cell mass are the focus of ongoing research, especially in light of evidence that tight blood glucose control can prevent and even reverse the secondary complications of diabetes.[185, 186]

Solid organ pancreas transplantation has been established as the gold standard with respect to reversal of diabetic hyperglycemia,[239] although the shortage of available donor organs has limited the clinical application of this therapeutic approach. Islet transplantation, it was thought, might represent a means to compensate for limited organ availability. Thus, given that diabetes only becomes evident with a ≥50% reduction in β-cell mass,[188] it was suggested that islet graft recipients would accordingly require far fewer islets to restore normoglycemia than would normally be found in the whole pancreas. The potential of improved donor:recipient ratios, coupled with the original notion that isolated islets were less immunogenic than the whole organ, led to significant efforts in the fields of islet isolation and transplantation. The landmark Edmonton protocol, which proved conclusively that diabetic hyperglycemia could be reversed with islets from two to three donors,[240] was followed by reports of restoration of normoglycemia in recipients of islets from a single donor.[244] However, recent data suggest that the half-life of graft function remains a constraint.[243]

One benefit of islet transplantation programs has been the increasing availability of high quality human islets for research. This has been instrumental in recent investigative efforts that have demonstrated that cultured islets may be induced to dedifferentiate, proliferate and redifferentiate, thereby providing a potential pathway to improving islet yield per donor organ.[245, 296, 394, 449, 450] Thus, while suitable donor pancreata are considered initially for solid-organ

transplantation, studies to identify ways to enhance islet isolation, survival and cell mass remain an important preoccupation. In fact, studies suggest that organs best suited for solid-organ grafts may differ from those that produce high islet yields.[475-477]

Prior to the implementation of a human islet replacement program at our institution, we have benefited from having received and processed cadaveric human donor pancreata for islet isolation for research purposes for a decade. In this report we describe the donor and isolation variables that we have identified as predictors of clinical islet isolation success.

2.3 Materials and Methods

2.3.1 Organ Procurement and Islet Isolation

Pancreata from adult human cadaveric organ donors were obtained through the local OPO. Islets were isolated according to established protocols.[400] Briefly, organs were flushed *in situ* with cold University of Wisconsin (UW) organ preservation solution and removed *en bloc* with the spleen and a stapled loop of duodenum. Organs were transported on ice to the islet isolation facility.

Upon receipt, organs were dissected free of spleen, duodenum and extraneous tissue, including fat. Next the main pancreatic duct was identified, cannulated and perfused for 10 minutes with a chilled solution containing Liberase HI or CI (Roche Diagnostics, Laval, QC) or Serva Collagenase (Helixx, Scarborough, ON). The distended organ was then cut into pieces, placed in a closed recirculating system (Bio-Rep, Miami, FL) and heated to 37°C to activate the enzyme blend. During the course of enzymatic and mechanical dissociation of the gland, samples were taken and evaluated in real-time, using dithizone (Sigma, St. Louis, MO). Following the appearance of islets unembedded in acinar tissue, the circulation circuit was cooled and the digestate collected in wash solution (Mediatech, Herndon, VA). After washing, the digestate was pooled and incubated on ice in UW solution to allow recovery.

Tissues were separated by continuous Ficoll (Biochrom AG, Berlin, Germany) density gradient centrifugation, using a 1.077 – 1.100g/mL gradient established in a cell processor (COBE DCT, Denver, CO). Aliquots determined to contain free ≥70% pure islets were collected, washed, and counted as IE. Isolations were considered successful if the final yield was ≥250,000IE.

2.3.2 β-Cell Volume and β-Cell Mass

Tissue samples were taken from the tail of the pancreas prior to isolation, and stored at 4°C in phosphate-buffered formalin. Following routine processing and embedding, 4μm sections were cut and dewaxed in xylene and petroleum ether. Slides were permeabilized by incubation in Tris-buffered saline with 0.1% Triton X-100 for 5 minutes, prior to antigen retrieval by heating for 10 minutes in 0.1M citrate buffer, pH 6. Slides were then incubated in blocking buffer (Zymed, San Francisco, CA) for 15 minutes before overnight incubation at 4°C with an α-insulin primary antibody (Dako, Carpinteria, CA). After washing in phosphate-buffered saline (PBS), staining was developed using a biotinylated broad-spectrum secondary antibody, streptavidin-conjugated horseradish peroxidase (HistoStain Plus kit; Zymed, San Francisco, CA) and 3,3'-diaminobenzidine substrate (Dako). Sections were analyzed using a Zeiss Axioskop 40 microscope and Northern Eclipse v6.0 (Empix Imaging, Mississauga, ON). Briefly, individual insulin$^+$ structures were traced and thresholded to determine insulin$^+$ area.[250] Total pancreatic tissue area was similarly determined, and β-cell volume was calculated relative to total pancreatic area. β-Cell mass was calculated by normalizing to pancreatic weight.

2.3.3 Statistical Analysis

Variables of interest were identified based on the literature[475-486] and empirical observations. Donor variables included age, sex, height, weight, body mass index (BMI = weight / height^2) and body surface area (BSA = $\sqrt{}$ (height * weight / 3600)), as well as a routine biochemical blood screen. Procurement variables included length of donor hospitalization, OPO coordinator, procurement team,

cause of death, peri-procurement hemodynamic measures and use of vasopressors. Organ and isolation variables included assessment (yes/no) of fatty infiltration and pancreas quality (capsule damage, edema or fibrosis); pancreatic weight; degree of distension after perfusion; enzyme type, lot and specific activity; duration of cold ischemia and enzymatic digestion; and the proportion of the yield derived from islets with diameters <150μm (small islets). There was little turnover over the 5-year study period with respect to laboratory members involved with islet isolations.

Results are presented as mean ± SEM. Statistical significance between means was determined using two-tailed Student's t-tests. Linear regression analysis was performed to relate continuous variables with islet yield, while χ^2 analyses were used to assess categorical variables with respect to isolation success. Stepwise logistic regression analysis was performed to establish a simple probability model to predict isolation success. Differences were considered significant at $p<0.05$.

2.4 Results

2.4.1 Islet Isolations

From January 2002 to June 2007, the Centre for Pancreatic Diseases at the McGill University Health Centre received 242 donor organs for islet isolation. Of these, 19 (7.9%) were from donors diagnosed with T2DM, and were excluded from this study. A further 52 organs were not processed for isolation because of donor- or procurement-related issues. As a result, 171 human islet isolations were available for analysis.

The average yield was 223,000±14,000IE, although 18 isolations did not produce any islet yield with a purity ≥70%. The majority (71.3%) of isolations produced yields of ≥100,000IE, including 38.0% with yields ≥250,000IE, considered to be successful.[240]

The number of organs received annually increased sharply at the beginning of the study period, but then leveled off, as did the average islet yield (Fig. 2.1A).

Increased organ numbers were associated with increased isolation success, and better discrimination of organ quality. This, in turn, led to a concomitant decrease in the number of isolations performed (Fig. 2.1B).

To identify factors that predict islet yield, we performed linear regression analysis between continuous variables and islet yield. Of the variables considered, donor age, height, weight, BMI and BSA were positively correlated with islet yield, though not strongly, while the duration of enzymatic digestion and proportion of the yield composed of small islets were negatively correlated with islet yield (Table 2.1). Based on these, we determined variable values that would correlate with a yield of 250,000IE.

To better understand the effect of these continuous variables on isolation outcome, we compared the mean values associated with successful and unsuccessful isolations. In this analysis, the effect of BMI was no longer significant (Table 2.2). However, with respect to the other continuous variables identified, the mean values associated with successful and unsuccessful isolations were found to separate around the calculated values associated with a yield of 250,000IE. Put differently, the predicted values calculated in Table 2.1 appear to serve well as threshold values for isolation success.

We next analyzed the average yields associated with categorical variables, and identified male donors and local procurement team as associated with higher islet yields. A similar trend was identified for pancreatic fatty infiltration (Fig. 2.1C). Male donors tended to be taller (171.8±0.7 vs. 160.5±0.9cm, $p<0.001$) and heavier (78.8±1.6 vs. 67.8±1.8kg, $p<0.001$) than female donors, resulting in increased BSA (1.94±0.02 vs. 1.73±0.03m^2, $p<0.001$) but no difference in BMI (26.9±0.5 vs. 26.2±0.6kg/m^2, n.s.). All these variables were associated with increased islet yield (Table 2.1). With respect to the effect of the procurement team, subjective assessment of organ quality, by blinded observers, was linked to local procurement (58.7 vs. 32.4%, $p=0.007$), while cold ischemia time did not differ between local and other procurement teams (7.6±0.5 vs. 7.3±0.5h, n.s.). In fact, all procurement teams in this study are based in the same city, suggesting that the observed effect is unrelated to logistic considerations.

All categorical data, including threshold values for continuous variables, estimated by linear regression, were analyzed for associations with isolation success (Table 2.3). Again, BMI was no longer associated with isolation success, although BSA remained significant. The only new categorical variable to be identified was that of fatty infiltration of the pancreas, which was more common in successful isolations.

2.4.2 Logistic Regression

To establish a model to predict isolation success, we performed stepwise logistic regression analysis, using the factors identified from the preceding analyses of both categorical and continuous variables. Working backwards to provide a simplified model for predicting isolation success, we identified three variables that, when combined, allowed for an accuracy of 72.3% with respect to predicting a successful isolation (Table 2.4).

2.4.3 Two-Layer Preservation and Serine Protease Inhibition

In the interest of maintaining an up-to-date isolation protocol, we assessed two recent improvements to the standard protocol. First, we assessed the two-layer method of pancreas preservation (n=4), using pre-oxygenated perfluorocarbons as an oxygen feeder layer, as described.[487] While this technique is designed for situations of longer preservation times, cold ischemia was kept relatively constant (6.9±1.3 vs. 7.6±0.3h) in order to study the direct effect on islet yield. Use of the two-layer method did not affect isolation yield (Fig. 2.1C), suggesting that there is no drawback to the use of this method in cases where longer cold ischemia times may be an issue.

Next, to examine the effect of serine protease inhibition, a commercially available inhibitor was added to the diluted enzyme blend, as described.[488] The inhibitor was used in only 3 isolations and then abandoned due to empirical observations of unsuccessful isolation. Indeed, average yield was significantly reduced relative to isolations performed using the same enzyme lot, but without the inhibitor added (Fig. 2.1C).

2.4.4 Enzyme Lot

The use of Liberase HI has become the accepted standard with respect to pancreatic digestion for clinical islet isolation.[489] Each lot of Liberase has a distinct enzymatic profile, as assessed by Wunsch and Caseinase activity. While the majority of isolations (93.0%) were performed using Liberase HI, a total of 22 lots were employed, for a minimum of 3 isolations each. The importance of enzyme lot was observed empirically, and indeed lot number was a strong predictor of isolation success ($p=0.009$). Moreover, while statistical analyses suggested that the duration of enzymatic digestion is a predictor of both isolation yield (Table 2.1) and success (Table 2.2), duration of enzymatic digestion tended to be associated with enzymatic lot ($p=0.048$), such that enzyme lot is a strong predictor of a digestion time ≥12min ($p=0.004$). There was no association, however, between Wunsch or Caseinase activity, and digestion length or isolation yield.

2.4.5 Enzyme Type

Based on experience, we identified young donors (≤25 years) as a population with sub-optimal isolation results; with islet yield being significantly reduced (101,000±27,000 vs. 234,000±15,000IE, $p=0.006$). While decreased β-cell mass likely affects yield in young donors,[490] we hypothesized that Liberase CI might provide a better enzymatic composition for the retrieval of islets from organs from young donors, based on a tissue architecture that is comparable to canine pancreata. We tested the effect of Liberase CI on a limited number of donor organs (n=8), including young donors (n=4). While the overall average yield did not differ between isolations performed with Liberase HI and Liberase CI, the average yield from young donors was significantly higher when Liberase CI was used (Table 2.5).

2.4.6 Pancreatic β-Cell Mass and Islet Size

We routinely obtained biopsies from the tail of donor organs prior to islet isolation, and we assessed β-cell volume and β-cell mass in a limited number of randomly selected organs (n=41). There was a significant correlation between isolation yield and β-cell volume, as determined by linear regression analysis (Table 2.1). Moreover, successful isolations were achieved from organs with significantly increased β-cell volume (Table 2.2).

The proportion of small islets (diameter <150μm) appeared to be a strong predictor of both isolation yield (Table 2.1) and success (Table 2.2). This variable was identified as a surrogate for digestion quality, given that over-digested islets tend to be smaller. It is not surprising that isolations that produced a higher proportion of small islets also had lower overall yields, given that yields were calculated based on IEs. However, analysis of average *in situ* insulin^{+} cross-sectional area, a measure of islet size prior to digestion, shows no correlation with the proportion of small islets. This suggests that yields comprised of small islets are a result of the isolation itself. Moreover, the proportion of small islets was associated with enzyme lot (p=0.015), suggesting that over-digestion is a characteristic of certain enzyme lots.

Table 2.1: Correlations Between Continuous Variables and Isolation Yield

Variable	Correlation	r^2	p	Value at 250,000IE
Age (years)	+	0.057	0.002	51.6
Height (cm)	+	0.028	0.029	167
Weight (kg)	+	0.063	0.001	74.1
BMI (kg/m^2)	+	0.025	0.038	26.7
BSA (m^2)	+	0.058	0.002	1.85
Enzyme digestion (min)	-	0.131	<0.001	12.0
Small islets (% yield)	-	0.173	<0.001	77.7
β-Cell volume (%)	+	0.104	0.040	2.28

Table 2.2: Mean Values for Continuous Variables Associated with Isolation Yield

Variable	Yield (IE)		p
	<250,000	**≥250,000**	
Age (years)	49.1±1.5	54.3±1.5	0.020
Height (cm)	165±1	169±1	0.002
Weight (kg)	70.6±1.6	78.4±1.8	0.002
BMI (kg/m^2)	26.1±0.5	27.3±0.6	0.107
BSA (m^2)	1.79±0.02	1.91±0.03	0.001
Enzyme digestion (min)	12.9±0.3	10.8±0.3	<0.001
Small islets (% yield)	81.2±1.2	73.2±1.6	<0.001
β-Cell volume (%)	0.97±0.11	2.20±0.35	0.002

Table 2.3: Frequencies of Categorical Variables Associated with Isolation Yield

Variable	Yield (IE)		p
	<250,000	≥250,000	
Male (%)	41.6	67.2	0.002
Age >45 years (%)	67.9	87.7	0.004
Height ≥167cm (%)	40.0	61.5	0.006
Weight ≥75kg (%)	40.0	66.2	0.001
BMI ≥27kg/m^2 (%)	41.3	52.3	0.164
BSA ≥1.85m^2 (%)	41.3	64.6	0.003
Fatty pancreatic infiltration (%)	62.0	79.2	0.039
Enzyme digestion <12min (%)	38.1	64.6	0.001
Small islets <75% yield (%)	19.6	43.1	0.001

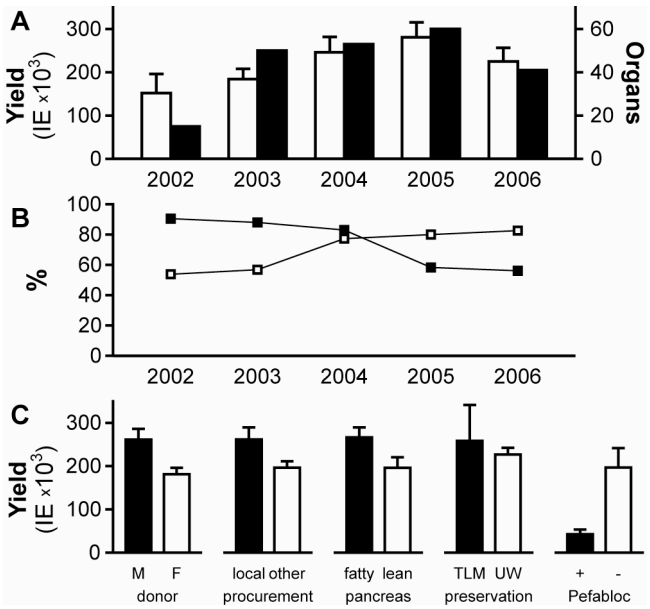
Table 2.4: Stepwise Multivariate Logistic Regression of Variables Predicting Isolation Success

Variable	B coefficient	p
Male	0.935	0.011
Age (years)	0.034	0.022
Enzyme digestion (min)	-0.295	<0.001
Constant	0.685	0.572
Accuracy	72.3%	

Table 2.5: Comparison of Enzyme Types in Young Donors

Variable	Liberase HI	Liberase CI	p
Yield (IE), all donors	228,000±15,000	229,000±45,000	n.s.
Yield (IE), donors ≤25 yrs	78,000±30,000	188,000±50,000	0.044
Wunsch (U/vial)	2230±20	1140±30	<0.001
Caseinase (U/vial)	51,800±3,800	67,000±500	<0.001

Figure 2.1: Isolation Yields and Success
A. Average yearly isolation yield (□) increased initially, but has since reached a plateau, as has the number of donor organs received yearly (■).
B. As isolation success rate (□) increases, the ability to predict isolation success improves, resulting in a decrease in the proportion of isolations performed (■).
C. Analysis of the effect of categorical variables on islet yield indicates that male donors (p=0.007) and local procurement team (p=0.030) are associated with increased isolation yield, while a trend is observed with respect to fatty infiltration of pancreata (p=0.066). No difference in islet yield was observed between organs preserved using the TLM and the traditional UW method. After only three attempts with a commercially available serine protease inhibitor (Pefabloc), this experiment was ceased, as isolation yield was adversely affected by protease inhibition, compared to isolations performed using the same enzyme lot (p=0.043).


A
Yield
(IE x10³)
300
200
100
0
60
40
20
0
Organs
2002
2003
2004
2005
2006
B
%
100
80
60
40
20
0
2002
2003
2004
2005
2006
C
Yield
(IE x10³)
300
200
100
0
M
F
donor
local
other
procurement
fatty
lean
pancreas
TLM
UW
preservation
+
-
Pefabloc

2.5 Discussion

While much progress has been made over the past 35 years in the development of islet transplantation as a cellular therapy for diabetes, outcomes are highly variable between centers, and successful outcomes are difficult to predict.[491] Moreover, sustained graft function appears to be difficult to maintain for reasons that remain to be fully elucidated.[243]

There are many variables associated with the islet transplant procedure, including those related to the donor, the pancreatic tissue, the isolation itself and, of course, the recipients. In the field of islet isolation, many attempts have been made to identify the critical variables that would predict high yields of high quality islets.[475-486] However, the utility of such studies rests on the identification of variables that can be used to screen donor organs prior to the isolation procedure. To this end, a recent study reported on identifying donor variables predicting pancreatic weight,[492] which serves as a surrogate for islet yield.[477] We did not observe such a correlation in our analyses (p=0.758). In fact, a review of the literature addressing isolation success indicates that few predictors are consistently identified, though these include donor age,[475, 477, 480] weight and/or BMI,[476, 477, 479, 480, 483] pancreatic quality[477, 481-483, 485] and enzyme lot and/or digestion duration;[477, 478, 480, 484, 486] the same factors found in our predictive model. Other groups have also reported an effect of increased cold ischemia,[475, 478, 493] which we did not observe. While islet yield and function are not necessarily related, we have previously analyzed islet viability and function,[494, 495] and our values are in keeping with those reported by other leading islet isolation facilities.[491]

A simple examination of the islet isolation procedure indicates that there are two requirements for a successful islet isolation; a large number of islets within the pancreas, and the ability to efficiently isolate them. Studies of donor and isolation variables are in fact identifying predictors of one or the other. Hence, donor variables associated with islet yield, namely age and weight, and/or BMI, are also associated with β-cell volume.[490, 496] We therefore sought to establish directly the association between β-cell volume and isolation yield. In fact, this is

the first report, to our knowledge, comparing β-cell volume and islet isolation yield from the same donor organs. Linear regression analysis of a limited number of samples demonstrated significance, and successful isolations were associated with donor organs with increased β-cell volume. Thus, while we anticipate that further analyses will indicate a strong correlation between β-cell volume and isolation yield, the influence of other isolation variables is such that an expanded sample size is required.

While the presence of islets within the pancreas is paramount, this fact alone does not guarantee their successful isolation. As we observed, enzyme type and lot are consistently identified as key variables in determining isolation success.[477, 478, 480, 484, 486] In fact, the issue of enzymatic activity is so critical to successful islet isolation that one report suggests that the selection between different enzyme preparations be based on general donor characteristics.[486] It is in keeping with this opinion that we propose the use of a different enzymatic preparation, Liberase CI, for young donors. Optimization of islet yield in this donor population is all the more important given that islets isolated from young donors appear to be the most functional.[480]

Since tissue quality is of critical importance for either whole pancreas or islet transplantation, methods to maintain or improve tissue preservation following organ harvest have engendered much attention. One such approach is the so-called two-layer method.[487] Although our experience with organs procured using this method has been limited, we can confirm that the two-layer method allows for successful islet isolation. While this technique of organ preservation now rivals UW as the standard approach to pancreatic preservation for the purposes of islet isolation,[491] the time and costs associated lead us to suggest that this technique only be applied for procurements in which the duration of cold ischemia may be excessively prolonged.

In this report, we analyzed variables to identify predictors of islet yield. Stepwise logistic regression provided a simple model for predicting isolation success based on donor sex and age and enzymatic digestion time. Analysis of β-cell volume suggests a correlation with islet yield, explaining the importance of

donor variables that have been associated with β-cell volume.[490, 496] However, our results, and a review of the literature, suggest that enzyme type and lot are in fact valuable predictors of isolation success, likely more so than donor variables. Moreover, we have also observed, empirically, an increase in the consistency of recent lots of available enzymes, suggesting that one of the key factors dictating isolation success may, in fact, be less of an issue moving forward. Finally, the results of continuing retrospective analysis of this type has allowed our laboratory to be more selective with respect to organs received, leading to improved cost-effectiveness. This selectivity, coupled with tailoring of the isolation procedure to specific variables, has led to ever increasing isolation success. However, the fact remains that organs for clinical islet isolation are in short supply, while the predictive value of isolation variables is limited. Taken together, islet isolation should be attempted whenever a suitable organ becomes available.

2.6 Acknowledgements

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Bridging Statement Between Manuscripts

The previous chapter established the ability of our islet isolation facility to purify human islets with a purity and quality that matches that of other sites. Furthermore, we demonstrated that the isolation yield is directly proportional to the β-cell mass found within the donor pancreas and that enzyme lot is a strong predictor of isolation success.

However, while these factors gauge isolation success, they do not serve as accurate predictors of the functional capacity, *in vitro* or *in vivo*, of the isolated islets. In fact, post-isolation apoptosis is a significant contributor to the paucity of human islets for transplantation.[242] Significant effort has been put into identifying the external factors that initiate this process, while more recent research suggests an autocrine role for the islets in initiating this response to isolation. The aim of Chapter 2, therefore, was to identify the role of endogenously produced TNFα in islet apoptosis post-isolation.

3 Islets Produce Pro-Apoptotic Factors in Response to Isolation

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3.1 Abstract

Recent successes in islet transplantation highlight the importance of islet isolation by experienced centers and minimization of cell injury as crucial to the achievement of insulin independence. Islet injury may manifest as cell death by apoptosis, shorter graft survival and the need for re-transplantation. While an inflammatory cytokine response at the graft site is known to inhibit engraftment, recent evidence indicates that islet cells may contribute to this response. Isolated human islets were cultured for up to one week in serum-free CMRL-1066 with 25μM of TNFα inhibitor RDP58. Gene expression was measured by RT-PCR, apoptosis and TNFα secretion by ELISA and ELISpot and islet function by stimulated insulin secretion. Isolation induced a 2-fold increase in TNFα expression between days 1 and 3 ($p<0.05$), while TNFα secretion peaked at day 1. RDP58 reduced TNFα secretion by 70.6% ($p<0.02$), though TNFα gene expression was unaffected. RDP58 reduced the frequency of TNFα-secreting islets by 64.4% ($p<0.05$) and reduced apoptotic levels by 26.4% within 24 hours post-isolation ($p<0.05$). The reduction in apoptosis was maintained throughout the week ($p<0.01$), while apoptosis increased in control cultures. Finally, RDP58-treated islets displayed increased insulin secretion in response to both elevated glucose (1915.0±396.6 vs. 825.3±261.1mU/L, $p<0.01$) and secretagogues (2294.3±529.5 vs. 939.8±333.7mU/L, $p<0.02$). These data demonstrate that intra-islet cytokine production should be considered as a factor leading to islet cell death post-isolation and post-engraftment, and strategies aimed at countering islet cytokine production represent a novel target for improving islet viability and function.

3.2 Introduction

The transplantation of isolated islets offers a promising therapeutic alternative to exogenous insulin or whole organ pancreas transplantation in the treatment of T1DM. Recent clinical achievements have highlighted the importance of careful islet isolation by experienced centers and minimization of cell injury as elements crucial to the achievement of insulin independence.[240, 497] Despite these successes, islet grafts display reduced viability compared to whole organ transplants, with a functional half-life of approximately three years.[498] This limited islet graft survival has been attributed, among other factors, to the slow erosion of an already marginal engrafted β-cell mass, as a result of the isolation, transplantation and engraftment procedures. A factor contributing to this loss may be a local inflammatory cytokine response,[420] possibly mediated by IL1β, IFNγ and TNFα release by recipient hepatic Kupffer cells.[421] As a result, many attempts have been made to reduce graft immunogenicity and/or prevent cytokine exposure by islet microencapsulation[499] as well as to protect islets from cytokine-mediated apoptosis by gene transfer[500] or pharmacologic pre-treatment.[501, 502]

While the above work is predicated on host-derived cytokine release, similar inflammatory responses are observed in and around syngeneic and autologous islet grafts,[503, 504] suggesting a role for the graft proper in cytokine production. Previous studies have established that islets produce pro-inflammatory cytokines during isolation and engraftment.[422-424] Resident macrophages, ductal cells and vascular endothelial cells contained within islet preparations have been proposed as the sources of intra-islet cytokine production.[502, 505]

It is well established that islets undergo apoptosis simply as a result of the isolation procedure.[242] While it has been proposed that exposure to endotoxin found in the collagenase blend induces islet cytokine production,[423, 427] cytokine production may also represent a response to the ischemic injury brought about by islet isolation. Thus, while reports suggest that inhibition of cytokine signalling to islets may improve graft function and viability,[500, 506-512] we report herein that inhibition of intra-islet cytokine production represents another therapeutic target

for improving islet graft viability with a view to improving the efficiency of the islet isolation and transplantation procedure.

3.3 Materials and Methods

3.3.1 Organ Procurement and Islet Isolation

Pancreata from adult human cadaveric organ donors were procured at the time of multi-organ procurement. Donor and isolation characteristics of pancreata used in these studies reflected the donor and isolation characteristics of all human pancreata received by this center over the same course of time (Table 3.1). Research consent was obtained through the local OPO, Québec-Transplant. Due to the current scale up from research to clinical islet program, islets from these isolations were prepared according to established clinical protocols,[400, 489] but were not used for human islet transplantation. Briefly, the organ was flushed and cold-preserved in Viaspan (Barr Laboratories, Pomona, NY) solution. The main pancreatic duct was cannulated and perfused with Liberase HI (Roche Diagnostics, Laval, QC). The perfused organ was placed in a closed system (Bio-Rep, Miami, FL) and heated to 37°C to activate the enzyme blend. Following the appearance of free islets by real-time visualization with dithizone (Sigma-Aldrich, St. Louis, MO), the system was cooled and free tissue was collected and washed. Tissue was applied to a continuous Ficoll (Biochrom AG, Berlin, DE) density gradient in a cell processor (COBE, Denver, CO). Free islets, were collected, washed and counted.[513] Mean islet purity, determined visually, was 84±1%, with a minimum of 70% purity, so as to reflect the composition of islet preparations isolated for transplantation.

3.3.2 Islet Culture

Islets were cultured in serum-free CMRL-1066 (Gibco, Burlington, ON), supplemented with 0.1% bovine serum albumin (Sigma-Aldrich) at 37°C, 5% CO_2. Where indicated, 10μg/mL lipopolysaccharide (LPS, Sigma-Aldrich) and/or 25μM H-*d*-Arg-*d*-Nle-*d*-Nle-*d*-Nle-*d*-Arg-*d*-Nle-*d*-Nle-*d*-Nle-Gly-*d*-Tyr-NH_2

(RDP58, Genzyme, Cambridge, MA) were added immediately following islet isolation. This concentration was chosen based on the supplier's information indicating significant post-transcriptional inhibition of TNFα production.

3.3.3 Analysis of TNFα Transcription

On days 0, 1, 3, 5 and 7 post-isolation, relative TNFα messenger ribonucleic acid (mRNA) production was evaluated by real-time polymerase chain reaction (RT-PCR). Briefly, samples were stored in Trizol (Invitrogen, Burlington, ON) until the time of RNA isolation. Following RNA extraction, as per the manufacturer's protocol, cDNA was reverse transcribed using an Omniscript RT kit (Qiagen, Mississauga, ON). Custom primers (Invitrogen) for TNFα, TNFR1, IL1β, IL1R2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesised (Table 3.2), and real-time PCR was performed using a QuantiTect SYBR green RT-PCR kit (Qiagen) in a MJ Research DNA Engine Opticon 2 (Bio-Rad, Mississauga, ON). Expression levels, normalized to GAPDH, are expressed relative to day 0.

3.3.4 Evaluation of TNFα Production

Relative islet TNFα production was determined by Western blot. Briefly, conditioned media samples were concentrated using centrifugal filter devices (Millipore, Nepean, ON). After protein quantification,[514] samples of equal protein content were separated by denaturing electrophoresis on 15% polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked (25 mM Tris, 150 mM NaCl, 0.1% Tween-20, 3% w/v bovine serum albumin) and probed with primary antibodies diluted in blocking buffer. α-TNFα and α-IL1β were used at 1:500 (Chemicon, Temecula, CA). After washing, blots were incubated with 1:5000 α-rabbit-HRP (Chemicon) diluted in blocking buffer. After another wash, blots were developed using chemiluminescent reagent (New England Nuclear, Boston, MA) and exposed to X-OMAT film (Fisher Scientific, Ottawa, ON).

TNFα secretion was determined by enzyme-linked immunosorbent assay (ELISA, ALPCO Diagnostics, Windham, NH) on 24-hour samples of conditioned media.

Following 24 hours incubation, the relative frequency of TNFα-producing islets was determined by ELISpot (BD Biosciences, San Diego, CA) using an Immunospot detector (Cellular Technology, Cleveland, OH). The assay is an ELISA-based method of detecting individual TNFα-producing cells, visualized as spots on a membrane.

3.3.5 Assessment of Apoptosis

Apoptosis was assessed on days 0, 1, 3, 5 and 7 post-isolation using a programmed cell death ELISA (Roche Diagnostics). The assay detects oligonucleosomal DNA fragmentation characteristic of apoptosis. Samples were normalized to total DNA content, evaluated fluorometrically.[515]

3.3.6 Glucose-Stimulated Insulin Secretion

Following 24h of culture, handpicked samples of 100 islets were washed in serum-free RPMI-1640 with 0.5% bovine serum albumin and incubated for 1h each with RPMI-1640 containing, sequentially, 2.2mM glucose, 22mM glucose and 22mM glucose + 50μM IBMX (Sigma-Aldrich), an inhibitor of cAMP phosphodiesterase. Finally, islets were washed in serum-free RPMI-1640 and incubated for one hour in 2.2mM glucose. Conditioned media samples were analyzed for insulin content using a commercially available insulin ELISA kit (ALPCO Diagnostics). Insulin secretion was normalized to sample protein content, determined as above. Stimulation index was calculated as insulin secretion at 22mM glucose ÷ insulin secretion at initial 2.2mM glucose.

3.3.7 Statistical Analysis

Analyses were performed in triplicate per donor organ, with results expressed as mean ± SEM. Statistical significance was determined using one-way ANOVA with *post hoc* Bonferroni's test, or Student's *t*-test, where appropriate. Linear

regression analysis was performed to assess trends in programmed cell death ELISA samples. In all cases, control and treatment samples were obtained from the same donor. Differences were considered significant at $p<0.05$.

3.4 Results

3.4.1 Isolation Induces Islet TNFα Production

Following isolation, TNFα transcription levels doubled, peaking between one and three days, and declined thereafter (Fig. 3.1). Similarly, IL1β transcription levels spiked following isolation, although IL1β expression was highly variable and did not approach significance. The receptors for these two cytokines, TNFR1 and IL1R2, displayed maintained expression levels. Treatment with RDP58 did not affect gene expression levels for any of the transcripts assessed.

Isolation induced a pronounced increase in TNFα secretion within 24 hours (Fig. 3.2). Again, these levels peaked at one day post-isolation and declined rapidly thereafter. Treatment with RDP58 diminished TNFα secretion by 70.6% at 24 hours ($p<0.02$). TNFα secretion returned to basal levels by day 3 post-isolation. In fact, TNFα secretion was barely detectable by day 5, and this trend was maintained until at least one week post-isolation. Thus, the effect of RDP58 was only observable in the first days following isolation.

Mirroring the transcription data, IL1β secretion was observed throughout the culture period, although no decline was observed as far as one week post-isolation. While RDP58 reduced islet TNFα secretion, no difference in IL1β secretion was observed.

Within 24 hours of isolation, 6.5±2.0% of islets secreted levels of TNFα detectable by ELISpot (Fig. 3.3). One day of culture with RDP58 was sufficient to significantly decrease the frequency of $TNF\alpha^+$ islets, by as much as 60%. Furthermore, while LPS stimulation induced a three-fold increase in $TNF\alpha^+$ islets, RDP58 treatment was sufficient to completely abrogate the TNFα-inducing effect of LPS.

3.4.2 Inhibition of TNFα Production Improves Islet Survival

Islet isolation induced a significant progressive increase in islet apoptosis. Apoptotic levels increased with successive days in culture, with day 7 levels as much as 3-fold higher than initial levels (Fig. 3.4). Within 24 hours of RDP58 treatment, apoptotic levels were significantly decreased ($p<0.05$). This effect persisted over the entire culture period such that the rate of apoptosis did not differ significantly over the course of post-isolation culture with RDP58, while control cultures showed a significant increase in apoptosis.

3.4.3 Inhibition of TNFα Production Improves Islet Function

Following 24 hours of culture with RDP58, islets displayed significantly increased basal, glucose-responsive and secretagogue-stimulated insulin secretion (Fig. 3.5). While the calculated stimulation index did not differ between control and RDP58-treated islets (2.45±0.10 vs. 2.57±0.37), this observation reflects the increases in both basal and stimulated insulin release.

Table 3.1: Donor and Isolation Characteristics

	Isolations 03-05 (n=103)	Study Isolations (n=18)	p
Age (yrs)	52.1±1.3	52.9±4.0	n.s.
Age >45yrs (%)	79.6±4.0	83.3±9.3	n.s.
Male (%)	52.5±5.1	52.9±12.9	n.s.
BMI (kg/m^2)	26.6±0.5	25.4±0.4	n.s.
BMI >30kg/m^2 (%)	22.5±4.2	22.2±10.4	n.s.
Traumatic cause of death (%)	30.1±4.6	27.8±11.2	n.s.
ICU duration (d)	2.8±0.3	2.3±0.5	n.s.
ICU duration >3d (%)	36.0±4.8	31.3±12.4	n.s.
Glucose (mM)	8.9±0.4	9.0±0.9	n.s.
Glucose >11.1mM (%)	15.5±3.7	11.8±8.3	n.s.
Amylase (U/L)	204.6±35.8	234.5±73.6	n.s.
Amylase >220U/L (%)	24.7±4.5	18.8±10.4	n.s.
Catecholamine use (%)	79.6±4.0	83.3±9.3	n.s.
Cold ischemia (h)	7.0±0.4	6.5±0.6	n.s.
Cold ischemia >8h (%)	32.7±4.8	27.8±11.2	n.s.
Yield (IE)	269,404±19,903	292,546±35,379	n.s.
Yield >250,000IE (%)	46.6±5.0	50.0±12.5	n.s.

Table 3.2: RT-PCR Primers and Conditions

Gene	Primers	Annealing (°C)	Amplicon (bp)
TNFα	5'-TGACAAGCCTGTAGCCCATGT-3' 5'-GCAATGATCCCAAAGTAGACCT-3'	60	440
TNFR1	5'-AAATGGGTCAGGTGGAGATC-3' 5'-GGCTTAGTAGTAGTTCCTTCC-3'	57	474
IL1β	5'-GGAAAAGAATCTGTACCTGTC-3' 5'-AATAGGGAAGCGGTTGCTCA-3'	55	621
IL1R2	5'-TCCTGCCGTTCATCTCATACC-3' 5'-TCCATGTGCAAATCCTCTCTT-3'	57	576
GAPDH	5'-CCTTCATTGACCTCAACTACATGGT-3' 5'-GAGGGGCCATCCACAGTCTTCTG-3'	60	469

Figure 3.1: Islet Isolation Induces TNFα Expression
Islets were cultured for up to one week post-isolation with (■) or without (□) 25μM RDP58. Total RNA was isolated and TNFα, TNFR1, IL1β and IL1R2 mRNA levels were assessed by RT-PCR, normalized to GAPDH levels and expressed relative to day 0 (* $p<0.05$ vs. day 0).

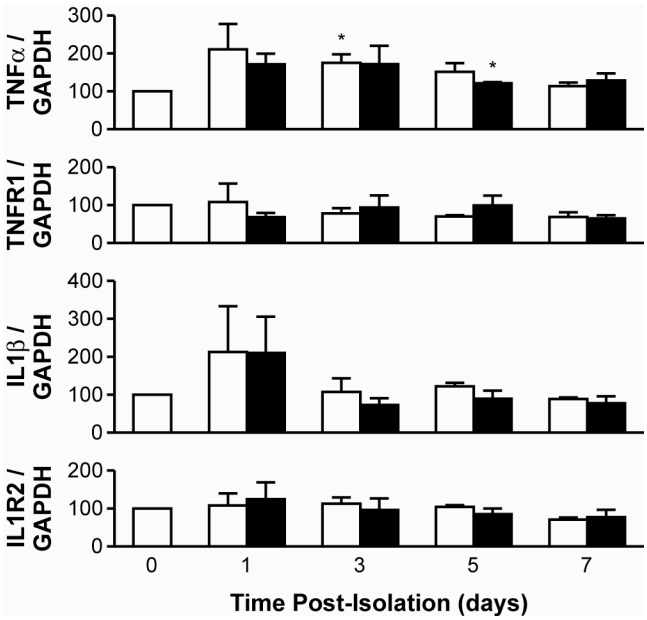

TNFα / GAPDH
300
200
100
0
*
*
TNFR1 / GAPDH
200
100
0
IL1β / GAPDH
400
300
200
100
0
IL1R2 / GAPDH
200
100
0
0
1
3
5
7
Time Post-Isolation (days)

Figure 3.2: Islet Isolation Induces TNFα Secretion
Islets were cultured for one week post-isolation with (■) or without (□) 25μM RDP58.
A. TNFα content in conditioned media was determined by ELISA.
B. Conditioned media were concentrated and TNFα and IL1β secretion evaluated by Western blot (* $p<0.05$ vs. same day control).

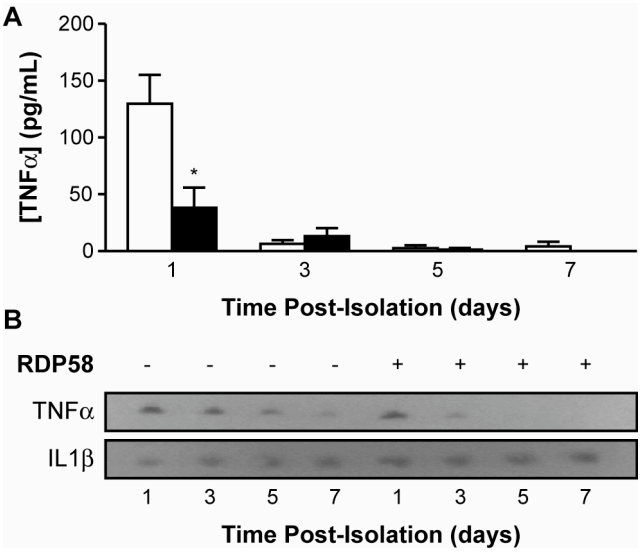

A
200
150
100
50
0
[TNFα] (pg/mL)
*
1
3
5
7
Time Post-Isolation (days)
B
RDP58
- - - - + + + +
TNFα
IL1β
1 3 5 7 1 3 5 7
Time Post-Isolation (days)

Figure 3.3: RDP58 Inhibits Islet TNFα Secretion
Islets were cultured with or without 10μM LPS and 25μM RDP58 for 24h. TNFα$^+$ islets were determined by ELISpot (* $p<0.05$ vs. control; ¶ $p<0.01$ vs. control; ‡ $p<0.01$ vs. LPS).

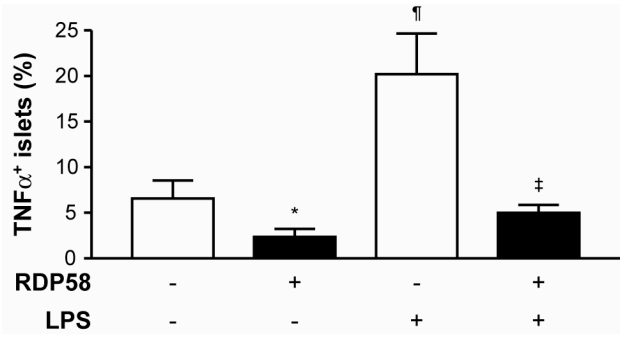

TNFα+ islets (%)
25
20
15
10
5
0
¶
*
‡
RDP58 - + - +
LPS - - + +

Figure 3.4: Inhibition of TNFα Secretion Improves Islet Viability
Islets were cultured for up to one week post-isolation with (■) or without (□) 25μM RDP58. Viability was assessed by cell death ELISA, normalized to sample DNA content (* $p<0.05$ vs. day 0 control; ¶ $p<0.05$ vs. same-day control; ‡ $p<0.01$ vs. same-day control).

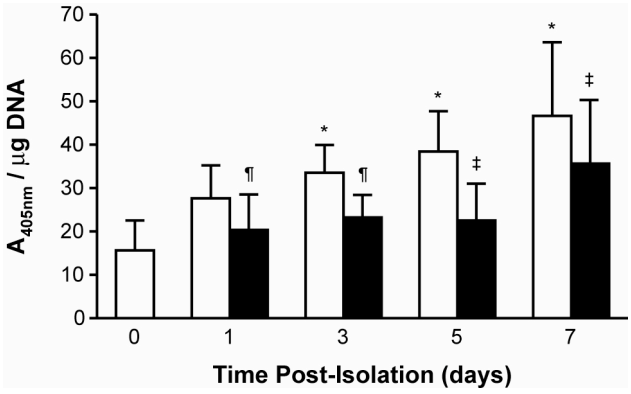
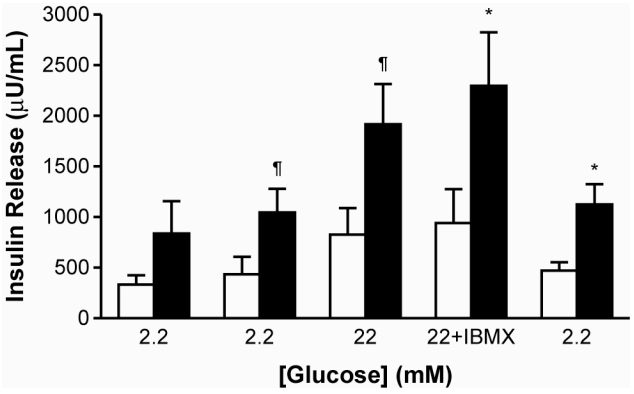

70
60
50
40
30
20
10
0
A_{405nm} / µg DNA
0
1
3
5
7
Time Post-Isolation (days)
*
¶
*
¶
*
‡
*
‡

Figure 3.5: Inhibition of TNFα Secretion Improves Islet Function
Islets were cultured for 24 hours with (■) or without (□) 25μM RDP58. Islets were then cultured sequentially with varying glucose concentrations (see Methods), and conditioned media assessed by ELISA for insulin secretion. Results were normalized to sample DNA content (* $p<0.05$ vs. same-concentration control; ¶ $p<0.01$ vs. same-concentration control).


Insulin Release (μU/mL)
3000
2500
2000
1500
1000
500
0
¶
¶
*
*
2.2
2.2
22
22+IBMX
2.2
[Glucose] (mM)

3.5 Discussion

It has long been established that islet isolation and engraftment induce cytokine production,[427, 516, 517] and that cytokines induce islet cell death that contributes to islet graft failure.[518] Islet-toxic TNFα has been shown to be produced locally at the graft site,[421] as well as within the islet as a response to endotoxin contained within impure collagenase blends.[423] However, islets used in the experiments described herein were isolated using low-endotoxin Liberase HI (11.1±2.3EU/mL), limiting the effect of collagenase-based endotoxin contamination on the islet response. Furthermore, reports attributing intra-islet cytokine production to collagenase-based endotoxin exposure examined the first 18 hours following isolation,[423] while we report that islet cytokine production peaks at one to three days post-isolation.

Data obtained from the ELISpot assay indicate that a small number of islets appear to secrete detectable levels of TNFα following isolation (Fig. 3.3). It is tempting to misconstrue these results as indicating that a small subset of islets are responsible for the significant amounts of TNFα secreted into islet cultures; we interpret these results as indicating that intra-islet cytokine levels are likely much higher, as any membrane-associated cytokines or cytokines acting in a juxtacrine or paracrine fashion within the same islet would not necessarily yield a positive result given the conditions of the assay.

The effects of the observed increase in TNFα transcription and secretion were immediately evident; apoptosis increased steadily over the course of culture, as has previously been observed for routinely isolated islets.[242] Inhibition of TNFα production by RDP58 demonstrated the role of TNFα in post-isolation apoptosis; levels were consistently and significantly decreased compared to control, to the extent that apoptosis did not significantly increase over one week in treated cultures, while control cultures demonstrated a three-fold increase in apoptotic levels (Fig. 3.4). Thus, while we previously reported the incidence of apoptosis post-isolation,[242] we now suggest that this cell death may be induced at least partially by the islet proper, via intrinsic cytokine production. This cytokine production may represent a response to the ischemic conditions present during

the isolation procedure; macrophages are known to produce TNFα in response to hypoxic insults in the context of transplantation.[519-521] Furthermore, the system may be self-amplifying; islet cytokine production is upregulated by cytokine exposure.[502] However, transcriptional data suggests that cytokine production is highest shortly after isolation, and returns to basal levels over the course of culture, although apoptotic rates continue to increase. Thus, while the trend in apoptotic rates would suggest a snowball effect, this effect is not reflected in TNFα or IL1β transcription or secretion levels. These data would suggest that while isolation induces cytokine production, which in turn initiates a self-propagating cycle of apoptosis, TNFα and IL1β do not represent the signals that propagate the apoptosis signal. Future work would therefore seek to determine whether transient inhibition of TNFα post-isolation would provide as dramatic an effect.

It is notable that RDP58 treatment for 24 hours significantly reduced TNFα secretion as well as the frequency of $TNF\alpha^+$ islets (Fig. 3.3). This translated into an immediate improvement in apoptosis rates. Furthermore, a 24-hour inhibition of TNFα secretion was sufficient to consistently double basal, glucose-responsive and secretagogue-stimulated insulin secretion, measured on a per-cell basis (Fig. 3.5). When the contribution of TNFα inhibition to cell survival is considered, the net effect of TNFα inhibition on islet function is even more dramatic, resulting in at least a five-fold increase in function relative to control islets.

These results demonstrate that inhibition of isolation-induced TNFα secretion can significantly increase islet viability and function. Recent findings have indicated that inhibition of cytokine production by other means, namely a pharmacological inhibitor of p38 phosphorylation, SB203580, also have dramatic effects on islet survival and function.[502] In fact, the authors showed that as little as an hour pre-treatment is sufficient to significantly improve graft function upon transplantation. Taken together, these results suggest that intra-islet cytokine production may play an important role in islet death and subsequent graft failure. While endotoxin contamination may play a role in isolation-induced cytokine

production, we propose that the isolation proper, with the inherent cellular stresses, induces a cytokine response from cells within the islet.

Many strategies being developed to improve islet survival target various levels of the TNFα signalling cascade, from cytokine production (RDP58), to receptor binding (DcR3,[522] etanercept[244]), to subreceptor signalling (SB203580,[502] SP600125[501, 523]). However, the current theory holds that islets are exposed to inflammatory cytokines at the graft site, and either culture-based pre-conditioning or systemic administration of cytokine inhibitors to the recipient represent the best opportunities to fortify islets and improve graft survival and function. While valid, this approach neglects the effect of the isolation proper. While there have been substantial improvements in preservation and isolation techniques, such as two-layer preservation[524] and iodixanol gradients,[525] there currently exists a therapeutic gap with respect to limiting the effect of the ischemic injury on isolated islets. Thus, while this study is limited in that it does not assess *in vivo* graft function or the effect of improved *in vitro* survival on islet mass requirements, it nevertheless suggests that strategies aimed at reducing the islet response to the ischemic insult that occurs during routine isolation represents a novel, and important, therapeutic target.

3.6 Acknowledgements

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Bridging Statement Between Manuscripts

In Chapter 2 we established that human islets produce TNFα in response to the isolation procedure, and that this signal contributes significantly to the apoptosis observed to occur in the first week following isolation.

In addition to establishing the advent of islet apoptosis post-isolation,[242] our group has also established the signaling patterns dictating the apoptotic response,[501] as well as the counteracting pro-survival pathways that are activated at a later stage in post-isolation recovery.[511] The delayed activation of these pro-survival pathways, without media supplementation, led us to hypothesize that another endogenous product could be acting as an islet survival factor. The aim of Chapter 4 was to establish whether insulin was acting as such an islet survival factor.

4 Islets Produce Pro-Survival Factors in Response to Isolation

Published as:

Aikin, R., Hanley, S., Maysinger, D., Lipsett, M., Castellarin, M., Paraskevas, S. & Rosenberg, L. Autocrine insulin action activates AKT and increases survival of isolated human islets. *Diabetologia* **49**: 2900-2909 (2006).

4.1 Abstract

The PI3K/AKT pathway plays a critical role in promoting the survival of pancreatic β-cells. AKT becomes activated in isolated human islets following overnight culture despite significant levels of cell death. The aim of the current study was to identify the cause for the observed increase in AKT phosphorylation in isolated islets. We hypothesised that a factor secreted by the islets in culture was acting in an autocrine manner to activate AKT. In order to identify the stimulus of the PI3K/AKT pathway in culture, we examined the effects of different culture conditions on AKT phosphorylation and islet survival during the immediate post-isolation period. We demonstrated that islet-conditioned medium induced AKT phosphorylation in freshly isolated human islets, whereas frequent media replacement decreased AKT phosphorylation. Following overnight culture, islet-conditioned medium contained significantly elevated levels of insulin, indicating that insulin may be responsible for the observed increase in AKT phosphorylation. Indeed, treatment with an α-insulin antibody or with inhibitors of IR/IGFR1 kinase activity suppressed AKT phosphorylation, leading to decreased islet survival. In addition, dispersion of islets into single cells also suppressed AKT phosphorylation and induced islet cell death, indicating that islet integrity is also required for maximal AKT phosphorylation. Our findings demonstrate that insulin acts in an autocrine manner to activate AKT and mediate the survival of isolated human islets. These findings provide new information on how culturing islets prior to transplantation may be beneficial to their survival by allowing for autocrine activation of the pro-survival AKT pathway.

4.2 Introduction

Although successful single-donor islet transplantations have been reported recently, the proportion of islet isolations producing sufficient viable islets for transplantation remains low.[244] Even with recent improvements to the isolation procedure, such as the use of the TLM and a less toxic iodixanol gradient, many experienced groups isolate enough islets for transplantation from only 50% of isolations. Furthermore, a significant number of cells are lost due to apoptosis and necrosis during the immediate post-isolation period.[242, 402] Thus, a better understanding of the mechanisms controlling isolated islet cell death may lead to the development of strategies to improve islet survival following isolation.

The v-akt murine thymoma oncogene homologue (AKT) is a serine/threonine kinase that regulates several cellular processes, including cell cycle progression, transcription, glucose uptake and apoptosis. In particular, AKT plays a critical role in mediating cell survival in response to growth factor stimuli.[526] AKT is recruited to the plasma membrane following activation of phosphatidylinositol 3-kinase (PI3K) and is activated through phosphorylation by phosphoinositide-dependent kinase-1 (PDK1) and 2.[527, 528] Activated AKT can directly phosphorylate several pro-apoptotic proteins, such as BAD, human caspase-9, apoptosis signal-regulating kinase-1 and forkhead transcription factor like-1, leading to suppression of apoptotic signals (reviewed in [529]). Transgenic mice expressing a constitutively active form of AKT1 under the control of the *insulin* promoter display increased β-cell mass, improved glucose tolerance and resistance to STZ-induced diabetes.[530, 531] Adenovirus-mediated overexpression of constitutively active AKT1 in isolated human islets increased *in vitro* β-cell proliferation, decreased apoptosis induced by serum/glucose withdrawal or high glucose, and decreased the number of transplanted islets required to reverse STZ-induced diabetes in mice.[532] In addition, PI3K/AKT signalling has been shown to mediate survival of isolated human islets,[501, 533] canine islets[511] and insulinoma cell lines.[534-537] Taken together, these findings indicate that the PI3K/AKT pathway plays a critical role in promoting the survival of β-cells.

Although the original Edmonton protocol involved transplanting islets immediately following isolation, the recovery of islets in culture prior to transplantation has once again become common practice.[244, 431] One reason for culturing islets prior to transplantation is that a significant amount of islet cell death due to the isolation occurs during the 48h following isolation, and thus it is advantageous to allow these cells to die off in culture so as not to provoke additional local inflammation at the graft site. Indeed, porcine islets that have been cultured for at least 24h are significantly better at reversing hyperglycemia when transplanted into diabetic nude mice compared with freshly isolated islets.[432] In addition, there is a much higher relative expression of inflammatory genes during the immediate post-isolation period when compared with islets cultured for 1 week.[433] Culturing islets also provides an opportunity for therapies aimed at improving islet survival, inducing islet expansion, and reducing islet immunogenicity. We and others have demonstrated that immediately following isolation, human islets display low levels of AKT phosphorylation.[501, 533] However, following overnight culture in either serum-containing or serum-free medium, AKT becomes highly phosphorylated in a PI3K-dependent manner.[501, 511, 533] This increased AKT phosphorylation affords islets a certain degree of protection against insults such as inflammatory cytokines.[501] The aim of the current study was to identify the cause of the observed increase in AKT phosphorylation in isolated human islets.

4.3 Materials and Methods

4.3.1 Human Islet Isolation

Pancreata (n=19) were retrieved from heart-beating cadaveric organ donors (mean age: 50.4±2.9yrs, mean BMI: 26.6±1.0kg/m^2, mean blood glucose: 9.1±0.8mM, mean amylase: 254.4±67.8U/L, mean pancreas weight: 86.8±4.4g) at the time of multi-organ harvest for transplantation. Consent for donation of tissues for research was obtained by the local OPO (Quebec-Transplant). Warm ischemia time was approximately 5min and cold ischemia time, using refrigerated

UW solution perfusion, was 3–8 h. Islets were isolated using the method of Ricordi *et al*.[400] as described previously.[501] The average islet yield was 389,983±48,809IE. GSIS was routinely assessed to ensure islet functionality.

4.3.2 Islet Cell Culture

Isolated islets (>90% purity) were cultured in CMRL-1066 medium (Gibco, Burlington, ON, Canada) containing 10% fetal bovine serum (FBS, Montreal Biotech Inc., Montreal, QC, Canada) at 37°C in a humidified atmosphere of 5% CO_2. Medium was changed every other day. In some experiments, medium was supplemented with insulin (Eli Lilly, Indianapolis, IN, USA) at the indicated concentration. Azide-free monoclonal α-insulin (Acris Antibodies, Hiddenhausen, Germany) and α-insulin-like growth factor-I (IGF-I) (Upstate, Lake Placid, NY, USA) were added to culture medium to a final concentration of 1:50. Triciribine (TCN), AKTi-1/2 and CB5233705 (all from Calbiochem, San Diego, CA, USA) were used at both 1 and 10μM. The IR/IGF receptor-1 (IGFR1) inhibitors AG1024 and AGL2263 (both from Calbiochem) were used at both 1 and 10μM. IBMX (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada) was used at 50μM. For all compounds prepared in dimethylsulfoxide (DMSO), the final concentration of DMSO in the culture medium was kept below 0.1%. Vehicle controls were prepared for all treatments. In some cases, islets were dispersed by first washing with Dispersion Solution (Gibco) followed by a 10min incubation at 37°C with trypsin/EDTA (Gibco). Trypsinized islets were washed with cold CMRL-1066 medium containing 10% FBS, pipetted gently, and resuspended in CMRL-1066 containing 10% FBS. The insulin content of islet medium was assessed using a commercially available insulin ELISA kit (ALPCO Diagnostics, Windham, NH, USA).

4.3.3 MTT Assay

Mitochondrial metabolic activity was used as an indicator of islet viability,[538] and was performed with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously .[501] Four independent samples

were analyzed per experiment and each experiment was performed at least three times.

4.3.4 Immunofluorescence

Samples were stored at 4°C in phosphate-buffered formalin until cell pellets were embedded in 2% agarose. Following routine processing and embedding, 4μm sections were cut and dewaxed in xylene and petroleum ether. Slides were then incubated in blocking buffer (Zymed, San Francisco, CA, USA) for 15 min before being exposed overnight at 4°C to an α-insulin antibody (Dako, Carpinteria, CA, USA) diluted 1:100 in blocking buffer. Following washing in PBS, slides were incubated with a broad-spectrum biotinylated secondary antibody (Zymed) for 30min at room temperature, washed, and re-incubated with a streptavidin–rhodamine conjugate (Molecular Probes Inc., Eugene, OR, USA) diluted 1:200 in blocking buffer for 30min at room temperature. Slides were washed again and re-probed with an α-phospho-AKT antibody (Cell Signalling, Danvers, MA, USA) diluted 1:100 in blocking buffer overnight at 4°C. A fluorescein isothiocyanate (FITC)-conjugated α-rabbit secondary antibody (Molecular Probes) was used at 1:200 in blocking buffer for 30min at room temperature. Slides were coverslipped with mounting medium containing DAPI (Vector, Burlingame, CA, USA), and analyzed using Northern Eclipse software (Empix Imaging, Mississauga, ON, USA). Confocal microscopy was performed using a Zeiss LSM 510 Confocal Laser Scanning Microscope (Carl Zeiss Canada Ltd, Toronto, ON, Canada).

4.3.5 Propidium Iodide Staining

Whole islets were dispersed (see above) and resuspended in PBS containing 2μM propidium iodide (PI) and 0.67μM fluorescein diacetate (FDA, both from Molecular Probes). The stained islets were placed on glass slides with coverslips and incubated for 15min at room temperature in a humid foil-covered container. Slides were visualized under a fluorescent Olympus BX60 microscope connected via a digital video camera to a PC. Five hundred cells were counted per slide,

with three slides per group for at least three independent experiments. The percentage of PI^+ cells was determined by dividing the number of PI^+ cells by the total number of cells counted.

4.3.6 Western Blotting

Western blotting was performed as described previously.[501] α-AKT and α-phospho-AKT (Ser-473) (both from Cell Signalling) were used at a dilution of 1:1000. Densitometric analysis was performed using Scion Image (Scion Corp., Frederick, MD, USA).

4.3.7 Statistical Analysis

All results are expressed as mean ± SEM. Statistical significance was determined using a one-way ANOVA with a post-hoc Bonferroni test as well as the Student's *t* test using SYSTAT 9 (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $p<0.05$.

4.4 Results

4.4.1 AKT Phosphorylation is Detectable 3h After Isolation and Reaches Maximal Levels After 12h

We have previously demonstrated that AKT becomes highly phosphorylated in isolated human and canine islets after 12–16h in culture.[501, 511] In order to determine the kinetics of this signal, we examined the levels of AKT phosphorylation in human islets over the course of the first 24 h following isolation in CMRL-1066 medium (5.5mM glucose) containing 10% FBS. Using Western blot analysis, an increase in AKT phosphorylation was detectable as early as 3h following isolation (Fig. 4.1A). The level of AKT phosphorylation reached a maximum by 12h, consistent with our previous findings.[501, 511] To examine whether the AKT phosphorylation detected in whole-cell lysates was occurring in insulin-producing islets, we co-stained islets cultured overnight for phospho-AKT and insulin. Indeed, significant colocalization was observed

between insulin and phospho-AKT staining (Fig. 4.1B), indicating that AKT is phosphorylated in insulin-producing cells of the islet.

4.4.2 Islet-Conditioned Medium Induces AKT Phosphorylation in Freshly Isolated Islets

If isolated islets were secreting a factor that was acting in an autocrine manner to induce AKT phosphorylation, then addition of islet-conditioned medium to freshly isolated islets would be expected to induce AKT phosphorylation. To address this possibility, conditioned medium was collected from islets that had been cultured for 24h, a point at which AKT is highly phosphorylated. Islets from the ensuing isolation were then treated with conditioned medium within 1h after isolation and AKT phosphorylation was examined after 30min. Islets treated with conditioned medium showed increased AKT phosphorylation compared with islets which received fresh, untreated medium (Fig. 4.1C). Conditioned medium that was heated to 65°C for 30min lost the ability to induce AKT phosphorylation, suggesting that a heat-labile factor was responsible for augmenting AKT phosphorylation.

4.4.3 Regular Medium Replacement Decreases AKT Phosphorylation

Since AKT becomes highly phosphorylated after 12–16h in culture, it is possible that the autocrine factor must accumulate in the medium to have its maximal effect. We therefore examined whether frequent medium replacement would prevent the accumulation of the secreted factor in the medium, resulting in lower AKT phosphorylation. Replacing the islet culture medium every 6h over a 24h period resulted in decreased AKT phosphorylation compared with islets that were replated with the same medium (Fig. 4.1D). This finding supports the notion that a local secreted factor is indeed responsible for the increase in AKT phosphorylation in isolated islets.

4.4.4 Pharmacological Inhibition of the AKT Pathway Leads to Cell Death in Isolated Human Islets

In order to demonstrate a pro-survival role for AKT signalling in freshly isolated islets, we employed three small molecule inhibitors of the AKT pathway. TCN is a tricyclic nucleoside that inhibits the activation of all three AKT isoforms, possibly by interacting with their pleckstrin homology (PH) domains.[539] AKTi-1/2 is a novel selective allosteric inhibitor of both AKT1 and AKT2, but not AKT3, which also acts via interactions with the PH domains of AKT1/2.[540] A benzimidazole compound, CB5233705, also blocks AKT pathway activation by targeting an unknown kinase upstream of AKT.[541] All three inhibitors decreased islet viability and caused an increase in cell death after 24h (Fig. 4.2), indicating that AKT activation following isolation mediates islet cell survival. One inhibitor, CB5233705, caused greater cell death than the others, perhaps due to the fact that it blocks an upstream kinase of AKT and is not specific for AKT per se.

4.4.5 Treatment With an α-Insulin Antibody Decreases AKT Phosphorylation and Human Islet Survival

Since insulin is secreted by β-cells, is a strong activator of AKT[542] and is heat-sensitive,[543] we examined whether insulin was the factor responsible for the observed rise in AKT phosphorylation. Using an insulin ELISA, we found that after 24h, islet-conditioned medium contained significantly elevated levels of insulin (conditioned medium 59.2±9.6mU/mL; fresh medium 1.08±0.062μU/mL) (Fig. 4.3A). Freshly isolated human islets were then cultured with or without an α-insulin antibody to sequester insulin secreted into the islet culture medium. Addition of α-insulin antibody significantly decreased AKT phosphorylation after 24h in culture (Fig. 4.3B). However, treatment with a similar concentration of an α-IGF antibody, which binds both IGF-I and IGF-II, had no effect on AKT phosphorylation (Fig. 4.3B). Treatment with α-insulin antibody also resulted in a 27% reduction in islet viability (Fig. 4.3C). On the other hand, an α-IGF antibody had no significant effect on islet viability. Furthermore, addition of the α-insulin antibody to the islet culture medium also caused an almost two-fold increase in

the number of PI^+ cells (Fig. 4.3D). Thus, isolated islets release high levels of insulin into the surrounding medium, which activates AKT and improves islet survival.

4.4.6 Inhibition of IR/IGFR1 Suppresses AKT Phosphorylation and Induces Cell Death in Isolated Human Islets

In order to confirm that insulin signalling is responsible for the observed increase in AKT phosphorylation in cultured islets, we examined whether inhibition of insulin reception affected AKT phosphorylation and islet survival. Since at high concentrations insulin can also activate IGFR1,[544] we employed two inhibitors of both IR and IGFR1 tyrosine kinase activity: AGL2263 and AG1024. AGL2263 inhibits both IR and IGFR1 activity with an IC_{50} of 0.4μM and is 100-fold more specific for these receptors than for AKT itself.[545] AG1024 can also inhibit both IR and IGFR1, but appears to inhibit IGFR1 activity to a greater extent.[546] Addition of either inhibitor immediately following isolation led to decreased islet viability and increased cell death after 24h in culture (Fig. 4.4A&B). Furthermore, IR/IGFR1 inhibition abolished AKT phosphorylation after 24h in culture (Fig. 4.4C).

4.4.7 Insulin, But Not Glucose, Leads to Increased AKT Phosphorylation in Isolated Human Islets

Immediately following isolation, AKT phosphorylation levels are low, while JNK is highly phosphorylated. Over the ensuing 24h, JNK phosphorylation levels decrease concomitantly with a rise in AKT phosphorylation.[501, 547] Since JNK is known to suppress insulin signalling,[548] it is possible that the insulin signalling pathway is suppressed in islets immediately following isolation and AKT only becomes activated following suppression of the JNK pathway. We therefore examined whether exogenous insulin could induce AKT phosphorylation in islets immediately following isolation, at a time when JNK is highly activated. Within 1h after isolation, human islets were treated with increasing concentrations of insulin for 15min and AKT phosphorylation was assessed. Insulin was able to cause an

increase in AKT phosphorylation at a concentration of 100nM (Fig. 4.5A), which is within the concentration range found in islet-conditioned medium (59.2mU/mL, ~360nM). Therefore, signalling to AKT via the IR is functional in isolated human islets, supporting our previous finding that, in fact, the PI3K/AKT pathway suppresses JNK activation.[501] This point is further demonstrated by the finding that the treatment of isolated islets with insulin decreases JNK phosphorylation.[549] Previous studies have suggested that glucose-induced activation of intracellular signalling pathways is mediated by the autocrine action of secreted insulin. Treatment of βTC3 cells for 2min with 15mM glucose resulted in increased IR tyrosine phosphorylation and increased association of IRS1 with the 85kDa subunit of PI3K,[550] and similar findings were observed in isolated rat islets.[551] Furthermore, 16.7mM glucose was able to activate AKT after only 5min in the HIT-T15 β-cell line.[552] Based on our finding that basal insulin secretion activates AKT following isolation, we examined whether inducers of insulin secretion can accelerate AKT phosphorylation following isolation. Treatment of freshly isolated human islets with 22.2mM glucose for 15min had no effect on AKT phosphorylation when compared with islets cultured in serum-containing CMRL-1066 medium, which contains 5.5mM glucose (Fig. 4.5B). Similarly, a combination of 22.2mM glucose and IBMX, an inhibitor of cAMP phosphodiesterase that causes elevated intracellular cAMP levels that lead to increased exocytosis of insulin,[553] had no significant effect on AKT phosphorylation. Therefore, in our hands, glucose was unable to induce AKT phosphorylation in human islets immediately following isolation. Similarly to these findings, treatment of isolated rat islets with high glucose had no effect on AKT phosphorylation, although 100nM insulin did.[554, 555]

4.4.8 Addition of Exogenous Insulin to Islet Culture Medium Has No Significant Effect on Islet Viability

Based on the finding that autocrine insulin action improves islet survival, we examined whether addition of exogenous insulin immediately after isolation could improve cultured human islet survival. However, islets treated with up to 100nM

insulin displayed no significant improvement in survival after 48h in culture (Fig. 4.5C&D).

4.4.9 Dispersion of Islets Into Single Cells Suppresses AKT Phosphorylation and Induced Islet Cell Death

Although diminished, AKT phosphorylation was still observed in the presence of α-insulin antibody, suggesting that the local concentration of insulin within the islet might be higher than in the surrounding medium and that α-insulin antibody is not able to completely sequester intra-islet insulin. We therefore examined whether dispersion of isolated human islets into single cells affects AKT phosphorylation. Isolated human islets were trypsinized and dispersed into single cells immediately after isolation. An identical number of islets were cultured in both the dispersed and control cultures (1000IE/mL medium). Therefore, the dispersed islet culture (1x) contained the same number of cells/mL as the undispersed control islets. Dispersion of islets into single cells significantly decreased AKT phosphorylation and increased cell death (Fig. 4.6). If the decrease in AKT phosphorylation caused by dispersion was due to reduced local concentration of insulin, then increasing the cell density would increase the insulin concentration and AKT phosphorylation. However, increasing the cell density up to eightfold had no significant effect on AKT phosphorylation (Fig. 4.6A). These findings demonstrate that islet integrity is required for maximal AKT activation and cell survival.

Figure 4.1: Islet-Conditioned Medium Contains A Heat-Labile Factor That Induces AKT Phosphorylation in Freshly Isolated Human Islets

A. Whole-cell lysates were taken at the indicated times following islet isolation and analyzed by Western blotting. Densitometric analysis was performed and the mean ratio ± SEM of phospho-AKT (P-AKT) to total AKT is shown for four independent experiments.

B. Islets cultured for 24h were stained for phospho-AKT (green) and insulin (red) as described in Materials and Methods. Yellow staining indicates regions co-staining for both phospho-AKT and insulin. Nuclei were visualized by DAPI staining (blue). The bar represents 50μm.

C. Following isolation, human islets were cultured for 1h, after which the medium was replaced with either fresh medium (Ctrl), islet-conditioned medium (ICM) or heat-inactivated conditioned medium (HCM). After 30min of treatment, whole-cell lysates were prepared and analyzed by Western blotting. Densitometric analysis was performed and the mean ratio±SEM of phospho-AKT to total AKT is shown for three independent experiments.

D. Isolated islets were placed in culture and the culture medium was replaced every 6h (MR) or control cells were replated with the same medium (Ctrl). Whole-cell lysates were prepared after 24h and analyzed by Western blotting. Densitometric analysis was performed and the mean ratio±SEM of phospho-AKT to total AKT is shown for three independent experiments (*$p<0.05$ compared with untreated controls).

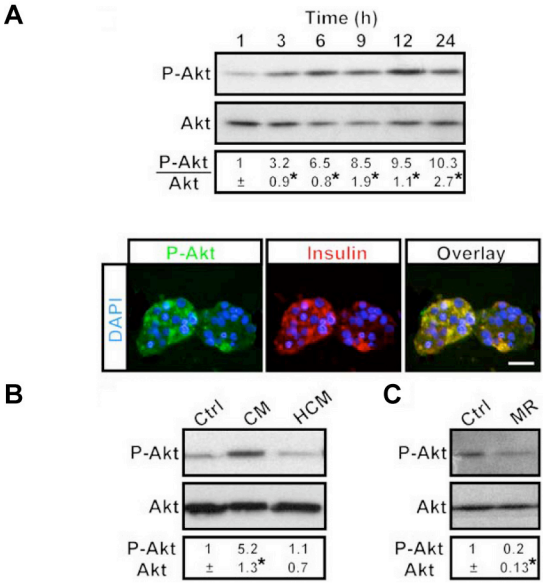

A
Time (h)
1 3 6 9 12 24
P-Akt
Akt
P-Akt / Akt
1 3.2 6.5 8.5 9.5 10.3
± 0.9* 0.8* 1.9* 1.1* 2.7*
P-Akt
Insulin
Overlay
DAPI
B
Ctrl CM HCM
P-Akt
Akt
P-Akt / Akt
1 5.2 1.1
± 1.3* 0.7
C
Ctrl MR
P-Akt
Akt
P-Akt / Akt
1 0.2
± 0.13*

Figure 4.2: AKT Inhibition Leads to Human Islet Cell Death
Freshly isolated islets were treated immediately following isolation with either 1 or 10μM of the indicated inhibitor (TCN; AKTi-1/2, a novel selective allosteric inhibitor of both AKT1 and AKT2, but not AKT3; or CB5233705 (CB52), which blocks AKT pathway activation by targeting an unknown kinase upstream of AKT).
A. Islet viability was assessed by the MTT assay after 24h. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from four independent experiments.
B. Membrane permeability was assessed following 24h in culture using PI/FDA staining. The number of PI^+ cells was expressed as a percentage of the total cells counted. Bars represent the mean values ± SEM for three independent experiments (**p<0.01, ***p<0.001 compared with untreated controls (Ctrl)).

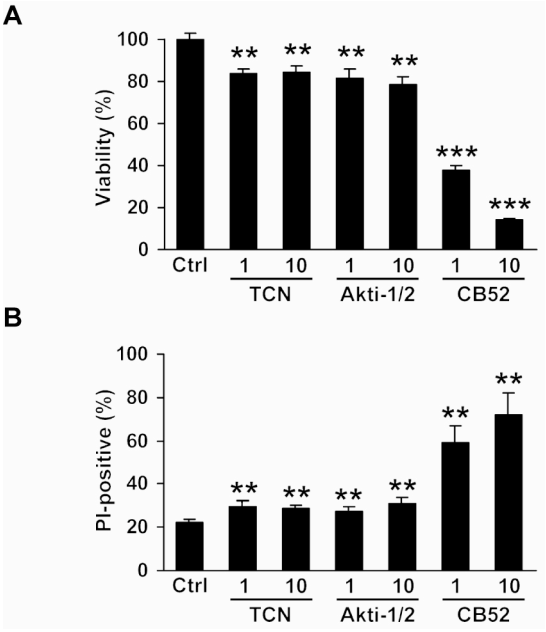

A
Viability (%)
100
80
60
40
20
0
**
**
**
**
***
***
Ctrl
1
10
1
10
1
10
TCN
Akti-1/2
CB52
B
PI-positive (%)
100
80
60
40
20
0
**
**
**
**
**
**
Ctrl
1
10
1
10
1
10
TCN
Akti-1/2
CB52

Figure 4.3: α-Insulin Antibody Decreases AKT Phosphorylation and Induces Cell Death of Isolated Human Islets

A. Insulin content of fresh medium (Ctrl) and medium from islets cultured for 24h was assessed by ELISA. Bars represent the mean insulin content ± SEM for duplicate samples from three independent experiments.

B. Immediately following isolation, islets were cultured with or without the addition of α-insulin antibody (α-ins) (1:50) or α-IGF (1:50) to the culture medium. Whole-cell lysates were prepared after 24h and assessed by Western blot analysis. Densitometric analysis was performed and the mean ratio of phospho-AKT to total AKT is shown ± SEM for three independent experiments.

C. Islet viability was assessed by the MTT assay after 24h. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from three independent experiments.

D. Membrane permeability was assessed following 24h in culture using PI/FDA staining. The number of PI^+ cells was expressed as a percentage of the total cells counted. Bars represent the mean values ± SEM for three independent experiments (*$p<0.05$, **$p<0.01$ compared with untreated controls).

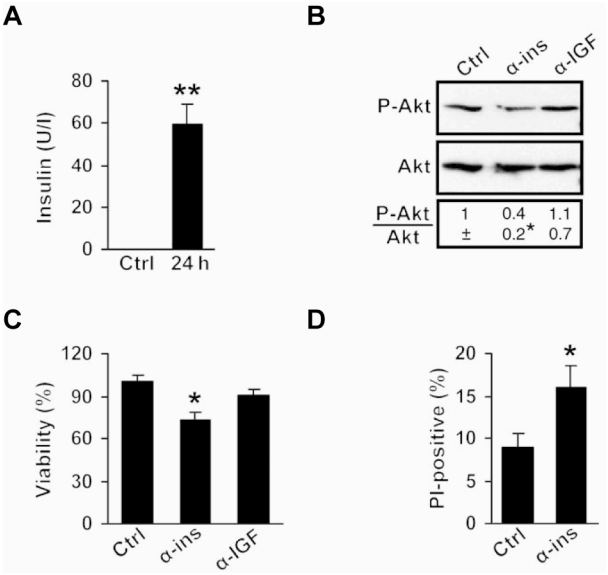

A
Insulin (U/l)
80
60
40
20
0
**
Ctrl
24 h
B
Ctrl
α-ins
α-IGF
P-Akt
Akt
P-Akt / Akt
1
0.4
1.1
±
0.2*
0.7
C
Viability (%)
120
90
60
30
0
*
Ctrl
α-ins
α-IGF
D
PI-positive (%)
20
15
10
5
0
*
Ctrl
α-ins

Figure 4.4: Inhibition of IR/IGFR1 Suppresses AKT Phosphorylation and Induces Cell Death in Isolated Human Islets

Freshly isolated islets were treated immediately following isolation with either 1 or 10μM of the indicated inhibitor (AGL2263 or AG1024).

A. Islet viability was assessed by the MTT assay after 24h. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from four independent experiments.

B. Membrane permeability was assessed following 24h in culture using PI/FDA staining. The number of PI^+ cells was expressed as a percentage of the total cells counted. Bars represent the mean values ± SEM for three independent experiments.

C. Whole-cell lysates were prepared after 24h and analyzed by Western blotting. Densitometric analysis was performed and the mean ratio of phospho-AKT (P-AKT) to total AKT is shown ± SEM for three independent experiments (*$p<0.05$, **$p<0.01$ compared with untreated controls (Ctrl)).

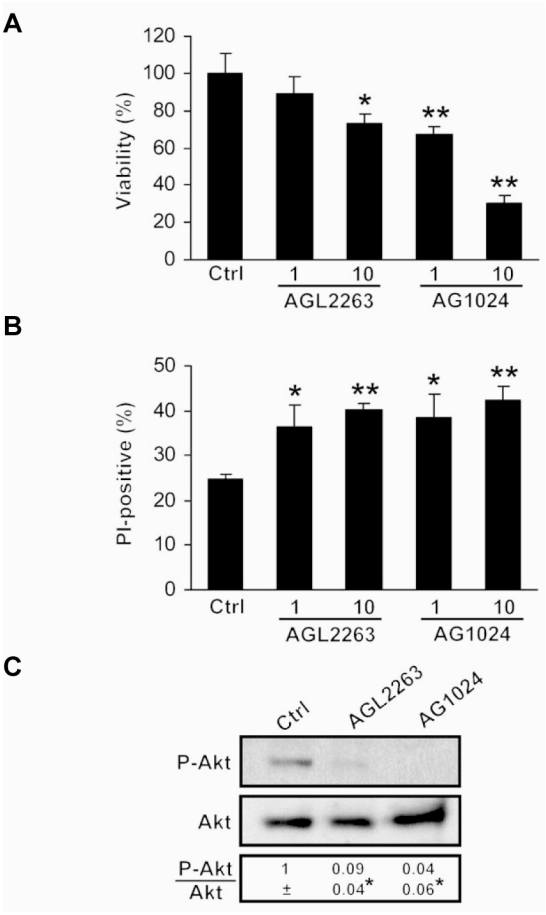

A
Viability (%)
120
100
80
60
40
20
0
*
**
**
Ctrl
1
10
1
10
AGL2263
AG1024
B
PI-positive (%)
50
40
30
20
10
0
*
**
*
**
Ctrl
1
10
1
10
AGL2263
AG1024
C
Ctrl
AGL2263
AG1024
P-Akt
Akt
P-Akt / Akt
1 ± 0.04
0.09 0.04*
0.04 0.06*

Figure 4.5: Exogenous Insulin Leads to Increased AKT Phosphorylation in Freshly Isolated Human Islets But Does Not Improve Islet Survival after 48h In Culture

A. Isolated islets were cultured for 1h following isolation and then treated with increasing concentrations of insulin. After 15min of treatment, whole-cell lysates were prepared and analyzed by Western blotting. Densitometric analysis was performed and the mean ratio of phospho-AKT (P-AKT) to total AKT is shown ± SEM for three independent experiments.

B. Freshly isolated islets were cultured for 1h, after which they were treated for 15min with glucose (22.2mM) or glucose in combination with IBMX (50μM). Whole-cell lysates were prepared and examined by Western blot analysis. Densitometric analysis was performed and the mean ratio of phospho-AKT to total AKT is shown ± SEM for three independent experiments.

C. Immediately following isolation, islets were cultured with the indicated concentration of insulin. Islet viability was assessed by the MTT assay after 48h. Bars represent the mean MTT reduction expressed as a percentage of the control ± SEM for quadruplicate samples from five independent experiments.

D. Immediately following isolation, islets were cultured with the indicated concentration of insulin. Membrane permeability was assessed following 24h in culture using PI/FDA staining. The number of PI^+ cells was expressed as a percentage of the total cells counted. Bars represent the mean values ± SEM for four independent experiments (*$p<0.05$ compared with untreated controls).

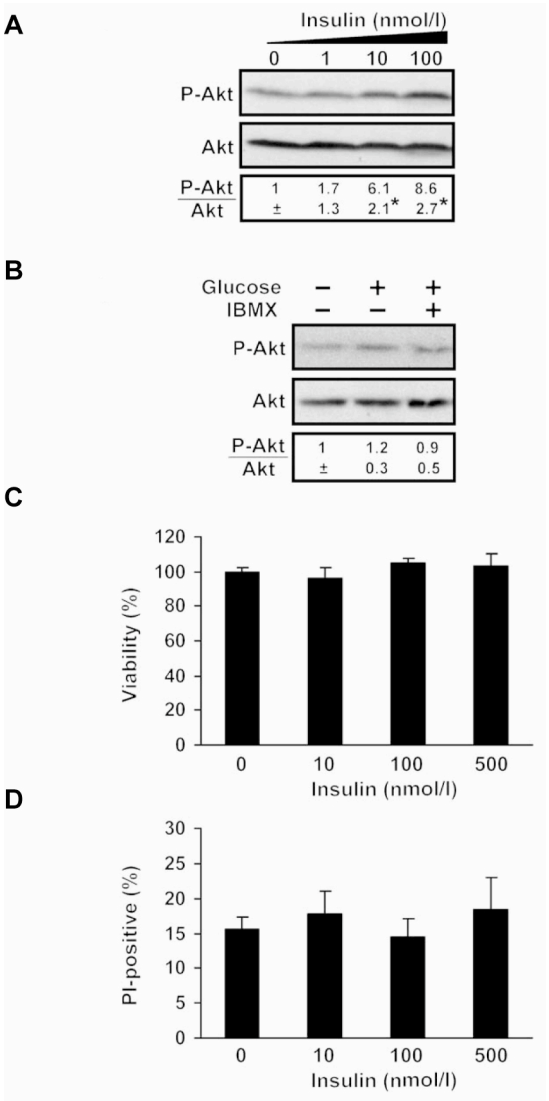
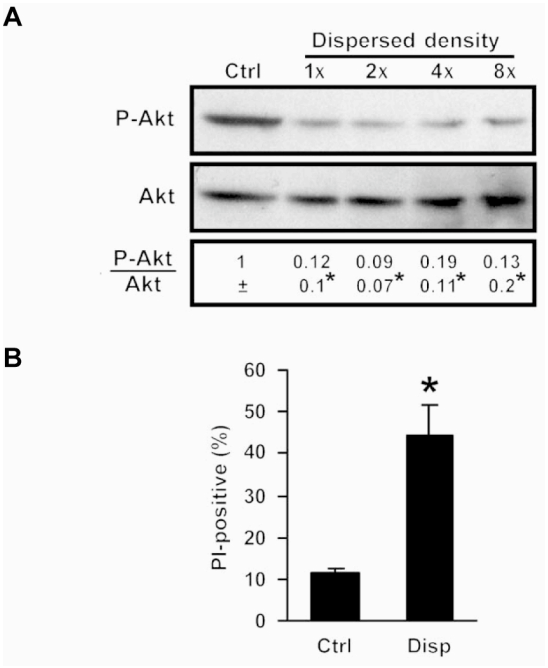

A
Insulin (nmol/l)
0 1 10 100
P-Akt
Akt
P-Akt / Akt
1 1.7 6.1 8.6
± 1.3 2.1* 2.7*
B
Glucose – + +
IBMX – – +
P-Akt
Akt
P-Akt / Akt
1 1.2 0.9
± 0.3 0.5
C
Viability (%)
120
100
80
60
40
20
0
0 10 100 500
Insulin (nmol/l)
D
PI-positive (%)
30
25
20
15
10
5
0
0 10 100 500
Insulin (nmol/l)

Figure 4.6: Dispersion of Islets Into Single Cells Suppresses AKT Phosphorylation and Induced Islet Cell Death

Immediately following isolation, islets were dispersed into single cells (see Materials and Methods). Dispersed cells were cultured at the indicated density, where the lowest density (1x) corresponds to the same number of cells/mL as the undispersed control islets (Ctrl).

A. Whole-cell lysates were prepared after 12h in culture and examined by Western blot analysis. Densitometric analysis was performed and the mean ratio of phospho-AKT (P-AKT) to total AKT is shown ± SEM for three independent experiments.

B. Membrane permeability was assessed following 24 h in culture using PI/FDA staining. The number of PI^+ cells was expressed as a percentage of the total cells counted. Bars represent the mean values ± SEM for three independent experiments (Disp: dispersed; *$p<0.05$ compared with untreated controls (Ctrl)).


A
Dispersed density
Ctrl
1x
2x
4x
8x
P-Akt
Akt
P-Akt / Akt
1
0.12 ± 0.1*
0.09 ± 0.07*
0.19 ± 0.11*
0.13 ± 0.2*
B
PI-positive (%)
60
50
40
30
20
10
0
*
Ctrl
Disp

4.5 Discussion

We previously demonstrated that the rise in AKT phosphorylation in cultured islets is dependent on PI3K activity and that inhibition of PI3K induced islet cell death.[501, 511] Here we present evidence that insulin released by the islets in culture activates AKT in an autocrine manner to mediate islet survival. We demonstrate that treatment with an α-insulin antibody decreased AKT phosphorylation and induced cell death, similar to findings in dispersed rat islets where treatment with α-insulin antibody increased the amount of terminal deoxynucleotidyl transferase-mediated X-dUTP nick end labelling $(TUNEL)^+$ cells.[556] We also demonstrate that inhibition of insulin reception blocks AKT phosphorylation and leads to cell death. Finally, we confirm that inhibition of AKT induces cell death in islets. Taken together, these findings outline an autocrine survival pathway in isolated human islets where secreted insulin improves islet survival by activating AKT.

The current findings provide new information on how culturing islets prior to transplantation may improve graft survival. Indeed, treatments that activate AKT during islet culture can improve graft survival,[533] indicating that elevated AKT activity could render islets less susceptible to injury during the immediate post-transplantation period. Therefore, it is plausible that culturing islets prior to transplantation allows for secreted insulin to activate AKT in an autocrine fashion, yielding islets that are more likely to survive the insults encountered immediately following transplantation. Moreover, it is tempting to speculate that the negative effects of calcineurin inhibitor-based immunosuppressants on islet survival are due to the fact that these compounds suppress insulin secretion,[557] thus decreasing autocrine insulin-mediated islet survival.

Addition of exogenous insulin immediately following isolation was not able to improve short-term islet survival. Despite the fact that exogenous insulin activated AKT immediately following isolation, AKT becomes highly activated in untreated islet after 3h and is maximally activated after 12–16h. Thus, the additional AKT activity afforded by exogenous insulin is minimal and would explain the lack of any observed effect on islet survival. However, this finding

does not rule out the possibility that exogenous insulin would have a beneficial effect in islets cultured for a longer period, where insulin expression and secretion could be diminished. In addition, it is possible that insulin signalling is partially suppressed immediately following isolation, since 100nM insulin only induced a mild increase in AKT phosphorylation. A combination of JNK inhibition and insulin treatment immediately following islet isolation may further increase AKT activation and survival.

We did not observe any increase in AKT phosphorylation following treatment with high levels of glucose. Similarly to our findings, treatment of isolated rat islets with high glucose had no effect on AKT phosphorylation, but 100nM insulin did.[554, 555] In addition, no effect of glucose on AKT was observed in INS-1 cells treated for under 1h.[535, 555, 558] However, high glucose has been shown to activate AKT after 1h in INS-1 cells,[352, 558] and after 2h in MIN6 cells and mouse islets,[537] which does not coincide with the kinetics of GSIS. Taken together, these data suggest that the ability of glucose to activate AKT in β-cells after at least 1h may be independent of GSIS.[554] It is also possible that during GSIS, the insulin signaling pathway is suppressed in β-cells for a short period of time to avoid over stimulation of insulin-mediated signalling.

Our findings demonstrate that dispersion of human islets into single cells suppresses AKT phosphorylation and induces islet cell death. Previous studies have also demonstrated high levels of cell death in dispersed islet cultures.[556, 559] One explanation for the increase in cell death upon dispersion of islets into single cells is the loss of contact with the ECM, which is mediated by the integrin receptor family. Integrin ligation is known to mediate survival through activation of AKT,[560] and the loss of ECM contact upon islet dispersion could explain the observed decrease in AKT (Fig. 4.6). Concordantly, culturing dispersed islets in the presence of ECM proteins decreases apoptosis.[561] It is also possible that dispersed islets secrete less insulin, since aggregates of β-cells have been shown to secrete more insulin than free β-cells.[562] Alternatively, it is possible that the local concentration of insulin within the islet could be higher than in the surrounding medium. However, if the decrease in AKT phosphorylation caused

by dispersion was due to reduced local concentration of insulin, then increasing the local insulin concentration by increasing the cell density would restore AKT phosphorylation, which it did not (Fig. 4.6). It is also possible that synergism between growth factor and integrin signalling could be affected in dispersed islets. Overall, the specific reasons for the poor survival of dispersed islets remain unclear, but it is probably due to the disruption of the islet microenvironment.

It is unclear why cell death is still occurring despite high levels of AKT activation following overnight culture of isolated islets.[242, 501, 533] This could be due to the energy requirements of AKT-mediated survival. Indeed, AKT-mediated survival in the absence of growth factors is dependent on the presence of glucose.[563] Furthermore, AKT cannot prevent apoptosis under hypoxic conditions.[564] Islets are highly vascularised structures whose function depends greatly on proper blood supply. Following isolation, islets are avascular, and revascularization is not complete until 10–14 days following transplantation.[418] During that time, oxygen delivery to the islets is limited to diffusion. Islets are exposed to varying degrees of ischemia throughout the isolation procedure, and culture techniques that are suitable for dispersed cell cultures may not be adequate for proper oxygen delivery throughout the entire islet. Therefore, efforts to improve oxygen delivery during culture should further improve islet survival.

4.6 Acknowledgements

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Bridging Statement Between Manuscripts

The previous two chapters support the increasing wealth of knowledge that indicates that post-isolation islet culture is a balance between survival and apoptotic signals. However, it appears that not only is maintenance of cell viability an issue in the post-isolation period, but so too is maintenance of cellular structure and function, as reports indicate that cultured islets are also prone to dedifferentiation.[436, 437, 452, 454, 455] Our group has also established a model of islet dedifferentiation post-isolation,[436, 437, 452, 454, 455] and the aim of the next chapter was to determine whether isolated human islets also contributed as active a role in the dedifferentiation process as these cells do in the balance between survival and death.

5 Islets Produce Phenotype Destabilizing Factors in Response to Culture

Submitted as:

Hanley, S., Assouline, B. & Rosenberg, L. Epidermal growth factor induces adult human islet cell dedifferentiation. *Endocrinology*.

5.1 Abstract

Given the inherent therapeutic potential of the morphogenetic plasticity of adult human islets, the identification of factors controlling their cellular differentiation is of interest. The EGF family has previously been identified in the context of pancreatic organogenesis. We examined the role of EGF in an *in vitro* model whereby adult human islets are embedded in a collagen gel and dedifferentiated into DLS. We demonstrated that DLS formation was EGF-dependent, while residual DLS formation in the absence of added EGF was abrogated by EGFR inhibitor treatment. While EGF administration led to acute increases in AKT and ERK phosphorylation, these signaling events were only observed later in the process of DLS formation, concomitant with the proliferation of dedifferentiated cells. Thus, EGF is necessary but not sufficient for DLS formation. While EGF may not be directly responsible for islet cell dedifferentiation, it plays a role nonetheless in the proliferation of these dedifferentiated cells.

5.2 Introduction

Recent studies of adult human islets have reported their ability to undergo dedifferentiation, proliferation and redifferentiation.[245, 296, 394, 449, 450] While still controversial, current research now focuses on identifying factors responsible for controlling the morphogenetic plasticity of these cells, once thought to be terminally differentiated. Initial examination suggests similarities with pancreatic endocrine development with respect to transcriptional regulators and the expression profile of the intermediate precursor-type cells.[296, 394, 449] Accordingly, interest has been directed towards factors identified as critical to pancreatic organogenesis. To this end, we have implicated TGFβ in the control of human islet plasticity.[474] Likewise, EGF ligands contribute to islet development, and therefore may represent candidates for modulating the differentiated state of adult human islet cells.

Fetal pancreatic explants respond to EGF with an increase in epithelial cell proliferation and a decrease in endocrine differentiation, while pharmacologic inhibition of the ERK pathway, a known mechanism of EGF signaling, elicits an opposite effect.[73] While a certain degree of redundancy likely exists within such a large family of molecules,[72] individual EGF ligands have differential effects on specified pancreatic endocrine progenitors; BTC appears to direct cells towards a β-cell phenotype, while neuregulin-4 seems crucial to the normal development of δ-cells, with both of these phenotypes developing at the expense of the α-cell.[74] $EGFR^{-/-}$ mice have disrupted pancreatic growth, most notably with respect to epithelial proliferation and branching. Moreover, β-cell frequency is significantly decreased during development, as is post-natal β-cell proliferation. The remaining islets are closely associated with ducts, and bud outwards, suggesting that islet migration is also adversely affected by defective EGF signaling.[75]

The importance of EGF signaling in the maintenance of adult islet function has also recently been highlighted. Pancreas-specific expression of DN-EGFR leads to overt diabetes caused by decreased β-cell proliferation and function, along with increased β-cell apoptosis.[76] Several EGF ligands have been reported as β-cell mitogens,[565] with BTC postulated as a signaling intermediate for the

mitogenic effects of GLP1.[360] However, EGF ligands are not only mitogenic to endocrine cells, but also induce duct cell proliferation.[295, 566] In fact, it is this latter effect that appears to prime duct cells for islet regeneration. It is therefore noteworthy that several studies have drawn attention to the regenerative effect of gastrin and EGF in combination.[348] Individually, these factors act mainly as duct cell mitogens,[340-342] whereas co-administration (or the production of double-transgenic animals) leads to a significant increase in β-cell mass.[286, 343, 344] The role of EGF ligands in this model, however, appears to be the generation of regeneration-competent metaplastic DLS,[307, 318] rather than the β-cell regeneration observed in response to gastrin alone.[310] In fact, EGFR signaling leads to the metaplastic transformation of acini into DLS, both *in vivo*[307] and *in vitro*.[318, 319]

With respect to cellular transformation, EGF signaling is linked to pancreatic adenocarcinoma,[181, 567, 568] characterized by expression of duct epithelial markers. There is debate as to the cell of origin,[165] suggesting EGF signaling may again induce cellular dedifferentiation into a duct-like phenotype. Interestingly, studies on isolated islets suggest that EGF ligands have little mitogenic effect in culture.[569] However, *in vivo* β-cell-specific overexpression of EGF leads to increased β-cell proliferation.[570] These contrasting effects may reflect a differential response of the islet to the microenvironment. For example, isolated human islets, when cultured in suspension, maintain a quiescent endocrine phenotype,[571] however, embedding islets in a collagen matrix facilitates cellular proliferation,[443] and culture with added EGF and cholera toxin induces dedifferentiation into DLS.[452] Although we have characterized the roles of the ECM and cholera toxin in this model of islet dedifferentiation,[454] we have not fully elucidated the role of EGF. In this paper, we now report the permissive effect of EGF on the dedifferentiation of cultured human islets, as well as the signaling pathways involved.

5.3 Materials and Methods

5.3.1 Islet Isolation and Culture

Pancreata from adult human cadaveric organ donors were obtained through the local OPO (Table 5.1). Islets were isolated according to established protocols.[400] Briefly, organs were flushed and stored in cold ViaSpan solution (Barr Laboratories, Pomona, NY) until islet isolation. The main pancreatic duct was cannulated and perfused with a solution of Liberase HI (Roche Diagnostics, Laval, QC). The distended organ was placed in a closed system (Bio-Rep, Miami, FL) and heated to 37°C to activate the enzyme blend. Following the appearance of free islets in samples, the system was cooled and free tissues were collected, washed and separated by continuous Ficoll (Biochrom AG, Berlin, Germany) density gradient centrifugation in a cell processor (COBE DCT, Denver, CO). Free islets, determined to be ≥75% pure by real-time staining with dithizone (Sigma, St. Louis, MO), a Zn^{2+} chelator, were collected, washed, and counted as IE (= islet with diameter of 150μm).

Isolated islets were cultured in suspension overnight in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Burlington, ON) containing penicillin, streptomycin, fungizone, 10% FBS (Montreal Biotech, Montreal, QC), 1μM dexamethasone, 10ng/mL EGF (Sigma) and 24mU/mL insulin (Lilly, Toronto, ON). Islets were then embedded in type I rat tail collagen, as per previous reports,[452, 454, 455] at a density of 2000IE/25cm^2, and cultured for 8 days in serum-free DMEM/F12 with penicillin, streptomycin, fungizone, 1μM dexamethasone, 24mU/mL insulin and 200ng/mL cholera toxin (Sigma), with media changes every other day. EGF was added over a range of concentrations, from 0.001 to 100ng/mL. When added, CB5233705, U0126, SP600125, and SB203580; pharmacologic inhibitors of AKT phosphorylation, ERK kinase, JNK and p38; were used at 10μM (Calbiochem, San Diego, CA).[572, 573] The EGFR inhibitor AG1478 was used at 5μM (Sigma). Inhibitors were prepared so that the final concentration of DMSO was 0.1%, and vehicle controls were used. To remove

tissue from collagen, samples were incubated with 0.25mg/mL collagenase XI (Sigma) for 30 minutes at 37°C.

5.3.2 Immunofluorescence and ELISAs

Samples were stored at 4°C in phosphate-buffered formalin until cell pellets were embedded in 2% agarose (Gibco). Following routine processing and embedding, 4μm sections were cut and dewaxed in xylene and petroleum ether. Slides were permeabilized by incubation in Tris-buffered saline + 0.1% Triton X-100 for 5min, prior to antigen retrieval by heating for 10min in 0.1M citrate buffer, pH 6. Slides were then incubated in blocking buffer (Zymed, San Francisco, CA) for 15min before overnight incubation with primary antibody in blocking buffer. Following washing in PBS, slides were incubated for 1h with secondary antibody in blocking buffer, and reprobed with a different primary antibody. Again, following washing slides were incubated with secondary antibody before washing and coverslipping with mounting medium containing DAPI (Vector, Burlingame, CA). Antibodies used were mouse α-EGFR (Abcam, Cambridge, MA), and rabbit α-insulin (Santa Cruz Biotechnology, Santa Cruz, CA), α-glucagon, α-somatostatin and α-PP (Dako, Carpintera, CA) primary antibodies, along with FITC-conjugated α-mouse and rhodamine-conjugated α-rabbit secondary antibodies (Abcam).

To determine protein phosphorylation levels, samples were washed thoroughly in PBS before sonication in lysis buffer (50mM Tris-HCl, pH 8, 1.37mM NaCl, 0.1mM Na_3VO_4, Complete protease inhibitor tablet (Roche), 1% v/v nonident P-40, 10% v/v glycerol). Substrate-specific phospho-protein ELISAs for AKT, ERK, JNK and p38 (Cell Signaling, Danvers, MA) were used, according to the manufacturer's instructions.

5.3.3 qRT- PCR

Briefly, samples were stored in RLT buffer (Qiagen, Mississauga, ON) until the time of RNA isolation. Following RNA extraction, as per the manufacturer's protocol, cDNA was reverse transcribed using an Omniscript RT kit (Qiagen). Custom primers (Invitrogen, Burlington, ON) were synthesized (Table 5.2),[566, 574]

and quantitative RT-PCR (qRT-PCR) was performed using a QuantiTect SYBR green RT-PCR kit (Qiagen) in a MJ Research DNA Engine Opticon 2 (Bio-Rad, Mississauga, ON). Expression levels were normalized to GAPDH.

5.3.4 Statistical Analysis

All experiments were paired such that control and treatment groups contained tissue from the same donor. Results are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA with post hoc Bonferroni's test, or paired Student's t-test, where appropriate. Differences were considered significant when $p<0.05$.

5.4 Results

5.4.1 EGF Induces Islet Dedifferentiation

Following overnight suspension culture, islets embedded in a type I rat tail collagen matrix and cultured with 10ng/mL EGF and 200ng/mL cholera toxin undergo islet cell dedifferentiation into DLS consisting of a layer of flattened or low cuboidal epithelium of one to several cells thickness (Fig. 5.1A), as previously reported.[452] To further investigate the role of EGF in the dedifferentiation process, islets were embedded and cultured in the absence of EGF. Under these circumstances, DLS formation was significantly reduced (18.8±4.1% of control, $p<0.001$). Moreover, EGF induced DLS formation in a concentration-responsive manner (Fig. 5.1B), with an EC_{50} of approximately 0.05ng/mL. This concentration is significantly less than that reported by others culturing fetal pancreatic and embryonic stem cells,[73, 575-577] suggesting that cells of the isolated adult islet are highly sensitive to EGF ligands. Nevertheless, in keeping with previous work, a concentration of 10ng/mL EGF was used for subsequent experiments.

While addition of EGF significantly improved DLS formation, we did observe a very low level of spontaneous DLS formation in the absence of added EGF. To determine whether this was the result of endogenous EGF ligands, islets were

embedded and cultured as described, with the addition of a pharmacological inhibitor of the EGFR tyrosine kinase, AG1478.[578] As expected, some DLS formation was observed in vehicle-treated samples (47.0±6.5% of control, $p<0.001$), while addition of EGF induced a significant increase in DLS formation (95.6±5.4%; Fig. 5.1C). However, inhibition of EGFR tyrosine kinase activity blocked DLS formation to a greater extent than simply omitting EGF (7.6±1.8%, $p<0.001$), suggesting that endogenous EGF ligands may be acting in this system. Co-administration of AG1478 and EGF also led to reduced DLS formation (16.4±5.0%, $p<0.001$), confirming the specificity of the inhibitor.

5.4.2 Islets Produce Endogenous EGF Ligands

We have previously reported that islets produce various factors in response to isolation and culture.[474, 494, 495] Given that the foregoing studies with AG1478 suggested that islets were indeed producing an EGF ligand, we sought to establish the nature of such a ligand, and whether production was responsive to embedding and culture. Accordingly, we examined mRNA expression levels of the EGF family ligands BTC, EGF and TGFα, as well as the EGFR family proteins EGFR, ErbB2 and ErbB4. The effects of embedding ± EGF administration were examined at 24h, given that DLS formation appears to be initiated within this time period.[454]

Based on cycle threshold values, EGF ligands were highly expressed by isolated human islets, while EGFRs were moderately expressed prior to embedding ± EGF administration (data not shown). In both suspension and embedded cultures, exposure to EGF for 24h did not induce any significant changes in gene expression, compared to untreated control (Fig. 5.2A). In cultures without EGF, the act of embedding islets significantly decreased BTC expression at 24h, while EGFR expression showed a similar trend. In EGF-treated cultures, embedding significantly reduced EGF and ErbB4 expression at 24h, while EGFR and ErbB2 expression displayed similar trends. The greatest changes in gene expression were incurred by islet embedding and EGF treatment, as these conditions led to significant reductions in BTC, EGF and

ErbB4 expression, while EGFR and ErbB2 were decreased, though not significantly.

5.4.3 AKT and ERK Signaling Mediate EGF-Induced Islet Dedifferentiation

With respect to the signaling pathways mediating DLS formation, we have previously observed an increase in JNK phosphorylation concomitant with the observed wave of cellular apoptosis, followed by an increase in AKT and ERK phosphorylation at the time of duct epithelial cell proliferation.[455] We hypothesized that the effect of EGF in this system was likely the activation of the latter two kinases, as these are known to act downstream of EGF binding.[76, 566] Moreover, the effect of EGF on pancreatic tissue appears to be the induction of cellular dysplasia and proliferation.[307, 318]

To further clarify signal transduction events during DLS formation, we sampled cultures 0, 2, 4, 6 and 8 days post-embedding (Fig. 5.2B) and assessed AKT, ERK, JNK and p38 phosphorylation by ELISA (Fig. 5.2C). AKT phosphorylation did not significantly change until day 6 of culture, by which time phosphorylation was double that found in unembedded islets. By day 8, the level of AKT phosphorylation was already returning to baseline. ERK phosphorylation was significantly decreased at day 2 of culture, and slowly recovered, peaking at day 8 of culture. Thus, increased AKT and ERK phosphorylation were observed after DLS formation, but at the time of DLS expansion and cell proliferation.[452, 455] Conversely, JNK phosphorylation spiked at day 2 of culture, the period of maximal DLS formation, thereafter returning to basal levels. In contrast, phosphorylation of p38 did not change during the time course of DLS formation.

To assess the direct signaling effects of EGF, cultured islets were treated with 10ng/mL EGF for 15, 30, 60, 120 and 240min, and AKT, ERK, JNK and p38 phosphorylation were again assessed by ELISA (Fig. 5.3A). Under these conditions, AKT and ERK phosphorylation peaked early; at 30 and 15min, respectively, before returning to baseline levels. Neither JNK nor p38 phosphorylation were affected by EGF administration. At 24h, no differences in

protein phosphorylation were observed for AKT, ERK, JNK or p38 (data not shown).

To confirm that EGF-mediated activation of these signaling pathways was in fact essential to the process of DLS formation, we examined DLS formation in the presence of pharmacological inhibitors of AKT, ERK, JNK and p38 (Fig. 5.3B). Inhibition of AKT, ERK or JNK significantly reduced DLS formation rates, while p38 inhibition did not alter DLS formation.

5.4.4 Islet Endocrine Cells Express EGFR

To determine the cellular target of EGF ligands within islets, we performed immunofluorescence localization studies of EGFR (Fig. 5.4). Double immunofluorescence indicated that EGFR is in fact expressed by the four principal endocrine cells of the islet, as indicated by co-expression of EGFR with insulin, glucagon, somatostatin and PP. This finding suggests that each of the four endocrine cell types may be implicated in the process of DLS formation.[579]

Table 5.1: Donor and Isolation Characteristics

n	13
Age (years)	55.8±3.0
Male (%)	30.8
BMI (kg/m^2)	28.4±1.5
Traumatic cause of death (%)	8.3
ICU duration (days)	1.8±0.3
Glucose (mM)	7.1±0.4
Amylase (U/L)	291±171
Cold ischemia (hours)	6.4±0.7
≥70% pure yield (IE)	332,438±87,304

Table 5.2: RT-PCR Primers and Conditions

Gene	Primers	Annealing (°C)	Amplicon (bp)
BTC	5'-TTCACTGTGTGGTGGCAGATGG-3' 5'-ACAGCATGTGCAGACACCGAT-3'	58	350
EGF	5'-TGCAACTGTGTTGTTGGCTACATC-3' 5'-TGGTTGACCCCCATTCTTGAG-3'	58	339
TGFα	5'-TCAGTTCTGCTTCCATGCAACC-3' 5'-TTTCTGAGTGGCAGCAAGCG-3'	58	317
EGFR	5'-CATGTCGATGGACTTCCAGA-3' 5'-GGGCACAGATGATTTTGGTC-3'	58	125
ErbB2	5'-ACTGCCTGTCCCTACAACTA-3' 5'-ATATTGGCACTGGTAACTGC-3'	58	194
ErbB4	5'-GCTACGTGTTAGTGGCTCTT-3' 5'-ACGTCCACATCCTGAACTAC-3'	58	311
GAPDH	5'-TGAGAACGGGAAGCTTGTCA-3' 5'-TCACACCCATGACGAACATG-3'	58	218

Figure 5.1: DLS Formation is EGF-Dependent
A. Representative photomicrographs depict the progression from solid islet to cystic DLS over the course of culture.
B. DLS formation, assessed at day 8 of culture, was EGF dose-dependent, with an $EC_{50} \approx 5x10^{-11}$g/mL.
C. Administration of an EGFR inhibitor (AG1478), with or without EGF co-administration, decreased DLS formation, assessed at day 8 of culture (* $p<0.001$ vs. DMSO + EGF, ¶ $p<0.001$ vs. DMSO).

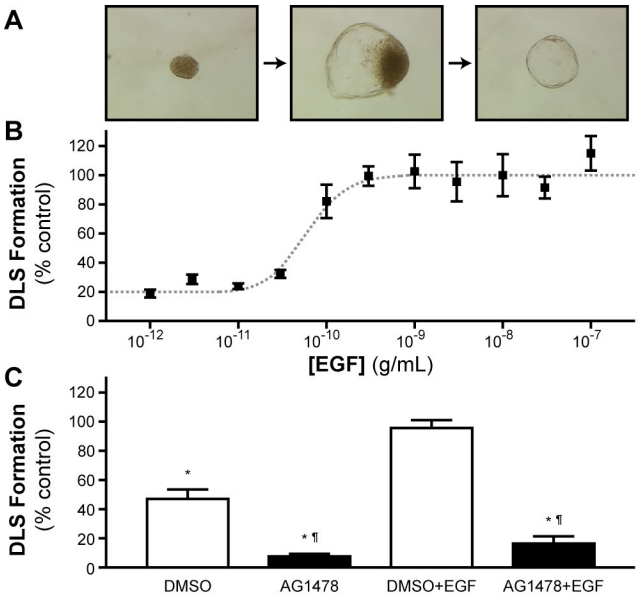

A
B
DLS Formation
(% control)
120
100
80
60
40
20
0
10^{-12}
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
[EGF] (g/mL)
C
DLS Formation
(% control)
*
* ¶
* ¶
DMSO
AG1478
DMSO+EGF
AG1478+EGF

Figure 5.2: DLS Formation Involves Early JNK and Late AKT and ERK Phosphorylation
A. Assessment of the 24-hour effect of islet embedding ± EGF administration on EGF ligand and receptor expression.
B. Time-course of DLS formation, assessed relative to maximal DLS formation.
C. Evaluation of the AKT, ERK, JNK and p38 phosphorylation states of cultures over the course of DLS formation (* $p<0.05$, ¶ $p<0.01$ vs. day 0).

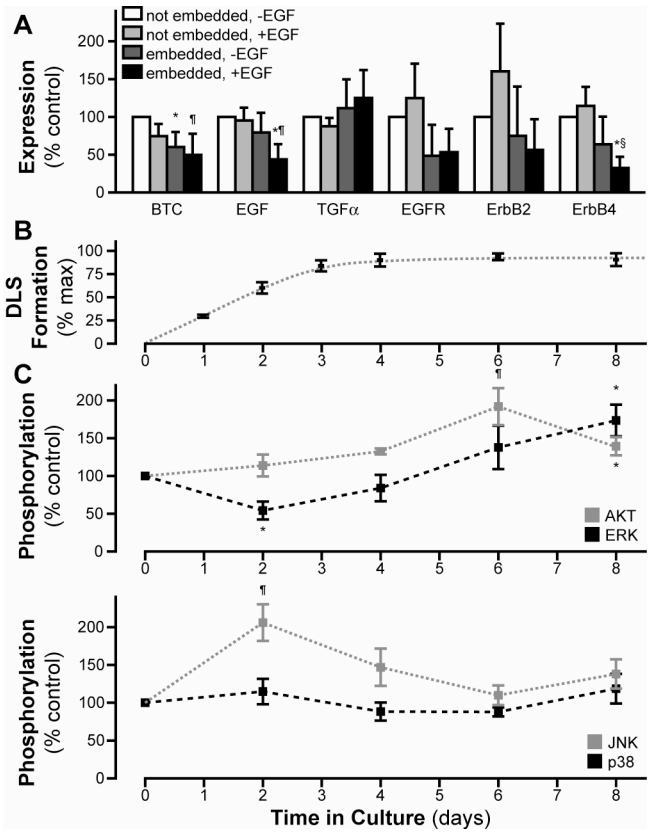

A
not embedded, -EGF
not embedded, +EGF
embedded, -EGF
embedded, +EGF
Expression (% control)
BTC
EGF
TGFα
EGFR
ErbB2
ErbB4
B
DLS Formation (% max)
C
Phosphorylation (% control)
AKT
ERK
JNK
p38
Time in Culture (days)

Figure 5.3: EGF Induces AKT and ERK Phosphorylation
A. Short-term time-course of the AKT, ERK, JNK and p38 phosphorylation states of islets following EGF administration.
B. The effects of AKT (CB5233705), ERK (U0126), JNK (SP600125) or p38 (SB203580) inhibition on DLS formation, assessed at day 8 of culture (* $p<0.05$, ¶ $p<0.01$ vs. control).

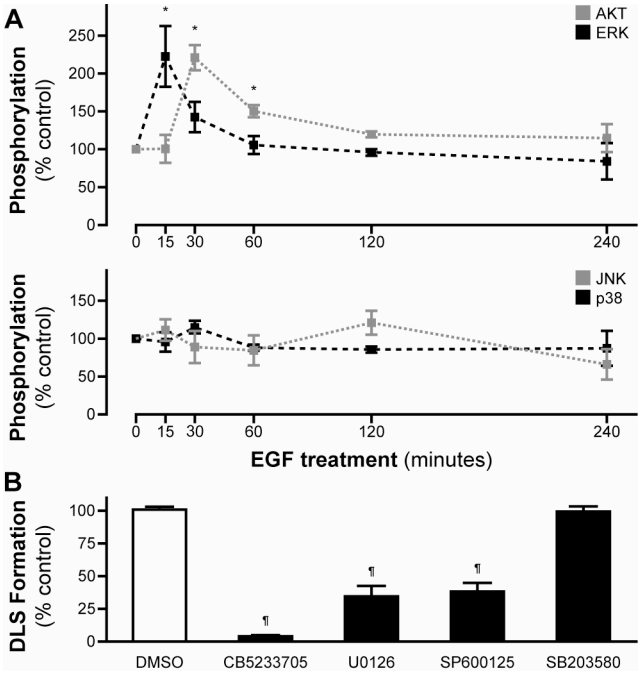
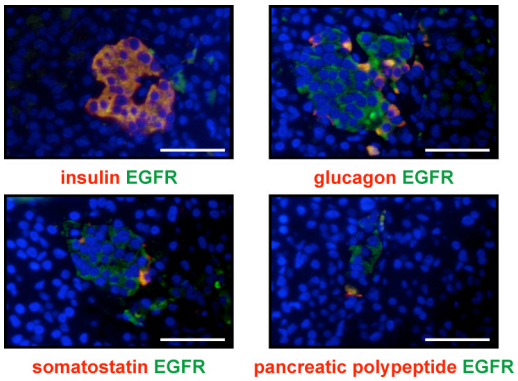

A
AKT
ERK
Phosphorylation (% control)
250
200
150
100
50
0
0 15 30 60 120 240
JNK
p38
Phosphorylation (% control)
150
100
50
0
0 15 30 60 120 240
EGF treatment (minutes)
B
DLS Formation (% control)
100
75
50
25
0
DMSO
CB5233705
U0126
SP600125
SB203580

Figure 5.4: EGFR is Expressed by Endocrine Cells
Immunofluorescent co-localization of EGFR with insulin, glucagon, somatostatin or PP in freshly isolated human islets.


insulin EGFR
glucagon EGFR
somatostatin EGFR
pancreatic polypeptide EGFR

5.5 Discussion

We have previously reported that islets do not simply react passively to the *ex vivo* environment following isolation, but can in fact produce autocrine factors to modulate the islet response to these new surroundings. These signals can act to enhance islet cell survival and to promote maintenance of the differentiated phenotype, as with insulin secretion in islets cultured in suspension,[494] or TGFβ production in embedded islets.[474] However, these signals may also be deleterious to preservation of islet phenotype and function, as with TNFα production in response to islet isolation.[495] In the case of embedded islets, it would appear that EGF ligand production acts in concert with the extracellular microenvironment to further destabilize islet phenotype, leading to islet cell dedifferentiation and the formation of highly proliferative precursor-like cells.[579]

Our studies on the islet response to collagen embedding ± EGF administration suggest that simple EGF administration induces little in the way of transcriptional feedback control of EGF ligands and receptors. While embedding of islets induced somewhat stronger transcriptional changes, these changes were still relatively minimal, even with concomitant addition of EGF. In fact, expression of ErbB4, a protein with limited affinity for EGF[580] and not required for EGF-mediated activation of the AKT and ERK signaling pathways,[581] was the only receptor transcript to be significantly downregulated in this context. Thus, isolated islets constitutively express significant levels of EGF ligands and receptors, while expression appears to be minimally responsive to collagen embedding and/or EGF exposure. These results do not appear to reflect differences in expression levels between the differentiated endocrine and primitive precursor cell phenotypes, as preliminary studies suggest that the latter dedifferentiated cells overexpress EGFRs relative to the islets from which they are derived (data not shown).

The role of EGF ligands in the control of islet morphogenetic plasticity is far from clear. Studies of pancreatic development have highlighted these proteins as essential to pancreatic duct epithelial development[73] and the subsequent differentiation and migration of endocrine cells.[75] Knock-out studies in adult

animals have indicated the EGFR expression is required in β-cells to maintain function and allow the limited proliferation that these cells undergo in the adult.[76] However, several groups have now reported that culture of isolated islets in medium containing EGF leads to the subsequent dedifferentiation of adult islet cells.[245, 394, 449] Moreover, in this paper we have presented evidence that suggests a critical role for EGF in this process, inducing the signaling pathways that lead to the subsequent proliferation of these dedifferentiated cells. However, the time-course of events leading to DLS formation suggests that the effect of EGF may be to modulate events later in the pathway. Accordingly, we have identified the key signal transduction responses of islet cells to EGF administration as ERK and AKT phosphorylation, and we have demonstrated the absolute requirement for these signals for islet cell dedifferentiation to occur. However, while time-course studies of isolated islets suggest that these pathways are activated early after EGF exposure, with peaks within 30 minutes, such activation signals are not observed during the transition from islet to DLS until at least day 6, that is, until after cellular dedifferentiation has occurred and when DLS cell proliferation is maximal. Thus, while essential to DLS formation, it appears that the primary role of EGF is the induction of proliferation of the dedifferentiating cells. While EGF is also known to activate the JAK/STAT signaling pathway, use of the EGFR tyrosine kinase inhibitor AG1478 confirmed the receptor-dependence of the EGF signal,[582] while JAK/STAT activation is known not to require such receptor kinase activity.[581]

EGF and other ligands of the same family seem to play contradictory roles in the control of pancreatic β-cell mass. In islet cells, EGF signaling appears to be key to the maintenance of β-cell function and survival in the native pancreas. However, in islet cells removed from the *in vivo* environment, EGF ligands act to induce islet cell dedifferentiation, highlighting the importance of the local microenvironment, namely the ECM, in directing the nature of the islet response to EGF. This niche-mediated effect of EGF ligands is not specific to mature islet cells. For example, EGF acts on primitive pancreatic ductal epithelium to inducing proliferation and branching morphogenesis during pancreatic

organogenesis, while later inducing islet cell differentiation from this same primitive pancreatic epithelium.[75] In fact, studies of transgenic and TGFα-administered animals suggest that EGF ligands may continue to act as inducers of islet differentiation in adults, through an apparent priming of otherwise differentiated ductal epithelium, which can then respond more strongly to a true differentiation stimulus.[286, 343, 344]

Finally, EGF signaling is also implicated in pancreatic carcinogenesis.[181, 567, 568] Here again, however, the mechanism of EGF is difficult to interpret, especially given that the cell of origin of pancreatic carcinogenesis remains a subject of debate. Regardless of an endocrine or duct epithelial origin, however, there is ample evidence to implicate EGF signaling in the metaplastic conversion of pancreatic tissues.

While work continues on identifying the factors and mechanisms that act to control the morphogenetic plasticity of adult human islets, it is tempting to speculate that the manipulation of such variables may allow the reprogramming of the endogenous pancreas to re-establish pancreatic β-cell mass. Such a feat would be invaluable in the treatment of diabetes. However, any manipulation of the proliferative potential and differentiation state of cells also carries with it the risk of neoplasia, and so care must be taken to fully understand the complex interrelationships of mechanisms that may be involved.[250]

5.5.1 Acknowledgements

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Bridging Statement Between Manuscripts

The previous chapter established that, as was the case with isolated islets in suspension, cultured islets also produce endogenous factors that can be detrimental to form and function. Given that EGF appears to be constitutively expressed by islets, and may in fact be essential to the normal function of islets *in vivo*,[76] it stands to reason that the same factor elicits a differential response under the altered microenvironment provided by *ex vivo* culture.

As with the response to isolation, however, it is also likely that there exist stabilizing factors that act to counter the deleterious effects. The aim of the next chapter was to establish whether any such stabilizing factors were involved during *ex vivo* culture.

6 Islets Produce Phenotype Stabilizing Factors in Response to Culture

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6.1 Abstract

Tissue plasticity is well documented in the context of pancreatic regeneration and carcinogenesis, with recent reports implicating dedifferentiated islet cells both as endocrine progenitors and as the cell(s) of origin in pancreatic adenocarcinoma. Accordingly, it is noteworthy that accumulating evidence suggests that TGFβ signalling is essential to pancreatic endocrine development and maintenance, while its loss is associated with the progression to pancreatic adenocarcinoma. The aim of this study was to examine the role of TGFβ in an *in vitro* model of islet morphogenetic plasticity. Human islets were embedded in a collagen gel and cultured under conditions that induced transformation into DLS. Addition of TGFβ caused a dose-dependent decrease in DLS formation. While it was demonstrated that collagen-embedded islets secrete low levels of TGFβ, antibody-mediated neutralization of this endogenously released TGFβ improved DLS formation rates, suggesting local TGFβ concentrations may in fact be higher. Time-course studies indicated that TGFβ signalling was associated with an increase in ERK and p38 MAPK phosphorylation, although inhibitor-based studies were consistent with an islet endocrine-stabilizing effect mediated by p38 alone. Localization of TGFβ signalling molecules suggested that the action of TGFβ is directly on the β-cell to inhibit apoptosis and thus stabilize endocrine phenotype.

6.2 Introduction

Until quite recently the consensus was that adult pancreatic cells were terminally differentiated, being neither stable nor labile. However, increasing evidence suggests that given the appropriate conditions, mature pancreatic cells can be directed to transdifferentiate into one of the other pancreatic cell types; endocrine, acinar or ductal.[173, 583, 584]

With respect to pancreatic endocrine regeneration, there is compelling evidence that β-cell mass exhibits morphogenetic plasticity in its adaptation to prevailing physiologic demands. For example, insulin resistance during pregnancy[248] or in obesity[22, 188] causes as much as a doubling of β-cell mass. Conversely, an argument could be advanced that T2DM is the result of defective β-cell mass dynamics, given the observed step-wise decline in β-cell mass from the non-diabetic state to impaired fasting glucose to clinically apparent T2DM.[22]

While the proliferation of existing endocrine cells has been proposed as the major mechanism of endocrine pancreatic regeneration,[276] the role of islet neogenesis, that is the *de novo* formation of endocrine cells from non-endocrine progenitors,[258, 259] cannot be discounted. While islet neogenesis has principally been attributed to islet precursors in the duct epithelium,[91, 258, 259, 288, 300] acinar cells[309, 310] and even intra-islet progenitors[327, 328, 330] have been proposed as alternate sources of new islets. Moreover, several recent reports have focused on the ability of adult islets to undergo *in vitro* dedifferentiation into proliferative cells/structures that can then redifferentiate into functional ILS,[245, 296, 394, 449] suggesting that the islet itself may be implicated in morphologic transformation in the pancreas through a process of transdifferentiation.

It is therefore noteworthy that transdifferentiation has also been associated with pancreatic carcinogenesis.[165] Thus, while pancreatic ductal epithelium constitutes less than 15% of all pancreatic cells, adenocarcinomas account for 90% of pancreatic cancer, suggesting the adoption of a ductal phenotype during the course of dysplasia.[585] Accordingly, both acinar-to-ductal[307, 586, 587] and islet-to-ductal transformation[165] have been proposed as early stages in carcinogenesis. Likewise, several groups have reported on the phenotypic

instability of cultured islets[436, 437, 452, 454, 455] as well as that of transplanted islet-derived cells,[588] suggesting that local factors are critical to the maintenance of the mature adult cellular phenotype.

One such factor, TGFβ, appears to play a crucial role in normal pancreatic development because it may be required to control the relative balance of pancreatic cell types. Furthermore, TGFβ has been demonstrated to limit the proliferation of acinar[589] and ductal cells,[590] while expression of a DN-TGFβRII results in increased acinar proliferation and differentiation.[84] Conversely, TGFβ has been proposed as a key mediator of islet morphogenesis[82] since the addition of TGFβ to mouse embryonic explants induces a reversal in relative frequencies to favour endocrine cell types over acinar and ductal.[86] Likewise, a recent report has indicated that inhibition of TGFβ signalling in the adult pancreas causes diabetes via the disruption of islet function.[88] TGFβ also appears to be an islet survival factor, given that it acts to protect islets from cytokine-mediated cell death.[591]

TGFβ may be equally important in pancreatic cancer[592] since the vast majority of pancreatic adenocarcinomas are defective in TGFβ signalling.[178] Although TGFβ receptor mutations have also been identified in pancreatic cancers,[177, 178] far and away the most frequent mutation in pancreatic cancer is that of SMAD4,[176] a key effector of TGFβ signalling.

Here we report novel findings on the important role of TGFβ in regulating islet plasticity in an *in vitro* model of islet-to-duct transdifferentiation. While we[452, 454, 455] and others[294, 456] have already described the ability of islets to transform into DLS, we now provide evidence that TGFβ regulates islet plasticity by stabilizing β-cell phenotype and inhibiting dedifferentiation.

6.3 Materials and Methods

6.3.1 Islet Isolation and Culture

Pancreata from adult human cadaveric organ donors were obtained through the local OPO (Table 6.1). Islets were isolated according to established protocols.[400] Briefly, following procurement, organs were flushed and stored in cold ViaSpan solution (Barr Laboratories, Pomona, NY) until islet isolation. The main pancreatic duct was cannulated and perfused with a solution of Liberase HI (Roche Diagnostics, Laval, QC). The distended organ was placed in a closed system (Bio-Rep, Miami, FL) and heated to 37°C to activate the enzyme blend. Following the appearance of free islets in samples, the system was cooled and free tissues were collected and washed. Tissues were separated by continuous Ficoll (Biochrom AG, Berlin, Germany) density gradient centrifugation in a cell processor (COBE DCT, Denver, CO). Free islets with diameters ranging from 75 to 400μm, determined to be greater than 70% pure by real-time staining with dithizone (Sigma, St. Louis, MO), a Zn^{2+} chelator, were collected, washed, and counted as IE. Immunofluorescent analysis determined that islet preparations contained 71.4±8.8% endocrine (insulin$^+$, glucagon$^+$, somatostatin$^+$, PP$^+$) and 3.4±0.9% ductal (CK$^+$) cells.

Isolated islets were cultured in suspension overnight in serum-free DMEM/F12 (Gibco, Burlington, ON) containing penicillin, streptomycin, fungizone, 1μM dexamethasone, 10ng/mL EGF (Sigma), 24mU/mL insulin (Lilly, Toronto, ON) and 200ng/mL cholera toxin (Sigma). Islets were then embedded in type I rat tail collagen, as per previous reports,[452, 454, 455] at a density of 2000IE/25cm^2, and cultured for 8 days in the above differentiation medium (DM), with media changes every other day. To remove tissue from collagen, samples were incubated with 0.25mg/mL collagenase XI (Sigma) for 30 minutes at 37°C. Control medium (CM) consisted of the above medium, without cholera toxin, with 10% FBS (Montreal Biotech, Dorval, QC).

Resultant structures were scored visually as being either islet (no evidence of DLS formation), DLS (with no residual islet) or islet-DLS (any combination of islet and DLS character). DLS formation was then calculated as a percentage:

$$\text{DLS formation} = \frac{\#\text{ islet-DLS} / 2 + \#\text{ DLS}}{\#\text{ islet} + \#\text{ islet-DLS} + \#\text{ DLS}}$$

6.3.2 Culture Additives

When added, concentrations of culture additives were 100pM for rhTGFβ1 (Sigma), and 10μg/mL for control and pan-neutralizing TGFβ antibodies (R&D Systems, Minneapolis, MN). U0126, SP600125, SB203580 and SB431542, pharmacologic inhibitors of ERK kinase, JNK, p38 and TGFβRI, respectively, were used at 10μM (Calbiochem, San Diego, CA).[572, 573, 593] Inhibitors were prepared so that the final concentration of DMSO was 0.1%, and vehicle controls were used.

6.3.3 Immunofluorescence

Samples were stored at 4°C in phosphate-buffered formalin until cell pellets were embedded in 2% agarose (Gibco). Following routine processing and embedding, 4μm sections were cut and dewaxed in xylene and petroleum ether. Slides were permeabilized by incubation in Tris-buffered saline + 0.1% Triton X-100 for 5 minutes, prior to antigen retrieval by heating for 10 minutes in 0.1M citrate buffer, pH 6. Slides were then incubated in blocking buffer (Zymed, San Francisco, CA) for 15 minutes before overnight incubation with primary antibody in blocking buffer. Following washing in PBS, slides were incubated for 1 hour with secondary antibody in blocking buffer, and reprobed with a different primary antibody. Again, following washing slides were incubated with secondary antibody before washing and coverslipping with mounting medium containing DAPI (Vector, Burlingame, CA). Antibodies used were rabbit α-insulin, α-glucagon, α-somatostatin and α-PP with mouse α-CK-AE1/AE3 (Dako, Carpinteria, CA), and mouse α-insulin and α-glucagon (Abcam, Cambridge, MA)

with rabbit α-TGFβRI, α-TGFβRII (Santa Cruz Biotechnology, Santa Cruz, CA) or α-SMAD2 (Cell Signalling, Danvers, MA) primary antibodies, along with FITC- and rhodamine-linked species-specific secondary antibodies (Cedarlane, Hornby, ON). To assess apoptotic cell death, the TUNEL kit (Roche Diagnostics) was used as per the manufacturer's instructions.

6.3.4 Insulin Content and Glucose-Stimulated Insulin Secretion

For evaluation of insulin content, samples were washed thoroughly in PBS before sonication in lysis buffer (50mM Tris-HCl, pH 8, 1.37mM NaCl, 0.1mM Na_3VO_4, Complete protease inhibitor tablet (Roche), 1% v/v nonident P-40, 10% v/v glycerol). Insulin content was assessed by enzyme-linked immunosorbent assay (ELISA; Alpco, Salem, NH), as per the manufacturer's instructions, and normalized to protein content, measured using the Bradford method.[514]

To assess islet function, cultures were washed thoroughly with Hank's buffered saline solution (HBSS) and incubated sequentially with HBSS + 2.2mM glucose for two consecutive 60 minute periods, HBSS + 22mM glucose for 30 minutes, HBSS + 22mM glucose + 50μM IBMX (a cAMP phosphodiesterase inhibitor; Sigma), and finally HBSS + 2.2mM glucose for 60 minutes. Conditioned media were collected and analyzed for insulin content by ELISA. Insulin secretion was normalized to baseline in freshly isolated islets from the same donor. Stimulation index was calculated as the ratio of insulin secretion at 22mM glucose to that observed at 2.2mM glucose.

6.3.5 TGFβ Secretion

Conditioned medium was collected every other day at the time of media change, and was frozen until analysis. TGFβ1 secretion was quantified by ELISA (Alpco), as per the manufacturer's instructions.

6.3.6 Western Blot and p38 Kinase Assay

Samples were sonicated in lysis buffer and centrifuged to remove cellular debris. After protein quantification, samples of equal protein content were separated by

denaturing electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked in tris-buffered saline + 0.1% Tween-20 + 3% bovine serum albumin and probed with primary antibody diluted in blocking buffer. After washing, blots were incubated with secondary antibody diluted in blocking buffer. After another wash, blots were developed using ECL (GE HealthCare, Piscataway, NJ) and exposed to X-OMAT film (Eastman Kodak, New Haven, CT). Membranes were stripped (62.5mM Tris-HCl, pH 6.7, 100mM β-mercaptoethanol, 2% w/v SDS) for 30 minutes at 65°C, blocked and reprobed with primary antibodies. Antibodies used were rabbit α-phospho-ERK, α-ERK, α-phospho-p38, α-p38, α-phospho-JNK, α-JNK, α-phospho-SMAD2 and α-SMAD2, as well as mouse α-GAPDH (Cell Signalling). Secondary antibodies were α-rabbit and α-mouse HRP conjugates (Cell Signalling). p38 kinase activity was assessed by a non-radioactive Western blot-based kinase assay (Cell Signalling), as per the manufacturer's instructions.

6.3.7 Statistical Analysis

All experiments were paired in that control and treatment groups from the same donor were compared. The number of biological replicates is indicated in the figure legends, and results are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA with post hoc Bonferroni's test, or paired Student's t-test, when applicable. Differences were considered significant when $p<0.05$.

6.4 Results

6.4.1 Embedded Islets Form DLS

Following embedding in collagen and culture for 8 days in DM, adult human islets formed cystic DLS lined by a layer of flattened or low cuboidal epithelium of one to several cells thickness (Fig. 6.1). Double immunofluorescence and electron microscopy studies indicated that islet-to-DLS conversion is characterised by the transdifferentiation of mantle islet cells into cells expressing duct epithelial

markers, while β-cells in the islet core are removed as a result of apoptosis.[394, 455] Recent lineage tracing studies confirmed the mantle cell origins of DLS.[594] We have previously characterised these islet-derived DLS as expressing high levels of epithelial and progenitor markers, low levels of endocrine markers, being highly proliferative and possessing the ability to regenerate ILS given appropriate stimulation.[394]

DLS formed from 64.2±2.5% of embedded islets, as determined visually. Morphological scoring was established as an appropriate indicator of endocrine (hormone$^+$) and epithelial (CK$^+$) cell frequency, as evaluated by immunofluorescence microscopic studies (Fig. 6.1G; endocrine: r^2=0.64, $p<0.001$; CK: r^2=0.74, $p<0.001$).

6.4.2 TGFβ Inhibits DLS Formation

Given the importance of TGFβ signalling in regulating the development of the endocrine pancreas,[82, 86] as well as the during pancreatic carcinogenesis,[177, 178, 595-597] seemingly from islet source tissue,[165] we sought to determine the role of TGFβ in islet-to-DLS transdifferentiation. While embedded islets formed DLS with relatively high frequency, the addition of TGFβ1 post-embedding blocked DLS formation (evaluated at day 8) in a concentration-responsive manner (Fig. 6.2A; $p<0.001$), with an EC_{50} of approximately 34pM. This concentration is similar to that reported for other cultured human cells.[598, 599] For subsequent experiments, a concentration of 100pM TGFβ was chosen based on the inhibition of DLS formation by approximately 70%.

6.4.3 TGFβ Maintains Islet Phenotype

In vitro dithizone staining suggested that cultures treated with TGFβ also maintained islet insulin content. To quantify this effect of TGFβ on islet cultures, insulin content was compared between freshly isolated and islets cultured for 8 days. Embedding and culture in DM decreased insulin content by 78.9±12.9% ($p<0.05$ vs. baseline), consistent with our previous reports (Fig. 6.2B).[452, 454] In cultures supplemented with TGFβ (DM+TGFβ), insulin content was decreased

relative to freshly isolated islets, but was preserved relative to DM cultures (Fig. 6.2B). It should be noted that embedded islets cultured in CM, with minimal DLS formation (16.7±5.5%), also had reduced insulin content (Fig. 6.2B; $p<0.01$), confirming our previous report that prolonged culture reduces islet insulin content.[454] Addition of TGFβ to CM improved overall insulin content (CM+TGFβ; Fig. 6.2B).

6.4.4 TGFβ Inhibits Islet Dedifferentiation

Evaluation of DLS formation over the course of culture suggested that the majority of DLS formation occurred by day 2 post-embedding (Fig. 6.3A), consistent with previous observations regarding the timing of the loss of endocrine phenotype[455] and function.[452, 454] TGFβ-treated cultures showed similar dynamics, though the overall DLS formation rate was reduced (Fig. 6.3A).
To determine whether the effect of TGFβ was to prevent islet dedifferentiation, embedded islets were cultured in DM for the first 4 days, then TGFβ was added for the final 4 days of culture. Under these conditions, TGFβ had no significant effect on the overall DLS formation rate (Fig. 6.3B), suggesting that TGFβ administered after 4 days acted only on those islets that had not yet undergone DLS formation.

In order to assess whether the effect of TGFβ was transient or whether acute TGFβ treatment conferred resistance to dedifferentiation, embedded islets were exposed to TGFβ for the first 2 days of culture - the period of maximal DLS formation - followed by a 6-day washout period. Under these conditions, DLS formation rates matched those of untreated islet cultures (Fig. 6.3B), suggesting that the effect of TGFβ is transient, and thus would require chronic exposure to stabilize islet phenotype.

Quantification of cell numbers after 8 days confirmed that TGFβ-treated cultures had an increased insulin$^+$ cell and decreased CK$^+$ cell frequency relative to DM cultures (Fig. 6.3C; $p<0.05$ vs. baseline). Moreover, this effect of TGFβ appeared to be mediated by a reduction in the frequency of β-cell apoptosis, as

assessed at day 2, the point of maximal DLS formation (Fig. 6.3D; $p<0.01$ vs. DM).

6.4.5 TGFβ Adversely Affects Islet Function

Based on the above observations regarding the effect of TGFβ on islet cell phenotype and insulin content, we hypothesised that TGFβ-treated cultures would also preserve islet function, as assessed by GSIS on day 8. As expected, islets cultured in DM had a stimulation index close to unity, indicating a lack of GSIS, especially when compared to the 2.4-fold glucose-stimulated increase in insulin secretion observed in freshly isolated islets (Fig. 6.4A; $p<0.05$). Interestingly, DM+TGFβ cultures exhibited a stimulation index of 0.36±0.07, suggesting a TGFβ-mediated dysregulation of glucose sensing and insulin secretion in the remaining islets, to the extent that insulin secretion was in fact reduced in response to elevated glucose concentrations. Investigation of islets cultured in CM confirmed these results; while CM cultures had a somewhat reduced stimulation index of 1.50±0.30, reflecting the effect of 8 days of culture, CM+TGFβ cultures also displayed an inverted stimulation index, at 0.40±0.24 (Fig. 6.4A; $p<0.05$).

After incubation in high glucose, cultures were washed and incubated in low glucose to confirm that insulin secretion was glucose concentration-dependent. TGFβ-supplemented cultures also displayed increased insulin secretion in this low glucose environment (Fig. 6.4B), suggesting that TGFβ causes a dysregulation of GSIS.

6.4.6 Endogenous TGFβ Acts on Islet Cultures

Based on previous reports that islets and acinar tissue produce TGFβ,[600, 601] we hypothesised that endogenous TGFβ may be acting to inhibit DLS formation. To determine the effect of endogenously produced TGFβ, cultures were treated with either a control or pan-TGFβ neutralizing antibody. While the control antibody

had no effect on DLS formation, antibody-mediated neutralization of endogenous TGFβ resulted in a significant increase in DLS formation (Fig. 6.5A; $p<0.05$).

Given the significant effect of endogenous TGFβ neutralization on DLS formation rates, it was surprising that the ELISA-determined concentration of secreted TGFβ1, at 0.85±0.22pM, was 40-fold less than the concentrations required to affect DLS formation rates (Fig. 6.2A). However, given our previous observation of increased intra-islet concentrations of islet secretory products,[494] the local concentration of TGFβ could in fact be significantly higher.

To identify the signalling pathways regulating TGFβ production and/or secretion, we undertook inhibitor-based studies. These experiments determined that the ERK and JNK mitogen-activated protein kinase (MAPK) pathways were required for TGFβ secretion (Fig. 6.5B), while p38 signalling was not. TGFβ release may be regulated in an autocrine manner, as inhibition of TGFβ signalling via TGFβRI inhibition caused a slight increase in TGFβ secretion that was not statistically significant (Fig. 6.5B).

Analysis of conditioned media samples also confirmed that significant levels of exogenously added TGFβ were still present for up to 2 days in culture (data not shown).

6.4.7 TGFβ Induces ERK and p38 Phosphorylation

Our previous inhibitor-based studies established that islet-to-DLS conversion is characterised by JNK-mediated β-cell apoptosis, followed by ERK-mediated duct cell proliferation.[455] We sought to determine what pathways were involved in transducing the TGFβ signal in this model.

Western blot analysis of islet cultures confirmed our previous report that embedding and culture led to an early spike in JNK phosphorylation, followed by a late rise in ERK phosphorylation (Fig. 6.6). Evaluation of p38 and SMAD2 phosphorylation states indicated that p38 followed the same pattern as ERK, with limited phosphorylation early in the cystic progression, followed by a late increase in phosphorylation, while there was limited SMAD2 phosphorylation throughout (Fig. 6.6).

Addition of TGFβ had a dramatic effect on all signalling pathways studied; SMAD2, p38 and ERK phosphorylation were all increased substantially at day 1 post-embedding, and this increase in phosphorylation followed through until at least day 6 in culture (Fig. 6.6). Conversely, JNK phosphorylation was decreased by TGFβ treatment, although this effect was less pronounced at day 6 (Fig. 6.6).

6.4.8 TGFβ Activates p38 Downstream of TGFβRI

Observation of phosphorylation states at 1 and 6 days post-embedding provides an indication of the signalling pathways involved in DLS formation, but gives little information as to the signalling pathways activated directly downstream of TGFβ treatment. To assess the immediate downstream effects of TGFβ, cultured islets were examined by Western blot and kinase assay 0, 15, 30, 60 and 120 minutes after administration of TGFβ. In this context, TGFβ was observed to have no discernable effect on ERK phosphorylation. However, a significant increase in SMAD2 phosphorylation was observed 15 to 30 minutes after TGFβ treatment (Fig. 6.7A). JNK phosphorylation was subsequently observed to decrease from 30 to 60 minutes post-treatment, and p38 kinase activity was observed to increase from 60 to 120 minutes post-treatment (Fig. 6.7). Co-administration of a TGFβRI kinase inhibitor completely abrogated the observed increase in p38 kinase activity, suggesting that p38 phosphorylation is downstream of TGFβRI (Fig. 6.7B).

6.4.9 DLS Formation Requires ERK and JNK while TGFβ Signalling Requires p38

The aforementioned studies established the effects of TGFβ on the signalling pathways involved in DLS formation, however these studies did not establish the dependence of the process on these pathways, nor the pathways by which TGFβ mediates its effect. To this end, studies were undertaken using pharmacologic inhibitors, either alone or in combination, with and without TGFβ co-administration.

Inhibition of the ERK and JNK pathways by U0126 and SP600125, respectively, confirmed our previous observation that DLS formation is dependent on ERK phosphorylation and JNK activity (Fig. 6.8).[455] Furthermore, the effect of TGFβ was observed to be additive to inhibition of either the ERK or JNK pathways (Fig. 6.8), suggesting that TGFβ does no inhibit DLS formation via inhibition of ERK or JNK signalling.

Under culture conditions without TGFβ, inhibition of p38 or TGFβRI kinase activity had no effect on DLS formation rates (Fig. 6.8). However, inhibition of either p38 or TGFβRI kinase activity did abrogate the effect of TGFβ on DLS formation, as did co-inhibition of p38 and TGFβRI (Fig. 6.8). Thus, it would appear that TGFβ stabilizes islet phenotype via activation of p38, downstream of TGFβRI.

6.4.10 TGFβ Signalling Molecules are Expressed by Islet β-Cells

While others have confirmed the expression of TGFβ receptors and SMAD signalling molecules in islets,[602] the cellular localization of these molecules remains to be elucidated. Likewise, while we have observed significant effects of TFGβ on stabilizing islet phenotype, the cellular targets of TGFβ also remain to be identified. Thus, we sought to examine, by immunofluorescent studies, the cellular localization of TGFβRI, TGFβRII and SMAD2; key mediators of TGFβ signalling. Double immunofluorescence studies localized these molecules almost exclusively to insulin$^+$ β-cells within the islet (Fig. 6.9), suggesting that TGFβ elicits its effects via direct effects on islet β-cells.

Double immunofluorescence studies indicated that the CK$^+$ cells comprising the DLS express TGFβRI and TGFβRII, but do not appear to express SMAD2 to an appreciable degree (Fig. 6.9), suggesting the possibility of a defective TGFβ signalling pathway.

Table 6.1: Donor and Isolation Characteristics

n	18
Age (years)	57.7±2.5
Male (%)	55.6
BMI (kg/m^2)	27.8±1.0
Traumatic cause of death (%)	11.1
ICU duration (days)	2.6±0.6
Glucose (mM)	9.1±1.2
Amylase (U/L)	211.4±83.0
Cold ischemia (hours)	6.5±0.6
≥70% pure yield (IE)	379,038±58,359

Figure 6.1: Embedded Islets Form DLS
(**A-C**) Inverted and (**D-F**) immunofluorescence microscopy demonstrates that fresh islets are solid spherical structures (**A**) comprised of insulin$^+$, glucagon$^+$, somatostatin$^+$ or PP$^+$ endocrine cells (**D**). By day 2 of the culture period, foci of DLS formation appear and expand (**B**, **E**), eventually replacing the islet. By day 8, these DLS are hollow, cystic structures (**C**) that are a heterogeneous population of CK$^+$ duct epithelial cells (**F**).
G. Morphological evaluation of cultures, including TGFβ-treated, shows that DLS formation correlates positively with ductal (green: CK$^+$) cell frequency (r^2=0.74, $p<0.001$) and correlates negatively with endocrine (red: insulin$^+$, glucagon$^+$, somatostatin$^+$, PP$^+$) cell frequency (r^2=0.64, $p<0.001$, n=7 individual donors).

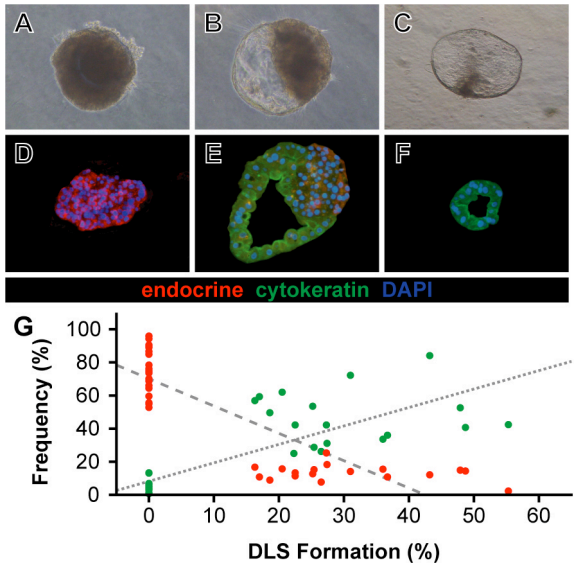

A
B
C
D
E
F
endocrine cytokeratin DAPI
G
Frequency (%)
100
80
60
40
20
0
0
10
20
30
40
50
60
DLS Formation (%)

Figure 6.2: TGFβ Inhibits DLS Formation
A. Culture medium was supplemented with TGFβ, causing a dose-dependent reduction in DLS formation, as evaluated morphologically at day 8 (§ $p<0.001$ vs. control, n=7).
B. Insulin content is reduced in islets cultured for 8 days, either forming DLS (DM: 64.2±2.5% DLS formation), or remaining as islets (CM: 16.7±5.5% DLS formation). In both cases, culture with added TGFβ partially maintains insulin content, as assessed by ELISA (* $p<0.05$, § $p<0.01$ vs. baseline, n=5).

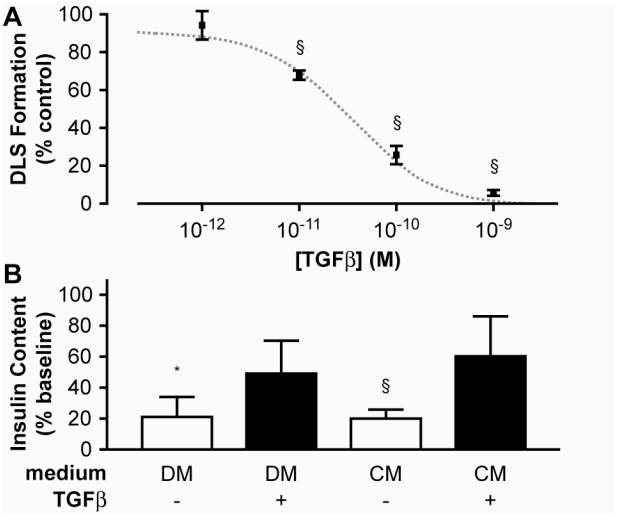

A
DLS Formation (% control)
100
80
60
40
20
0
§
§
§
10-12
10-11
10-10
10-9
[TGFβ] (M)
B
Insulin Content (% baseline)
100
80
60
40
20
0
*
§
medium
DM
DM
CM
CM
TGFβ
-
+
-
+

Figure 6.3: TGFβ Maintains Islet Phenotype
A. DLS formation occurs primarily within the first 2 days of culture, as determined morphologically. Furthermore, supplementing with TGFβ (- - -) reduces DLS formation relative to control (···), with this effect occurring within the first 2 days of culture (§ $p<0.01$ vs. time-matched control, n=4).
B. DLS formation, evaluated morphologically at day 8, is not affected if cultures are exposed to TGFβ for only the first 2 days, or the final 4 days of culture (n.s., n=3).
C. Supplementing with TGFβ results in an increased frequency of insulin^{+} β-cells and decreased frequency of CK^{+} ductal cells, determined by immunofluorescence at day 8 (* $p<0.05$ vs. DM, n=5).
D. The islet stabilizing effect of TGFβ is mediated via inhibition of β-cell apoptosis, as assessed by immunofluorescence and TUNEL-based detection of apoptosis at day 2 (§ $p<0.01$ vs. DM, n=3).

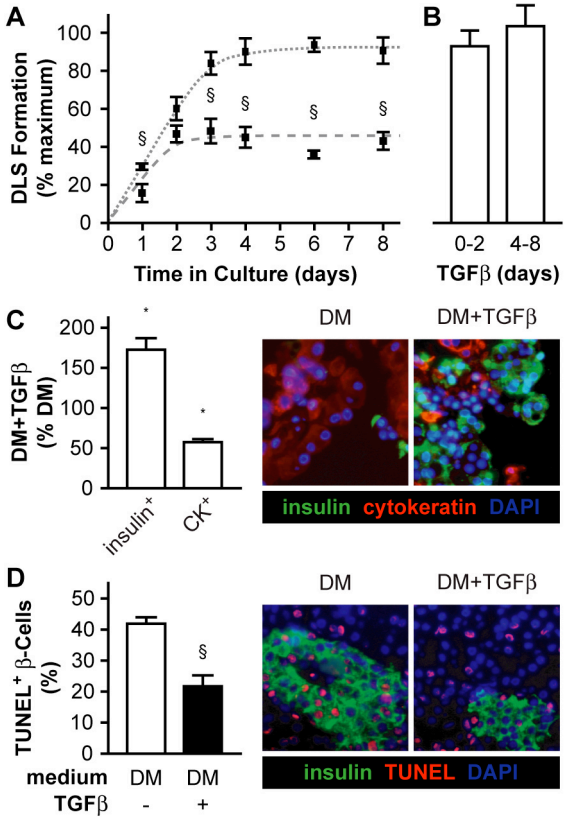

A
DLS Formation (% maximum)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8
Time in Culture (days)
§
B
0-2 4-8
TGFβ (days)
C
DM+TGFβ (% DM)
200
150
100
50
0
*
insulin+
CK+
DM
DM+TGFβ
insulin cytokeratin DAPI
D
TUNEL+ β-Cells (%)
50
40
30
20
10
0
medium DM DM
TGFβ - +
insulin TUNEL DAPI

Figure 6.4: TGFβ Adversely Affects Islet Function
A. While cultured islets (CM) have a reduced stimulation index relative to freshly isolated islets (fresh), islets cultured to produce DLS (DM) have further reduced function. However, culture with TGFβ reduces GSIS in both CM and DM cultures (* $p<0.05$ vs. fresh islets, n=3).
B. This effect of TGFβ is observed as an inversion of normal secretion kinetics (CM+TGFβ), as compared to freshly isolated (fresh) and cultured islets (CM; * $p<0.05$, § $p<0.01$ vs. equivalent fresh islet value, n=3).

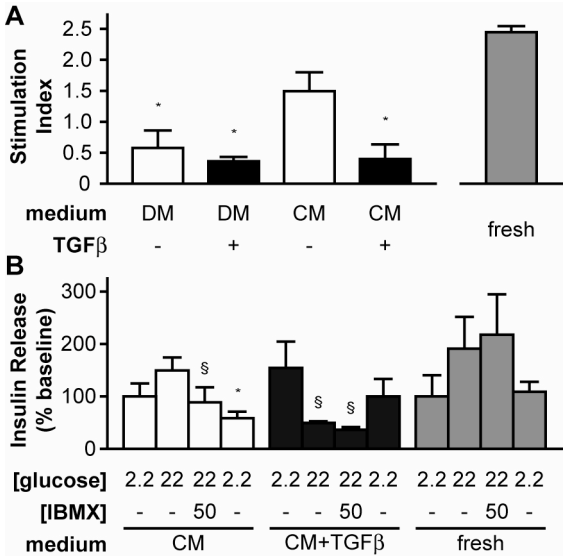

A
Stimulation Index
2.5
2.0
1.5
1.0
0.5
0
*
*
*
medium
DM
DM
CM
CM
fresh
TGFβ
-
+
-
+
B
Insulin Release (% baseline)
300
200
100
0
§
*
§
§
[glucose]
2.2 22 22 2.2 2.2 22 22 2.2 2.2 22 22 2.2
[IBMX]
- - 50 - - - 50 - - - 50 -
medium
CM
CM+TGFβ
fresh

Figure 6.5: Endogenous TGFβ Acts on Islet Cultures
A. Supplementing the culture medium with a pan-neutralizing α-TGFβ antibody increases DLS formation, while a control antibody has no effect (* $p<0.05$ vs. control, n=6).
B. Islet cultures secrete TGFβ at low levels, and this secretion is decreased by inhibition of the ERK (U0126) or JNK (SP600125) signalling pathways, while p38 (SB203580) or TGFβRI (SB431542) inhibition have no effect (* $p<0.05$ vs. vehicle, n=6).

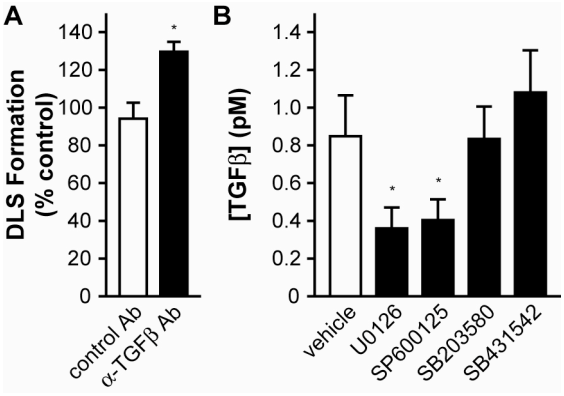

A
DLS Formation (% control)
140
120
100
80
60
40
20
0
*
control Ab
α-TGFβ Ab
B
[TGFβ] (pM)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
*
vehicle
U0126
SP600125
SB203580
SB431542

Figure 6.6: TGFβ Induces ERK and p38 Phosphorylation
Assessment of protein phosphorylation in embedded islets by Western blot suggests that one day of TGFβ exposure leads to an increase in ERK, p38 and SMAD2 phosphorylation levels, while JNK phosphorylation is decreased. These effects are maintained as far as 6 days in culture.

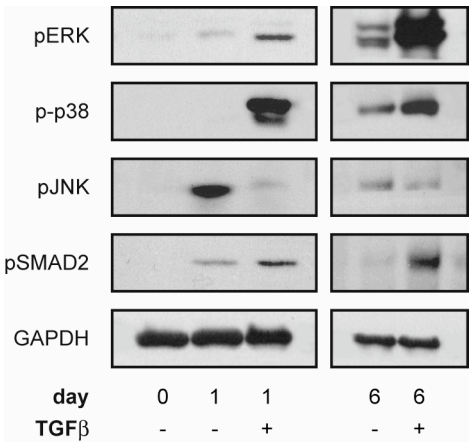

pERK
p-p38
pJNK
pSMAD2
GAPDH
day 0 1 1 6 6
TGFβ - - + - +

Figure 6.7: TGFβ Activates p38 Downstream of TGFβRI
A. Short-term TGFβ treatment of islets does not affect ERK or JNK phosphorylation levels, while p38 kinase activity and SMAD2 phosphorylation are induced, with peaks at 120min and 15min, respectively.
B. TGFβ treatment of islets causes a 4-fold increase in p38 kinase activity, as assessed by *in vitro* kinase assay, and this effect is blocked by SB431542-mediated TGFβRI inhibition (* $p<0.05$ vs. baseline, n=3).

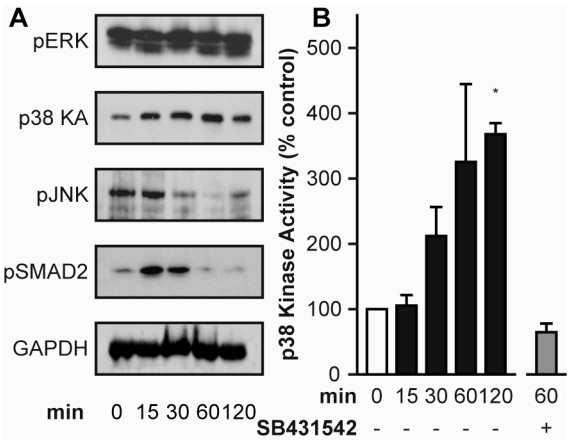

A
pERK
p38 KA
pJNK
pSMAD2
GAPDH
min 0 15 30 60120
B
p38 Kinase Activity (% control)
500
400
300
200
100
0
*
min 0 15 30 60120 60
SB431542 - - - - - +

Figure 6.8: DLS Formation Requires ERK and JNK While TGFβ Signalling Requires p38

Administration of inhibitors (U0126 - ERK; SP600125 - JNK; SB203580 - p38; SB431542 - TGFβRI), alone or in combination, with (■) or without (□) TGFβ co-administration, demonstrates that inhibition of ERK and/or JNK abrogates DLS formation, as does TGFβ treatment. The effect of TGFβ is additive with ERK and/or JNK. TGFβ-mediated inhibition of DLS formation is abrogated by inhibition of p38 and/or TGFβRI (* $p<0.05$ vs. TGFβ alone, § $p<0.01$ vs. untreated control, ¶ $p<0.01$ vs. same treatment without TGFβ, n=7).

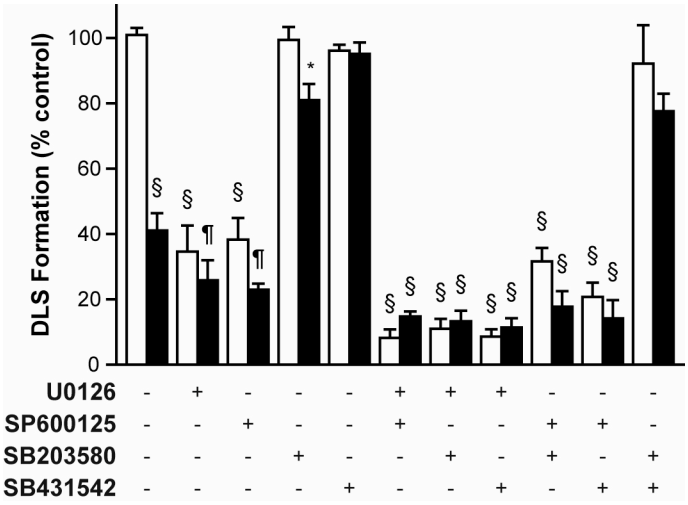
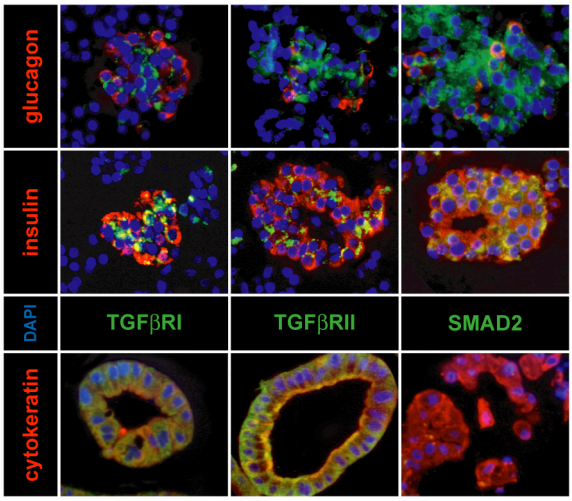

DLS Formation (% control)
100
80
60
40
20
0
§
¶
*
U0126 - + - - - + + + - - -
SP600125 - - + - - + - - + + -
SB203580 - - - + - - + - + - +
SB431542 - - - - + - - + - + +

Figure 6.9: TGFβ Signalling Molecules are Expressed by Islet β-Cells
Immunofluorescent co-localization studies demonstrate that TGFβRI, TGFβRII and SMAD2 are expressed predominantly in insulin$^+$ β-cells, rather than glucagon$^+$ α-cells. Immunofluorescence also demonstrates that CK$^+$ ductal cells of the DLS express TGFβRI and TGFβRII, but do not appear to express significant levels of SMAD2.


glucagon
insulin
DAPI
TGFβRI
TGFβRII
SMAD2
cytokeratin

6.5 Discussion

We report herein the ability of TGFβ to stabilize islet phenotype such that adult human islets do not undergo dedifferentiation into proliferative DLS. While this observation represents a novel effect attributable to TGFβ, it is not altogether unexpected given the role of TGFβ in directing islet development.[82, 86] However, while it is known that TGFβ favors endocrine development, the *de novo* formation of islets - islet neogenesis - represents the reverse of islet stabilization in that the former is represents a precursor-to-islet transition while the latter is characterised by islet-to-precursor dedifferentiation. That being said, our islet-to-DLS model has also been used to study islet neogenesis induced by various growth factors.[345, 394] Interestingly, in this context, administration of TGFβ was not observed to have any islet neogenic effect on DLS cultures (unpublished observations). This is somewhat contradictory to previous reports that suggest TGFβ favors endocrine differentiation in the developing pancreas,[86] although it has been argued that the mechanisms of β-cell formation are different in the developing and adult pancreas.[276] Furthermore, the endocrine potential of developing pancreatic ducts may be very different from that of the DLS formed from dedifferentiating islets.[91]

The effect of TGFβ on adult pancreatic cell types has been characterised to some extent. TGFβ inhibits the growth of ductal[603] and acinar[604, 605] tissue, and has also been suggested to induce apoptosis in the latter. Evidence also indicates that peri-acinar fibroblastoid cells, the so-called pancreatic stellate cells that act to regenerate the organ after pancreatitis, are activated by TGFβ[606-608] in a self-propagating loop.[609] This activation may lead to the ECM remodelling and fibrosis associated with this disease,[610] given that administration[611] or overexpression[612, 613] of TGFβ leads to pancreatitis-like fibrotic lesions, while administration of neutralizing antibodies limits excessive matrix deposition.[614]

TGFβ also appears to be an islet survival factor given that it acts to protect islets from cytokine-mediated cell death.[591] Furthermore, a recent report has indicated that inhibition of TGFβ signalling in the adult pancreas causes diabetes

via the disruption of islet function.[88] More specifically, targeted expression of SMAD7, a negative regulator of TGFβ signalling[615, 616], to PDX1$^+$ islet cells leads to a loss of gene expression related to β-cell function, resulting in decreased insulin content and hyperglycemia. Likewise, reinstatement of normal TGFβ signalling reverses the observed diabetes-like state. Other reports also suggest that TGFβ improves islet function, as measured by the insulin secretory response to glucose in isolated islets.[617, 618]

Interestingly, while we observed a TGFβ-mediated stabilization of islet phenotype, the effect of TGFβ on islet function was in fact the opposite (Fig. 6.4). This is in keeping with a recent report indicating a similar effect of TFGβ on isolated mouse islets.[619] Thus, there remains some debate as to the effect of TGFβ on islet function, while we present data indicating a regulatory effect on islet phenotype. Our study is only informative insomuch as it addresses the effect of TGFβ on islets cultured in a very specific microenvironment, therefore more definitive studies are required to establish the effect of TGFβ on isolated human islets.

Based on the islet expression profile of TGFβ signalling molecules (Fig. 6.9), as well as the mechanism of DLS formation, it appears likely that TGFβ elicits an islet stabilization effect by acting directly on β-cells. In fact, TGFβ appears to inhibit the β-cell apoptosis observed under these specific culture conditions (Fig. 6.3D). As mentioned, TGFβ is known to protect islets from cytokine-mediated apoptosis.[591] We observed TGFβ-induced p38 phosphorylation and activation, both acutely and chronically (Figs. 6.6&7). Furthermore, it appeared that the islet-stabilizing effects of TGFβ were p38-dependent (Fig. 6.8). There is no clear consensus on the role of p38 in islet survival and function. Conflicting reports have described p38 as being implicated in both pro-apoptotic[502, 547, 620] and pro-survival[549] pathways, as well as having positive effects on β-cell proliferation and gene expression,[352, 621] or no effect at all.[271, 622] While our studies cannot be extrapolated beyond the context of the islet dedifferentiation culture model, we can conclude that p38 activity is crucial to the islet-stabilizing effects of TGFβ.

The role of the SMAD signalling proteins is equally difficult to elucidate in our model. While TGFβ can signal through the SMADs or the MAPKs, these pathways are not mutually exclusive. In fact, it appears that the interaction between p38 and SMAD2 can be inhibitory or stimulatory, depending on the cellular context.[604, 623, 624] Thus, while the lack of pharmacological inhibitors of SMAD activity renders study of these molecules difficult in three-dimensional primary structures such as islets, the observed kinetics of SMAD2 and p38 phosphorylation suggest that TGFβ-mediated p38 activation is likely independent of SMAD activity, and as such the islet stabilizing effects of TGFβ are likely SMAD-independent. However, the experiments we describe cannot rule out an important role for SMAD signalling in islets.

Likewise, ERK and JNK signalling represent definite effectors of islet dedifferentiation,[455] although neither of these MAPKs is affected directly by TGFβ. As such, it would appear that the observed TGFβ-mediated changes in ERK and JNK phosphorylation may represent changes in the phenotypic state of the islets under study, thus increased ERK and decreased JNK phosphorylation are related to increased islet phenotypic stability, and TGFβ administration is one means of obtaining this effect.

Finally, definitions of endocrine pancreatic plasticity typically focus on β-cell mass as a unit; responding to the prevailing needs and conditions of the body by increasing or decreasing the net β-cell mass through a variety of mechanisms. While not incorrect, this view does not take into account the added plasticity of individual islets and/or other endocrine cell types. Thus, while likely negligible, one cannot discount the contribution of islet dedifferentiation to overall β-cell mass, especially if islet dedifferentiation, proliferation and redifferentiation[245, 296, 394, 449] is to be considered as a mechanism of islet regeneration.

6.6 Acknowledgements

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Bridging Statement Between Manuscripts

Having established an active role for islets in the regulation of their survival and differentiated state following isolation, we next addressed the question of tissue plasticity. Many recent studies reported the potential of isolated human islets to undergo dedifferentiation, proliferation and subsequent redifferentiation into structures with varying degrees of islet-like form and function.[245, 296, 448-450] Conversely, several studies in mice, including genetically engineered mice to allow lineage tracing, indicated that adult islet cells were not the prime contributors to any such regenerative mechanisms *in vitro*.[462-465] Having already established one such model of islet dedifferentiation and redifferentiation,[394] we next sought to identify the cellular origins of the populations of dedifferentiated cells we observed in culture. The next chapter addresses the cellular origins of such cells.

7 Cellular Origins and Regenerative Potential of Islet Plasticity

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7.1 Abstract

Cultured human islets can be dedifferentiated to DLS composed mainly of CK^+ and $nestin^+$ cells. Given that these structures possess the potential to redifferentiate into ILS, we sought to elucidate their specific cellular origins. Adenoviral vectors were engineered for β-, α-, δ- or PP-cell-specific expression. A double stranded system was designed whereby cultures were infected with two vectors; one expressed GFP behind the CR5 sequence, and the other expressed the requisite transactivator behind the human insulin, glucagon, somatostatin or PP promoter. This system labels $hormone^+$ cells in the islet in a cell-specific manner, allowing these cells to be tracked during the time-course of transformation from islet to DLS. Post-infection, islets were cultured to induce dedifferentiation. Fluorescence microscopy demonstrated that α-, δ- and PP-cells contributed equally to the CK^+ population, with minimal β-cell contribution, while the converse was true for $nestin^+$ cells. Complementary targeted cell ablation studies, using STZ or similar adenoviral expression of the BAX toxigene, validated these findings and suggested a redundancy between α-, δ- and PP-cells with respect to CK^+ cell derivation. These results call into question the traditional understanding of islet cells as being terminally differentiated, and provide support for the concept of adult islet morphogenetic plasticity.

7.2 Introduction

Pancreatic β-cell mass is tightly regulated,[22, 248, 250] reflecting the net effect of homeostatic mechanisms that include proliferation, neogenesis and apoptosis.[246]

With respect to neogenesis, small clusters of β-cells in close proximity to pancreatic ducts are taken as a surrogate measure,[258, 259] given that this is the mechanism by which islets form during pancreatic organogenesis.[54] Reports also suggest that pancreatic acinar tissue may serve as a source of new islets, either directly[309] or via duct-like intermediates.[310] Intra-islet progenitors have also been implicated.[327-330] In fact, varying degrees of islet-like morphology and function have been reported in cultures derived from non-endocrine pancreatic cell sources.[294, 319, 625] Unfortunately, the lineage relationships that exist between pancreatic cell types, and the extent to which this plasticity contributes to pancreatic β-cell mass dynamics, are highly controversial.[254, 255] For example, the physiological relevance of islet neogenesis as a mechanism of β-cell renewal or regeneration has been questioned by lineage tracing studies in mice.[276, 626] Nonetheless, elucidation of these cellular inter-relationships may be key to the development of novel diabetes therapies.

Notwithstanding this controversy, several groups,[245, 296, 448-450] including our own,[394] have recently reported that cultured adult human islets dedifferentiate into proliferative precursor-type cells, expressing either CK or nestin. These cells can in turn be induced to redifferentiate into ILS, given the appropriate stimulus. Preliminary results of ultrastructural,[394] immunofluorescence[245, 394, 450] and pulse-chase[448] experiments suggest that these precursor-type cells are of endocrine origin. To provide more conclusive evidence, recent studies have used islets from mice marked using *in vivo* lineage tracing techniques.[462-465] Chase *et al.* cultured islets isolated from mice engineered to express GFP in cells having had an active pdx1 promoter,[463] indelibly marking cells of all pancreatic lineages.[466] After culture, few if any GFP^+ precursor-type cells were identified, consistent with a non-pancreatic origin. However, minimal islet redifferentiation has been reported from cultured mouse islets,[462-465] suggesting an innate difference between mouse and human islet-derived cells.[22, 249] In fact, a most recent lineage

tracing study indicated that human β-cells were capable of *in vitro* dedifferentiation, while murine β-cells were not.[467]

The aim of this study was to establish the cellular origins of human islet-derived precursor-type cells, using adenoviral vectors to specifically label individual endocrine cell types. Identifying the exact source of precursor-type cells is a necessary prelude to the development of techniques for scaling up this process.

7.3 Materials and Methods

Adenoviral vectors were engineered for β-, α-, δ- or PP-cell-specific expression (Table 7.1). A double stranded system was designed whereby cultures were infected with two vectors; one expressed the marker (GFP or the Bcl-2-associated X protein (BAX)) behind the cumate-inducible promoter (CR5) sequence,[627, 628] and the other expressed the requisite transactivator behind the promoter sequence of interest. In this way, even low promoter activity would be sufficient to induce expression of the transactivator, in turn amplifying the signal[627] with minimal delay.[629] Similar co-infection systems have been used with some success in isolated islets.[630, 631] Comparison of this system to GFP expression controlled directly by the insulin promoter confirmed signal amplification (data not shown).

7.3.1 Cell Culture

The 293 cell line (ATCC, Manassas, VA), the 293CR5GFP cell line that stably expresses GFP under the control of CR5,[628] and the BMAdE1 cell line (Qbiogene, Irvine, CA), were grown in DMEM (Wisent, St-Bruno, QC) containing 5% serum and 2mM L-glutamine.

7.3.2 Plasmids

Plasmids containing the CR5 transactivator (cTA) behind the promoter sequence of interest (pAd-promoter-cTA) were constructed by replacing the cytomegalovirus (CMV) promoter portion of the pAdPS-CMV-cTA-DC-GFP

plasmid[628] with the promoter sequence of interest (Table 7.2), amplified by PCR from genomic DNA extracted from 293 cells using the DNeasy tissue kit (Qiagen, Mississauga, ON).

Plasmid containing BAX controlled by CR5 (pAd-CR5-BAX) was constructing by inserting the BAX coding sequence (a generous gift from Dr. B. Fang, University of Texas MD Anderson Cancer Center)[632] in place of GFP in the pAd-CR5-GFP vector.

7.3.3 Adenoviral Production

pAd-promoter-cTA plasmids were used to produce adenoviral vectors, using a positive selection method based on the rescue of PS-, E1- and E3-deficient adenoviruses (Qbiogene), as previously described.[633, 634] Homogeneously green fluorescent plaques on 293CR5GFP were picked and eluted.

Recombinant adenoviral vectors were amplified by consecutive passages in BMAdE1 cells, except for the last passage, which was in 293A cells, as per standard procedures. Adenoviruses were purified by centrifugation on CsCl gradients using standard procedures. Viral titer was determined by plaque assay on 293CR5GFP cells, as described previously.[635]

The adenoviral vector expressing GFP under the control of CR5 (Ad-CR5-GFP) has been described previously.[628] The adenoviral vector expressing BAX under the control of CR5 (Ad-CR5-BAX) was produced as described above.

7.3.4 Islet Isolation

Pancreata from adult human cadaveric organ donors were obtained through the local OPO (Table 7.3). Islets were isolated according to established protocols.[400] Free islets with diameters ranging from 75 to 400μm, determined to be greater than 75% pure by real-time staining with dithizone (Sigma, St. Louis, MO), a Zn^{2+} chelator, were collected, washed, and counted as IE.

7.3.5 Islet Infection

Islets were infected two days post-isolation, at a density of 2500IE/mL and a multiplicity of infection (MOI) of 5000/IE for Ad-CR5-GFP or Ad-CR5-BAX, and a MOI of 50,000/IE for Ad-promoter-cTA. After six hours, medium was added to a density of 1000IE/mL. Islets were further incubated for four days to allow marker expression to develop before culturing to form DLS. Controls included mock infection, infection with Ad-CR5-GFP or Ad-CR5-BAX alone (MOI of 5000/IE), or in combination with Ad-CMV-cTA (MOI of 50,000/IE).

7.3.6 Streptozotocin Treatment

In select groups, islet were pre-treated with 5mM STZ (Sigma), prepared in 0.1M citrate buffer, pH 4.5, for 30 minutes prior to embedding.

7.3.7 Islet Culture

Infected islets were washed in serum-free DMEM/F12 (Gibco, Burlington, ON) containing penicillin, streptomycin, fungizone, 1μM dexamethasone, 10ng/mL EGF (Sigma), 24mU/mL insulin (Lilly, Toronto, ON) and 200ng/mL cholera toxin (Sigma) prior to embedding. Islets were then embedded in type I rat tail collagen, as per previous reports,[452] at a density of 2000IE/25cm^2, and cultured for 8 days in the above medium, with media changes every other day. To remove tissue from collagen, samples were incubated with 0.25mg/mL collagenase XI (Sigma) for 30 minutes at 37°C. To disperse cultures into single cells, samples were washed twice in HBSS before incubation in 0.05% trypsin-EDTA (Invitrogen) for 10 minutes at 37°C. Samples were then washed in serum-containing medium before fixing in 4% phosphate-buffered formalin at 4°C.

In selected groups, spontaneous islet redifferentiation was assessed by switching DLS cultures to the above islet culture medium, with 10% serum but without cholera toxin, for four days (redifferentiation medium). Islet regeneration was also induced by adding 667nM INGAP peptide (Sheldon Biotech, Montreal, QC).[394]

7.3.8 Immunofluorescence

For immunofluorescence studies, sample of 4000IE were dispersed into single cells, and then aliquots applied to microscopes slides by cytocentrifugation. Slides were then processed for immunofluorescence as per standard protocols.[474] Antibodies used were rabbit α-insulin (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit α-glucagon, α-somatostatin and α-PP, mouse α-CK-AE1/AE3 (Dako, Carpinteria, CA) and mouse α-nestin (Abcam, Cambridge, MA) primary antibodies, along with FITC- and rhodamine-linked α-rabbit and α-mouse secondary antibodies (Abcam). To assess cell death, samples were processed using a TUNEL kit (Roche). Images were captured using a Zeiss Axioskop 40 microscope and Northern Eclipse v6.0 (Empix Imaging, Mississauga, ON), and at least 400 cells were counted per sample (528±27 cells).

7.3.9 Statistical Analysis

All experiments were performed using islets from a minimum of 3 donors (the number of biological replicates is indicated in the figures and tables) and experiments were paired in that control and treatment groups from the same donor were compared. Results are expressed as mean ± SEM. Statistical significance was determined by one-way ANOVA with a *post hoc* Bonferroni's test, or paired Student's t-test, when applicable. Differences were considered significant when $p<0.05$.

7.4 Results

7.4.1 Islet Purity and Composition

Immunofluorescence (Table 7.4) determined cells to be 72.7±5.0% hormone$^+$ and 3.0±0.9% CK$^+$. While 0.3±0.4% were hormone$^+$/CK$^+$, 23.2±8.7% were hormone$^-$/CK$^-$ and likely consisted of acinar and dead cells. Preparations also contained 5.2±1.1% nestin$^+$ cells, although these cells were in fact nestin$^+$/insulin$^+$. Cell frequencies (53:28:13:5 for β:α:δ:PP) were in keeping with previous reports for human islets.[39]

7.4.2 GFP Expression

Following adenoviral infection, islet samples were collected to assess labeling efficiencies and specificities. Immunofluorescence microscopy analysis determined that cell type-specific vectors had highly reproducible labelling efficiencies with specificities that approached 100% (Table 7.5, Fig. 7.1A). It is the reproducibility of these labelling efficiencies, and not the specific labelling efficiency that allows subsequent analysis. Moreover, these vectors appear to be highly specific, as few promoter-driven GFP^+ cells were observed that did not also express the hormone of interest (Table 7.5).

7.4.3 DLS Formation

Following collagen embedding and over 8 days of culture, islets became cystic DLS, composed of one to several layers of low cuboidal-type cells in thickness (Fig. 7.1B). Previously, we reported that DLS appeared to form through a combination of β-cell apoptosis and α-, δ- and PP-cell transdifferentiation. Furthermore, DLS express high levels of epithelial and progenitor markers, low levels of endocrine markers, and have the ability to regenerate ILS given the appropriate stimulus.[394] It is noteworthy that cells that comprise the DLS appear to be similar to those found in islet monolayers,[245, 296, 448-450] both with respect to the required culture conditions, as well as the presence of $nestin^+$/$vimentin^+$ and CK^+/$E\text{-}cadherin^+$ cells.[394]

To confirm that selective β-cell apoptosis was in fact occurring during the process of DLS formation, we assessed apoptosis prior to embedding and at day 2 of culture (Fig. 7.1C), the point of maximal DLS formation.[474] While little apoptosis was observed in endocrine cells prior to embedding, DLS formation was associated with a significant increase in apoptosis in all endocrine cells. However, β-cell apoptosis was the most prevalent, affecting nearly half of β-cells. Of note, non-endocrine cells also appeared to be affected by ongoing apoptosis.

We have previously reported that the process of DLS formation is complete by 8 days of culture.[474] Given the apparent unidirectional differentiation occurring

in this model, as well as the objective of determining cellular origins, we chose to analyze DLS cultures at this single time point (Fig. 7.2A). By immunofluorescence microscopy, we determined that 40.3±3.7% of cells in DLS cultures were CK^+ and 17.6±2.4% were $nestin^+$. Only 11.3±2.0% of cells retained hormone expression (Table 7.4), indicating that the majority of cells either died or became CK^+ or $nestin^+$.

7.4.4 Islet Cell Fate

To evaluate the fate of specific islet cell types, DLS cultures were assessed by immunofluorescence microscopy to determine the relative frequencies of GFP^+ cells (Fig. 7.2B,C). Only a minority of GFP^+ cells were also $hormone^+$, indicating that most GFP-labeled cells had ceased to express hormone. GFP-labeled β-cells, however, were more likely than other endocrine cells to retain hormone expression. Moreover, in all samples assessed, no cells were found that co-expressed promoter-driven GFP and any other islet hormone. This suggests that no transdifferentiation from one endocrine cell type to another was occurring in our model. Adenoviral infection did not affect the transformation of islets into DLS (Fig. 7.1D).

With respect to CK^+ cell formation, a dichotomy was apparent between β-cells and α-, δ- and PP-cells: 5.0±5.0% of GFP^+ β-cells expressed CK upon DLS formation, as compared to ~75% of labeled α-, δ- or PP-cells. These results support our previous observations of CK^+/$glucagon^+$ and CK^+/$somatostatin^+$ double immunofluorescence, without any indication of a CK^+/$insulin^+$ cell population.[394] The opposite was true with respect to nestin expression: 84.2±5.1% of GFP^+ β-cells became $nestin^+$, as compared to only ~4% of labeled α-, δ- or PP-cells.

While few $insulin^+$/$nestin^+$ cells are observed in freshly isolated human islets,[303] we noted that islets cultured in suspension for one week contained 5.2±1.1% $nestin^+$ cells (Table 7.4), most of which were also $insulin^+$. One explanation is that β-cell dedifferentiation is initiated in islets even prior to embedding.[437]

7.4.5 DLS Cell Origins

To establish the contributions of individual endocrine cell types to the sub-populations of the DLS, the frequencies of GFP^+/CK^+ and $GFP^+/nestin^+$ cells were calculated relative to the frequencies of CK^+ and $nestin^+$ cells (Table 7.6).

Accounting for labelling efficiencies, the overall contribution of β-cells to the CK^+ cell population was minimal. The contributions of α-, δ- and PP-cells were nearly equal, with each cell type providing approximately one-third of the final CK^+ cell population. Moreover, based on labelling efficiencies, α-, δ- and PP-cells appeared to be the main contributors to the population of CK^+ cells.

Conversely, an adjusted 74.4±0.5% of $nestin^+$ cells were of β-cell origin, with a minimal contribution from the α-, δ- and PP-cells. It should be noted, however, that at 6.1±1.1%, the PP-cell contribution, while minor compared to that of β-cells, was nevertheless more than that of either α- or δ-cells. Additionally, a significant proportion of $nestin^+$ cells could not be accounted for by the dedifferentiating β-cell population, suggesting a possible non-endocrine origin to some of these cells.

7.4.6 BAX Targeting

To confirm the contribution of specific endocrine cell types to DLS formation, islet cell types were targeted for apoptosis by promoter-mediated BAX expression. Post-infection samples were assessed for specificity of cell death induction (Fig. 7.3). Cell type-specific BAX expression induced a ~75% reduction in targeted cell types, without adversely affecting the frequency of other cell types, though overall cell numbers and frequencies were affected accordingly.

BAX-infected cultures were evaluated for DLS formation (Fig. 7.4A) and for the frequencies of the constituent DLS cell subtypes (Fig. 7.4B). Selective β-cell removal resulted in decreased DLS formation, suggesting an important role for β-cells in this process. Likewise, β-cell-depleted cultures also exhibited a relative decrease in the frequency of $nestin^+$ DLS cells, while the frequency of CK^+ cells increased proportionally.

Targeted depletion of α-, δ- or PP-cells was also associated with decreased DLS formation, although CK^+ and $nestin^+$ cell frequencies were unaffected, suggesting a redundancy in the roles of these non-β-cells in CK^+ DLS cell formation in that the remaining cell types are able to compensate for the absence of a single specific cell type.

To further clarify the role of individual cell types in DLS formation, additional experiments were carried out in which islets were infected with multiple adenoviral vectors to remove three endocrine cell types, theoretically leaving only one cell type remaining within the islet (Fig. 7.4A). Under these conditions, islets containing primarily α-, β-, δ- or PP-cells all displayed reduced rates of DLS formation, coincident with the notion that there is no one cell type that is entirely responsible for DLS cell formation, but rather an interdependence between cell types. Accordingly, removal of one cell type affected the ability of the islet as a whole to form a DLS.

7.4.7 STZ Treatment

As an alternative approach to targeted β-cell death, islets were treated with STZ prior to embedding. Pre-embedding samples were analyzed to confirm the specificity of cell death induction (Fig. 7.5A). STZ treatment led to a ~50% reduction in the frequencies of β- and PP-cells, without affecting the frequencies of α- and δ-cells. Assessment of apoptotic indices further confirmed the specificity of β- and PP-cell death (Fig. 7.5B).

STZ-treated islets were evaluated for DLS formation (Fig. 7.5C) and the frequencies of DLS cell types (Fig. 7.5D). Surprisingly, STZ treatment had no effect on DLS formation. This result appears at odds with the results of the BAX expression experiments (Fig. 7.4A) that suggested that the removal of any single endocrine cell type impairs DLS formation. As BAX expression reduced target cell type frequencies by ~75%, while STZ treatment reduced β- and PP-cell numbers by only ~50%, it is possible that a threshold exists whereby islets can withstand limited cellular depletion without differentiation being affected.

As for DLS cell types, the removal of ~50% of PP-cells did not adversely affect the CK^+ cell frequency, which was to be expected given the apparent redundancy of α-, δ- and PP-cells with respect to their contribution to dedifferentiation. STZ-mediated removal of ~50% of β-cells in islets translated to a significant reduction in the frequency of $nestin^+$ cells within the DLS, confirming earlier observations of DLS cell origin.

7.4.8 Islet Redifferentiation

We have previously established that DLS can be induced to redifferentiate into ILS given the appropriate stimulus.[394] To identify the precursor-type cell(s) responsible for islet redifferentiation in this model, we fed cultures of DLS from STZ-treated islets with a redifferentiation medium, with or without INGAP peptide, for four days (Fig. 7.5E). While control cultures responded to the redifferentiation medium with a 14.2±5.3% increase in the frequency of $dithizone^+$ structures, INGAP peptide administration increased this effect to 26.2±4.4%. Conversely, STZ-treated cultures did not respond to either the redifferentiation medium alone, or with INGAP peptide, indicating a deficiency in redifferentiation in DLS formed from STZ-treated cultures.

Table 7.1: Adenoviral Vectors

Vector	Adenovirus		Target	Effect
CMV-GFP	Ad-CMV-cTA	+ Ad-CR5-GFP	all cells	GFP label
INS-GFP	Ad-INS-cTA	+ Ad-CR5-GFP	β-cells	GFP label
GCG-GFP	Ad-GCG-cTA	+ Ad-CR5-GFP	α-cells	GFP label
SST-GFP	Ad-SST-cTA	+ Ad-CR5-GFP	δ-cells	GFP label
PP-GFP	Ad-PP-cTA	+ Ad-CR5-GFP	PP-cells	GFP label
0-BAX		Ad-CR5-BAX	no cells	apoptosis
CMV-BAX	Ad-CMV-cTA	+ Ad-CR5-BAX	all cells	apoptosis
INS/GCG/ SST/PP-BAX	Ad-INS-cTA + Ad-GCG-cTA + Ad-SST-cTA + Ad-PP-cTA	+ Ad-CR5-BAX	endocrine cells	apoptosis
INS-BAX	Ad-INS-cTA	+ Ad-CR5-BAX	β-cells	apoptosis
GCG/SST/ PP-BAX	Ad-GCG-cTA + Ad-SST-cTA + Ad-PP-cTA	+ Ad-CR5-BAX	α-, δ-, PP-cells	apoptosis
GCG-BAX	Ad-GCG-cTA	+ Ad-CR5-BAX	α-cells	apoptosis
INS/SST/ PP-BAX	Ad-INS-cTA + Ad-SST-cTA + Ad-PP-cTA	+ Ad-CR5-BAX	β-, δ-, PP-cells	apoptosis
SST-BAX	Ad-SST-cTA	+ Ad-CR5-BAX	δ-cells	apoptosis
INS/GCG/ PP-BAX	Ad-INS-cTA + Ad-GCG-cTA + Ad-PP-cTA	+ Ad-CR5-BAX	β-, α-, PP-cells	apoptosis
PP-BAX	Ad-PP-cTA	+ Ad-CR5-BAX	PP-cells	apoptosis
INS/GCG/ SST-BAX	Ad-INS-cTA + Ad-GCG-cTA + Ad-SST-cTA	+ Ad-CR5-BAX	β-, α-, δ-cells	apoptosis

Table 7.2: Promoter Sequences

Promoter	Position	Primers
INS[636, 637]	-717 to -170	5'-TTTCTCGAGACAGGGGTGTGGGGACAG-3' 5'-GCTTTGATATCCCTGACGCAAAGGCCCTG-3'
GCG[638, 639]	-3660 to -2931	5'-CGGAACTCGAGAAACAGAACACATAGGGGT-3' 5'-CTGAGATATCCTTCACTGTCCGCCAAAC-3'
SST[640, 641]	-1075 to +158	5'-CCGCTCGAGGCTTCCTGACATAAAACTATGC-3' 5'-CTGGATATCGTCGAGGGAGTCTCCTTACC-3'
PP[642]	-1740 to -766	5'-GCGGCGGGCGGATATCGTGGGAAATTATAACAATAC-3' 5'-GGCGGAGATCTAGAGTAAATGGGCACTAGAAC-3'

Table 7.3: Donor and Isolation Characteristics

	Study Isolations	Isolations 2005 – 2007*	p
N	17	61	
Age (years)	51.5±3.5	52.2±1.8	n.s.
Sex (% male)	47	58	n.s.
BMI (kg/m^2)	29.4±1.0	27.4±0.6	n.s.
Cause of death (% traumatic)	38	27	n.s.
ICU duration (days)	2.8±0.5	2.5±0.3	n.s.
Glucose (mM)	9.5±0.7	9.1±0.4	n.s.
Amylase (U/L)	193±64	213±39	n.s.
Cold ischemia (hours)	7.7±1.1	6.7±0.7	n.s.
Yield ≥70% pure (IE)¶	306,727±30,268	272,267±22,009	n.s.

* Indicates all isolations performed during the calendar years 2005 to 2007.

¶ Indicates the yield (in IE) of islets having a minimum purity of 70%.

Table 7.4: Cell Frequencies

Marker	Islet – day 0 (%)	DLS – day 8 (%)	p	n
Insulin	38.9±2.5	2.4±0.9	<0.001	7
Glucagon	20.1±1.4	3.1±0.7	<0.001	7
Somatostatin	9.2±1.1	2.9±0.6	<0.001	7
PP	4.0±0.5	2.9±0.5	0.056	7
Total endocrine	72.7±5.0	11.3±2.0	<0.001	7
CK	3.0±0.9	40.3±3.7	<0.001	8
Nestin	5.2±1.1	17.6±2.4	<0.001	8

Table 7.5: Labelling Efficiencies and Specificities

Vector	Efficiency (%)*	Specificity (%)¶	n
INS-GFP	35.0±3.4	97.1±1.7	4
GCG-GFP	49.8±0.8	99.1±1.1	3
SST-GFP	46.3±3.2	100	4
PP-GFP	43.9±1.9	100	3

* Indicates the fraction of hormone$^+$ cells (for the promoter of interest) also found to be GFP$^+$, and indicates the cell type-specific labeling efficiency of the individual vectors. General infection efficiency was 46.5±1.0%, as assessed using a CMV-GFP infection (n=3).

¶ Indicates the fraction of GFP$^+$ cells also found to be hormone$^+$ (for the promoter of interest), and indicates the cell type specificity of the individual vectors.

Table 7.6: DLS Cell Origins

Vector	GFP^+/CK^+ (% CK^+)	×	$Efficiency^{-1}$ (%)	=	Origin (%)
INS-GFP	0.5±0.6		35.0±3.4		1.4±4.2
GCG-GFP	19.1±4.1		49.8±0.8		38.4±4.1
SST-GFP	15.4±3.4		46.3±3.2		33.3±5.2
PP-GFP	15.9±2.1		43.9±1.9		36.2±2.8
Vector	**$GFP^+/nestin^+$ (% $nestin^+$)**	**×**	**$Efficiency^{-1}$ (%)**	**=**	**Origin (%)**
INS-GFP	26.0±0.7		35.0±3.4		74.4±0.5
GCG-GFP	1.6±1.1		49.8±0.8		3.3±1.1
SST-GFP	1.0±0.7		46.3±3.2		2.3±0.6
PP-GFP	2.7±1.1		43.9±1.9		6.1±1.1

Figure 7.1: Embedded Islets Form DLS
A. Fluorescence microscopy of islet cells, post-infection but pre-embedding, confirms the specificity and efficiency of the GFP-expressing adenoviral vectors.
B. Isolated human islets are solid spherical structures comprised of hormone$^+$ cells. After collagen embedding and culture, islets form DLS; cystic structures comprised of CK$^+$ and nestin$^+$ cells, as determined by inverted and immunofluorescence microscopy.
C. Quantification of apoptotic cell death prior to embedding and during the course of DLS formation indicates that β-cell preferentially undergo apoptosis during DLS formation.
D. Visual assessment of DLS formation after 8 days in culture confirms that adenoviral infection does not affect the ability of islets to form DLS (n=3; * $p<0.05$, ¶ $p<0.01$, § $p<0.001$ vs. day 0; bar = 100μm).

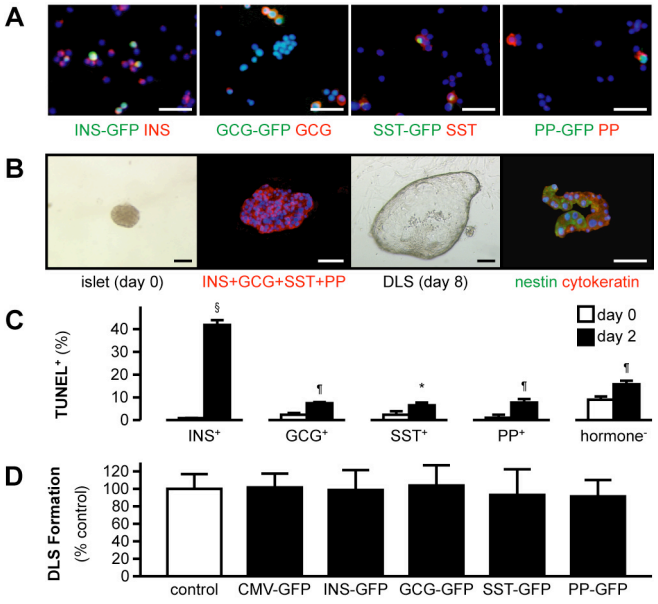

A
INS-GFP INS
GCG-GFP GCG
SST-GFP SST
PP-GFP PP
B
islet (day 0)
INS+GCG+SST+PP
DLS (day 8)
nestin cytokeratin
C
TUNEL+ (%)
day 0
day 2
INS+
GCG+
SST+
PP+
hormone-
D
DLS Formation
(% control)
control
CMV-GFP
INS-GFP
GCG-GFP
SST-GFP
PP-GFP

Figure 7.2: GFP Labelling Allows *In Vitro* Cell Tracking
A. Live fluorescence microscopy of embedded islets, both initially and after 8 days in culture, confirms that the GFP label is retained in cells and structures.
B. Fluorescence microscopy of islet-derived cells can be used to follow the fate of labeled cell types.
C. Quantification of GFP^+ cell types indicates that few cells continue to be $hormone^+$, while many begin to express either CK or nestin (n=3-4; * $p<0.05$, § $p<0.001$ vs. INS-GFP; bar = 100μm).

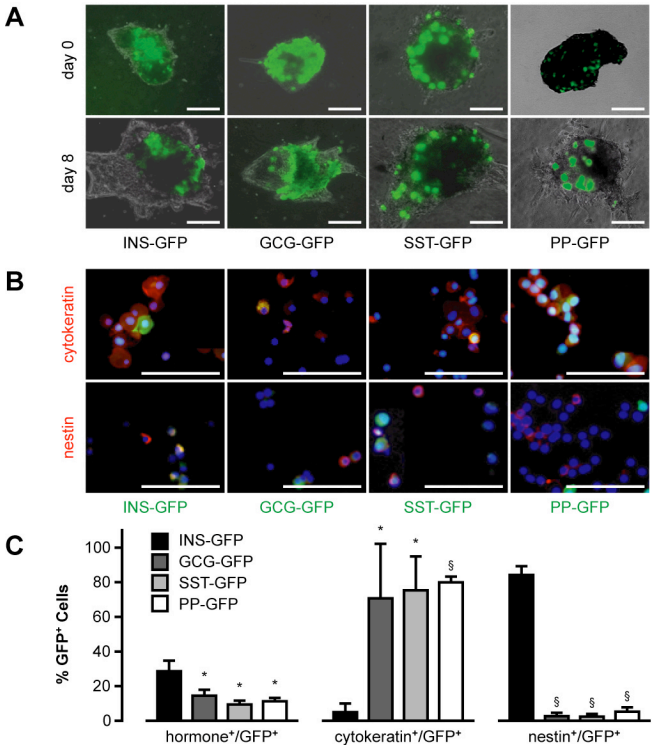

A
day 0
day 8
INS-GFP
GCG-GFP
SST-GFP
PP-GFP
B
cytokeratin
nestin
INS-GFP
GCG-GFP
SST-GFP
PP-GFP
C
% GFP+ Cells
100
80
60
40
20
0
INS-GFP
GCG-GFP
SST-GFP
PP-GFP
*
*
*
*
*
§
§
§
hormone+/GFP+
cytokeratin+/GFP+
nestin+/GFP+

Figure 7.3: BAX Expression Allows Inverse Cell Tracking
A. Quantification of islet cell frequencies, post-infection but prior to embedding, confirms the specificity and efficiency of the BAX-expressing adenoviral vectors. **B.** Quantification of the frequency of hormone^{+} islet cells, as well as overall cell numbers, post-infection but prior to embedding, confirms the specificity and efficiency of the BAX-expressing adenoviral vectors employed (n=3; * $p<0.05$, ¶ $p<0.01$, § $p<0.001$ vs. control, all others n.s.).

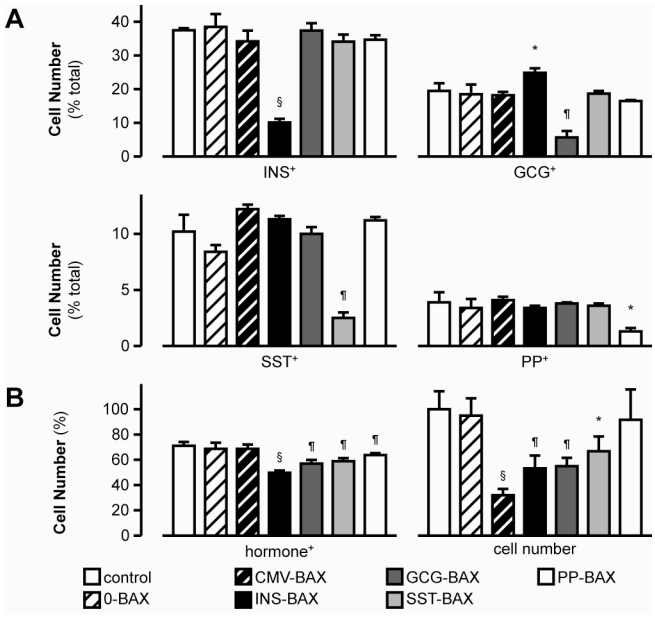

A
Cell Number (% total)
40
30
20
10
0
§
*
¶
INS+
GCG+
Cell Number (% total)
10
5
0
¶
*
SST+
PP+
B
Cell Number (%)
100
80
60
40
20
0
§
¶
¶
¶
§
¶
¶
*
hormone+
cell number
control
0-BAX
CMV-BAX
INS-BAX
GCG-BAX
SST-BAX
PP-BAX

Figure 7.4: Islet Cell Inter-Dependence and Redundancy
A. Visual assessment of DLS formation after 8 days in culture confirms the inter-dependence of islet cell types in that selective depletion of any individual cell type adversely affects DLS formation.
B. Quantification of CK^+ and $nestin^+$ islet-derived cell frequencies indicates that α-, δ- and PP-cell-derived CK^+ cells increase in number to compensate for the reduced frequency of β-cell-derived $nestin^+$ cells in DLS derived from β-cell-depleted islets. Moreover, redundancy exists in that α-, δ- and PP-cells appear to compensate for a lack of any of these cell types so as to maintain the frequency of CK^+ cells in DLS derived from α-, δ- or PP-cell-depleted islets (n=3; * $p<0.05$, ¶ $p<0.01$, § $p<0.001$ vs. control).

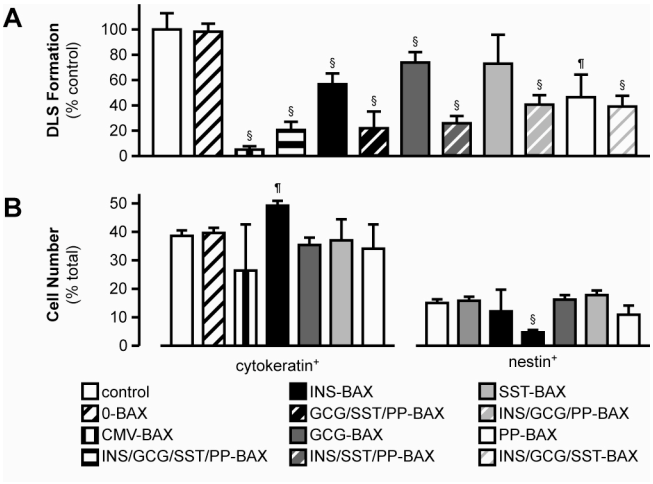
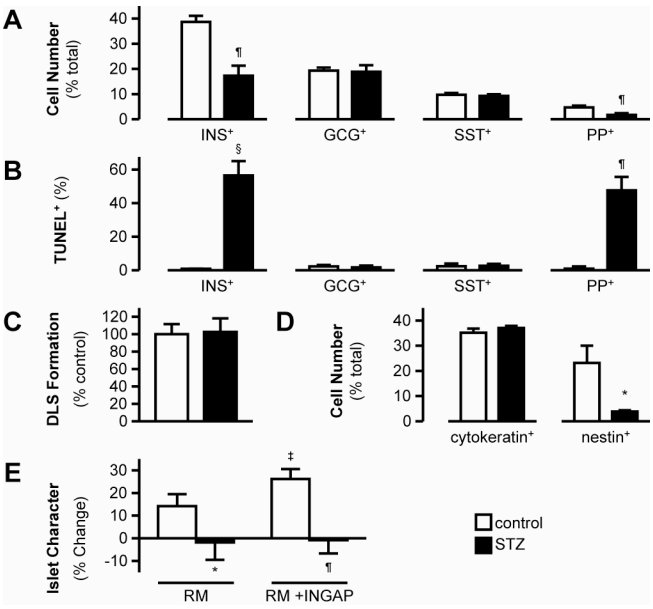

A
DLS Formation
(% control)
100
80
60
40
20
0
§
¶
B
Cell Number
(% total)
50
40
30
20
10
0
cytokeratin+
nestin+
control
0-BAX
CMV-BAX
INS/GCG/SST/PP-BAX
INS-BAX
GCG/SST/PP-BAX
GCG-BAX
INS/SST/PP-BAX
SST-BAX
INS/GCG/PP-BAX
PP-BAX
INS/GCG/SST-BAX

Figure 7.5: STZ Treatment Confirms Inverse Cell Tracking
A. Quantification of islet cell frequencies and (**B**) apoptotic cell death after STZ treatment but prior to embedding, indicates that STZ selectively targets β- and PP-cells.
C. STZ treatment does not affect DLS formation, as evaluated visually after 8 days in culture.
D. The frequency of CK^+ DLS cells is unaffected by islet STZ treatment prior to embedding, but the frequency of $nestin^+$ DLS cells is reduced.
E. After culture to form DLS, switching to redifferentiation medium allows a spontaneous reversion of DLS into $dithizone^+$ ILS, which is potentiated by INGAP peptide. STZ treatment of islets prior to culture abrogates the redifferentiation response of DLS, both with and without INGAP peptide (n=4; * $p<0.05$, ¶ $p<0.01$ vs. same conditions for control, ‡ $p<0.05$ vs. RM for control).


A
Cell Number
(% total)
40
30
20
10
0
¶
¶
INS+
GCG+
SST+
PP+
B
TUNEL+ (%)
60
40
20
0
§
¶
INS+
GCG+
SST+
PP+
C
DLS Formation
(% control)
120
100
80
60
40
20
0
D
Cell Number
(% total)
40
30
20
10
0
*
cytokeratin+
nestin+
E
Islet Character
(% Change)
30
20
10
0
-10
‡
*
¶
RM
RM +INGAP
control
STZ

7.5 Discussion

Under specific culture conditions, cultured human islets appear to lose their endocrine phenotype and are replaced by a heterogeneous population of seemingly primitive, highly proliferative cells.[245, 296, 394, 437, 447-450, 452] Recently, investigators have identified culture conditions under which these cells can be induced to redifferentiate into ILS, albeit with varying degrees of endocrine phenotype and function,[245, 296, 394, 449, 450] thereby lending support to the hypothesis that islet-derived cells can act as precursors. While encouraging, these reports have been met with controversy.

Previous reports have presented evidence, including electron microscopy of individual cells with both endocrine (endosecretory granules) and ductal (microvilli) characteristics, and double immunofluorescence microscopy of cells co-expressing endocrine hormones and duct cell markers, suggesting that these precursor-type cells derive from adult endocrine cell types.[296, 394, 437] However, lineage tracing and pulse-chase studies in mice have suggested that the primary mechanism of both β-cell renewal and regeneration in the endogenous pancreas is β-cell replication.[276, 626] More recently, yet another lineage tracing study concluded that the fibroblast-like cells observed in mouse islet cultures do not derive from cells of any pancreatic lineage.[463] These discrepancies between human and rodent studies led us to employ other methods to confirm our previous double immunofluorescence-based observations of an α- and δ-cell origin for the CK^+ population that is observed to arise.[394]

In the present study, we employed a newly validated adenoviral co-infection technique to serve as a signal amplifier.[628] In this way, we transiently labeled individual endocrine cell types with reproducible efficiency and high specificity. Given the strength and cumulative half-lives of the transactivator and GFP, we were able to follow specific endocrine cell types, even after the promoters of interest were no longer active in the dedifferentiated cells. Thus, we observed that in our culture system, both CK^+ epithelial cells and $nestin^+$ fibroblast-like cells derive from hormone-expressing endocrine cells, albeit from different endocrine cell types.

Our results suggest that the CK^+ epithelial cells of DLS derive primarily from glucagon$^+$, somatostatin$^+$ and PP^+ cells, with approximately equal contributions from each, while very few CK^+ cells derive from insulin$^+$ cells. Conversely, nestin$^+$ cells derive mainly from insulin$^+$ cells, while mantle cells contribute little. The possibility exists, however, that nestin$^+$ cells also derive from non-endocrine cell origins, as a significant number of nestin$^+$ cells were not accounted for by labeled endocrine cells.

As with any promoter-based expression system, it is important to note that cell types were defined based only on the activity of a single promoter sequence.[276] Thus, a β-cell was defined based on the presence of an active insulin promoter at the time of culture. Accordingly, data from such studies must be interpreted with this in mind, because sub-populations of islet cells that are insulin$^+$, but would not be considered adult β-cells, have been reported previously.[5] To our knowledge, this is the first report of human promoter sequences being used to label human islets.

With respect to lineage tracing studies, Dor *et al.* acknowledged the possibility of an insulin$^+$ precursor cell, which would be labeled as a β-cell in lineage tracing studies.[276] This notion is especially appealing in our *in vitro* islet culture system, because we followed not only insulin$^+$ cells, but rather the four principal endocrine cells of the islet. Our studies led to the novel observation that there was a dichotomy with respect to the cellular origins of the two primary cell types of the DLS: CK^+ cells derive primarily from α-, δ- and PP-cells, and nestin$^+$ cells derive mainly from β-cells.

Targeted depletion of specific cell types by adenoviral expression of BAX or STZ treatment was originally performed with the intention of identifying the cell(s) responsible for the redifferentiation potential of DLS. As such, the lack of redifferentiation from DLS derived from STZ-treated islets suggests an important role for β- and PP-cell-derived cells. However, these results also serve to highlight the interdependence of endocrine cell types with respect to DLS formation and, presumably, islet regeneration. Thus, no cell type can yet be proposed as an *in vitro* islet precursor without true lineage tracing of the islet

regeneration process. In fact, it may well be that cells exist as facultative precursors only within the niche defined by DLS or monolayer cultures.[643] It is for this reason that studies of redifferentiation of BAX-treated cultures could not be carried out, for in an attempt to remove an individual cell type from the niche, the niche itself was disrupted.

Characterization of the starting tissue in these experiments indicates a slight presence of CK^+ and $nestin^+$ cells from the beginning, and thus the proliferation of these cells could have possibly contributed to the resultant cell populations. However, correcting for labeling efficiencies suggests that virtually all CK^+ cells were in fact derived from endocrine cells over the course of culture. Likewise, BrdU labeling studies carried out previously indicated that the proliferation of CK^+ cells does not begin immediately, but only after several days in culture, following the main wave of dedifferentiation.[455, 474] Finally, we do not presume to exclude a contribution of some inherent ductal or fibroblastic cells in our preliminary cultures, but rather we are reporting on the origins of cells that seem to account for a majority of the CK^+ and $nestin^+$ cells present at the end of the culture period. In fact, to pinpoint the exact source of *in vitro* islet neogenesis, long-term lineage tracing studies using indelible cell markers would be required. These experiments are beyond the scope of these studies, however, as we sought only to identify the endocrine cell origins of cells within the DLS.

While other groups have reported on monolayers formed from cultured islets,[245, 296, 449, 450] our model involves three-dimensional organotypic culture.[394] Nonetheless, it seems that the precursor-type cells we observe are very similar to those reported in monolayer culture, especially when the culture conditions and expression profile of both cell populations are considered. As in the monolayer, we observe two predominant cells types: the majority of cells are CK^+ and epithelial in nature, while a less frequent $nestin^+$ fibroblast-like cell also exists within the DLS. Additionally, there is heterogeneity of both PDX1 and vimentin expression, as in the monolayer.[394]

At first glance, our results may appear to contradict those reported by others who have conducted lineage tracing studies in mice.[463] However, evidence is

accumulating to suggest that the non-human pancreas is not always an appropriate model to study phenomena occurring in the human organ.[644] For example, recent reports aimed at elucidating the islet regenerative response in humans and mice indicated that humans increase β-cell mass primarily through neogenesis,[22] while the mouse regenerative response involves β-cell replication.[249] Likewise, cultured human islets have been reported to form precursor cell cultures that can be induced to redifferentiate into ILS.[245, 296, 449, 450 394] When similar culture conditions were applied to mouse islets, little if any redifferentiation was observed.[462-465] Taken together, these data suggest a difference with respect to the mechanisms of regeneration that predominate in human and non-human tissue. Consequently, while certainly informative, the results of lineage tracing studies in mice may not necessarily be informative of what transpires in man. In fact, a recent study using a similar lineage tracing technique confirmed that human, but not murine, β-cells were capable of significant *in vitro* dedifferentiation and proliferation.[467]

Finally, a debate persists over the cellular source of *in vitro* islet redifferentiation. Given the presence of β-cells, non-β- endocrine cells and non-endocrine cells in islet cultures, the mechanisms involved may represent β-cell neogenesis, proliferation or both. For example, if ILS were observed to form from the CK^+ epithelial cell source, which is almost exclusively of non-β-cell origin, this could be termed islet neogenesis. Immunofluorescence and BrdU pulse-chase studies have provided support for this pathway.[394, 448] Conversely, if β-cell-derived $nestin^+$ cells are observed to give rise to ILS, then this could be defined as β-cell replication, albeit indirect, given the presence of an intermediate cell type.[254] Immunofluorescence studies provide tentative support for this mechanism as well.[245] Thus, our findings do not rule out either pathway of β-cell formation. Additional longer-term lineage tracing studies would undoubtedly prove useful in elucidating a predominant mechanism.

7.6 Acknowledgements

We thank Béatrice Assouline, Emily Austin, Mauro Castellarin, Jieping Ding, Deborah Driver, Xinfang Li, Mark Lipsett, Julia Makhlin and Ryan Scott for technical assistance, as well as Québec-Transplant for coordination of organ availability. This work was supported in part by the Canadian Institutes of Health Research (CIHR) and the Stem Cell Network of Canada. S. Hanley is supported by fellowships from the Canadian Diabetes Association (CDA) / CIHR and Fonds de Recherche en Santé du Québec (FRSQ). L. Rosenberg is a chercheur national (national scientist) of the FRSQ.

Bridging Statement Between Manuscripts

In the previous chapter, we identified the cellular origins of the dedifferentiated cell types observed in DLS cultures. Without ruling out the possibility of minor contributions of other, unidentified cell types, it appears that α-, δ- and PP-cells are the main contributors to the CK^+ cell population of the DLS, while the $nestin^+$ cell population appears to derive primarily from β-cells. Moreover, limited STZ-mediated cell ablation studies suggest that β-cells are essential to the redifferentiation process, either directly or indirectly. This supports other reports of a critical role for β-cells in *in vitro* redifferentiation.[245, 296, 448-450]

The clinical relevance of these studies rests on the possibility of amplifying the number of islets derived from a donor organ. The intention, therefore, would be to culture isolated human islets in such a way as to allow the expansion of the isolated β-cell mass to the extent that multiple islet grafts could be obtained from a single donor organ. Several key questions remain to be addressed, not the least of which is a more formal evaluation of the plasticity of human β-cells.

Next, if β-cell plasticity is in fact the mechanism of the observed islet plasticity, a logical follow up question is whether such a phenomenon is simply an artifact of tissue culture, or whether such a process can also occur *in vivo*.

Finally, assuming that the endogenous pancreas does possess such plasticity, it is reasonable to ask whether a defect in such a process contributes to the pathogenesis of diabetes.

Unfortunately, studies of the *in vivo* environment are typically static in nature, while studies of isolated islets do not necessarily correlate with *in vivo* events. Nevertheless, a recent study sought to establish the mechanisms at play in regulating β-cell mass in human obesity and T2DM.[22] The aim of the next chapter was to ascertain whether islets from T2DM donor organs do in fact possess a defect in islet cell plasticity.

8 Relationship Between Type 2 Diabetes Mellitus and Islet Plasticity

Submitted as:

Hanley, S., Assouline, B., Petropavlovskaia, M. & Rosenberg, L. Is islet-derived regeneration defective in type 2 diabetes? *Diabetes.*

8.1 Abstract

Cultured human islets from non-diabetic organ donors give rise to precursor-type cells with the potential to regenerate ILS. While it is unknown whether such a phenomenon can be reproduced with islets from T2DM organ donors, it is known that β-cell mass is reduced in T2DM, mainly via apoptosis. We compared islets from T2DM (n=8) and age- and BMI-matched non-diabetic (n=14) donors with respect to cellular constituents, and the ability to give rise to precursor-type cells that can regenerate ILS. β-Cell volume was reduced in T2DM pancreata (1.50±0.34 vs. 2.87±0.81%, $p<0.05$) and these organs yielded fewer islets upon isolation (217,000±55,000 vs. 505,000±88,000IE, $p<0.01$). T2DM islets were smaller and contained more apoptotic β-cells (18.2±1.3 vs. 9.7±0.9%, $p<0.001$). Non-diabetic and T2DM islet cultures produced equal numbers of putative precursor-type cells, reflected by the frequencies of CK^+ and $nestin^+$ cells. On exposure to a redifferentiation medium, cells derived from non-diabetic cultures developed $dithizone^+$ buds, however this did not occur with cells from T2DM cultures. Gene expression analysis indicated that precursor-type cell formation was associated with increased *ngn3* expression in non-diabetic cultures, whereas T2DM cultures were characterized by increased expression of Notch signaling molecules. These results suggest that a defect in islet morphogenetic plasticity may also contribute to the deficit in functional β-cell mass in T2DM, along with β-cell apoptosis. Moreover, the process of *in vitro* redifferentiation of adult human islets may be controlled by mechanisms similar to those implicated in the regulation of islet formation during pancreatic organogenesis.

8.2 Introduction

T2DM is a heterogeneous disease marked by both extra- and intra-pancreatic dysfunction related to insulin signaling.[191, 192] While the extra-pancreatic component of the disorder comprises insulin resistance in target tissues, T2DM is equally a disease of β-cell dysfunction, marked by suboptimal insulin secretion.[135, 136]

The pathogenesis of T2DM involves both genetic[193] and environmental factors, including obesity.[194, 195] However, recent data indicate that T2DM is characterized by a relative β-cell mass deficit, regardless of weight,[22] suggesting a general defect in the maintenance of a functional β-cell mass.[136, 214] Indeed, the primary mechanism by which this β-cell mass deficiency occurs, increased β-cell apoptosis, is common to lean and obese subjects.[22] In addition, clinical studies have highlighted the impaired first-phase insulin secretion that is common in T2DM.[645-647] In fact, correlations between β-cell mass and glucose tolerance are not direct,[215] suggesting that sufficient insulin$^+$ tissue does not necessarily equate with normoglycemia.

Studies of human islets isolated from the pancreata of T2DM organ donors indicate that islet insulin content and GSIS are decreased.[648-651] In fact, genetic comparison of T2DM and non-diabetic islets suggests that much of this dysfunction may be attributed to reduced expression of the aryl hydrocarbon receptor nuclear translocator (ARNT), a bHLH transcription factor that appears to regulate the expression of many β-cell functional effectors.[652] Thus, transcriptional defects have been linked to reduced β-cell function in T2DM.[135, 136]

Notch signaling is one of the key pathways dictating pancreatic endocrine differentiation. Activation of the Notch pathway is critical for the maintenance of the progenitor pool between the so-called first and second transitions of pancreatic development.[108] In fact, disruption of Notch signaling, at the level of the ligand, receptor, intracellular mediator or target gene, results in pancreatic hypoplasia and precocious endocrine differentiation.[108, 118, 653, 654] These studies indicate that Notch signaling normally maintains progenitors in a dedifferentiated state until sufficient expansion of the progenitor pool, whereupon endocrine

differentiation occurs. Conversely, constitutive Notch activation leads to pancreatic epithelial hyperplasia, with an associated deficit in mature endocrine and exocrine cells.[655]

A main target of the Notch signaling cascade is the bHLH transcription factor NGN3. HES1, the main Notch transcriptional target, represses NGN3 expression, itself the first step in directed endocrine differentiation. NGN3 overexpression mimics the phenotype of attenuated Notch signaling.[116, 656] While NGN3 is transiently expressed by all islet endocrine precursors, and heads the cascade of transcription factors leading to the formation of mature endocrine cells,[654] NGN3 is rarely expressed in adult pancreatic islets.

With respect to islet regeneration, it has been suggested that NGN3 re-expression is not required for the endocrine regeneration induced in the 50% partial pancreatectomy model.[304] However, the primary mechanism of regeneration in this model is β-cell replication, a process that would not be expected to lead to NGN3 re-expression. Thus, the roles of Notch and NGN3 in islet regeneration remain to be more clearly defined. It is noteworthy, however, that Notch is re-expressed in pancreatic adenocarcinoma.[567] This is in keeping with the notion that the neoplastic process involves a return of adult cells to a more proto-differentiated state. Moreover, primary rat β-cells and insulinoma cells respond to IL1β and IFNγ, cytokines associated with diabetes[213], with upregulated Notch signaling,[657-659] suggesting a role for this pathway in the dedifferentiation of mature endocrine cells.

We[394, 452] and others[245, 296, 448-450] have reported that cultured human islets can be induced to dedifferentiate into proliferative precursor-type cells that have the potential to redifferentiate into ILS. While lineage tracing studies using islets from genetically engineered mice have called into question the endocrine[462, 464, 465] and even pancreatic[463] origins of these cells, our and others' adenoviral-based studies in human islets indicate that endocrine cells are indeed the main contributors to the *in vitro* derivation of these precursor-type cells.[467, 660]

The goal of this study was to investigate whether cultured islets from T2DM donor organs can generate precursor-type cells, and whether such cells possess

the ability to undergo islet regeneration. In addition, we sought to determine whether Notch-mediated regulation of the progenitor pool is implicated, as it is during pancreatic organogenesis.

8.3 Materials and Methods

8.3.1 Islet Isolation

Pancreata from adult human cadaveric organ donors were obtained through the local OPO. Islets were isolated according to established protocols.[400] Briefly, following procurement, organs were flushed and stored in cold ViaSpan solution (Barr Laboratories, Pomona, NY) until islet isolation. The main pancreatic duct was cannulated and perfused with a solution of Liberase HI (Roche Diagnostics, Laval, QC). The distended organ was placed in a closed system (Bio-Rep, Miami, FL) and heated to 37°C to activate the enzyme blend. Following the appearance of free islets in samples, the system was cooled and free tissues were collected and washed. Tissues were separated by continuous Ficoll (Biochrom AG, Berlin, Germany) density gradient centrifugation in a cell processor (COBE DCT, Denver, CO). Free islets determined to be greater than 70% pure by real-time staining with dithizone (Sigma, St. Louis, MO), a Zn^{2+} chelator, were collected, washed, and counted as IE.

8.3.2 Islet Culture

Isolated islets were cultured in suspension overnight in serum-free DMEM/F12 (Gibco, Burlington, ON) containing penicillin, streptomycin, fungizone, 1μM dexamethasone, 10ng/mL epidermal growth factor (Sigma), 24mU/mL insulin (Lilly, Toronto, ON) and 200ng/mL cholera toxin (Sigma). Islets were then embedded in type I rat tail collagen, as per previous reports,[452] at a density of 2000IE/25cm^2, and cultured for 8 days to form DLS.

Islet redifferentiation, as defined by the budding of dithizone$^+$ structures, was induced by switching DLS cultures to the above medium, with 10% FBS but

without cholera toxin, for 4 days. Islet redifferentiation was also potentiated by adding 667nM INGAP peptide (Sheldon Biotechnology Centre, Montreal, QC).[394]

8.3.3 Immunohistochemistry and Immunofluorescence

Tissue samples were taken from the tail of the pancreas prior to isolation, and stored at 4°C in phosphate-buffered formalin. Islet and DLS samples were stored at 4°C in phosphate-buffered formalin until cell pellets were embedded in 2% agarose (Gibco). Following routine processing and embedding, 4μm sections were cut and dewaxed in xylene and petroleum ether. Slides were permeabilized by incubation in Tris-buffered saline with 0.1% Triton X-100 for 5 minutes, prior to antigen retrieval by heating for 10 minutes in 0.1M citrate buffer, pH 6. Slides were then incubated in blocking buffer (Zymed, San Francisco, CA) for 15 minutes before overnight incubation at 4°C with primary antibody in blocking buffer.

For immunohistochemical determination of β-cell volume, slides were incubated with an α-insulin primary antibody (Dako, Carpinteria, CA). After washing in PBS, staining was developed using a biotinylated broad-spectrum secondary antibody, streptavidin-conjugated horseradish peroxidase (HistoStain Plus kit; Zymed, San Francisco, CA) and 3,3'-diaminobenzidine substrate (Dako). Sections were analyzed using a Zeiss Axioskop 40 microscope and Northern Eclipse v6.0 (Empix Imaging, Mississauga, ON). Briefly, individual insulin$^+$ structures were traced and thresholded to determine insulin$^+$ area.[250] Total pancreatic tissue area was similarly determined, and β-cell area was calculated relative to total pancreatic area.

For immunofluorescence, after incubation with a primary antibody, slides were washed in PBS, incubated with secondary antibody in blocking buffer for one hour at room temperature, and reprobed with a different primary antibody. Again, following washing, slides were incubated with secondary antibody before washing and coverslipping with mounting medium containing 4',6-diamidino-2-phenylindole (Vector, Burlingame, CA). Antibodies used were mouse α-insulin and α-glucagon (Abcam, Cambridge, MA), rabbit α-somatostatin and α-PP,

mouse α-CK-AE1/AE3 (Dako, Carpinteria, CA), and rabbit α-nestin (Chemicon, Temecula, CA) primary antibodies, along with FITC- and rhodamine-conjugated species-specific secondary antibodies (Abcam). To assess apoptotic cell death, the TUNEL kit (Roche Diagnostics) was used as per the manufacturer's instructions.

8.3.4 Insulin Content

Samples were washed thoroughly in PBS before sonication in lysis buffer (50mM Tris-HCl, pH 8, 1.37mM NaCl, 0.1mM Na3VO4, Complete protease inhibitor tablet (Roche), 1% v/v nonident P-40, 10% v/v glycerol). Insulin content was assessed by ELISA (Alpco, Salem, NH), as per the manufacturer's instructions, and normalized to protein content, measured using the Bradford method.

8.3.5 qRT-PCR

Briefly, samples were stored in RLT buffer (Qiagen, Mississauga, ON) until the time of RNA isolation. Following RNA extraction, as per the manufacturer's protocol, cDNA was reverse transcribed using an Omniscript RT kit (Qiagen). Custom primers (Invitrogen, Burlington, ON) were synthesized (Table 8.2), and qRT-PCR was performed using a QuantiTect SYBR green RT-PCR kit (Qiagen) in a MJ Research DNA Engine Opticon2 (Bio-Rad, Mississauga, ON). Expression levels were normalized to GAPDH.

8.3.6 Statistical Analysis

Results are expressed as mean ± SEM. Statistical significance was determined by unpaired Student's t-test. Differences were considered significant when $p<0.05$.

8.4 Results

8.4.1 Donors and Isolations

From January 2003 to February 2007, the Centre for Pancreatic Diseases at the McGill University Health Centre received 216 donor organs for islet isolation. Over that time, 15 of the organs (6.9%) were from T2DM donors. Islet isolation was attempted on 11 of the T2DM organs, and islets with a minimum purity of 70%, as evaluated by dithizone, were obtained from 8 organs. Fourteen age-, sex- and BMI-matched non-diabetic controls were retrospectively selected from the same time period. As indicated (Table 8.1), no differences in major isolation determinants were detected, with the exception of pancreatic weight, which showed a trend towards heavier organs in the T2DM group. In fact, when normalized to body weight, T2DM donors had significantly heavier pancreata (1.49±0.16 vs. 1.16±0.07g/kg, $p<0.05$). Although glycemia was also slightly, though not significantly, higher in the T2DM group, it has been suggested that peri-procurement glycemia does not reflect glycemic control, but rather the physiologic disturbances associated with cause of death.[649]

Recent evidence indicates that T2DM is characterized by a deficit in β-cell mass.[22] In keeping with this observation, our morphologic assessment of β-cell volume identified a 48% reduction in β-cell mass in T2DM donors ($p<0.05$, Fig. 8.1A). Coincidentally, we found islet yield on isolation to be reduced by 57% ($p<0.01$, Fig. 8.1B). Moreover, the yield was further reduced when normalized to pancreatic weight ($p<0.001$, Fig. 8.1C). Although a negative correlation has been reported between duration of T2DM and islet yield,[649] we did not observe such a relationship (data not shown). However, the correlation would have to be quite strong to be apparent given the small sample size.

8.4.2 Islets

Islets isolated from T2DM donor organs tended to be smaller. The proportion of islets with a diameter less than 150μm was significantly higher in preparations from T2DM organs compared to non-diabetic organs ($p<0.01$, Fig. 8.2A). We also

examined islet cross-sectional area in pancreatic tissue sections from the same organs, and determined that average *in situ* islet area was indeed reduced in the pancreas of T2DM donors ($p<0.05$, Fig. 8.2B), suggesting that the decreased size of isolated islets was not a consequence of the isolation process.

To determine whether the reduction in islet area was a result of β-cell atrophy, we measured the cross-sectional area of individual β-cells in tissue sections from T2DM and non-diabetic organs (n=6 organs/group, 11 cells/organ). No difference was observed (Fig. 8.2C), suggesting a deficiency in the number of β-cells as the cause of reduced islet size.

We next asked whether the frequency of β-cells was reduced within T2DM islets relative to non-diabetic controls. Samples of isolated islets were processed for evaluation of the frequencies of endocrine cell types by immunofluorescence microscopy. As expected, there was a significant reduction in the frequency of β-cells in T2DM islets, with an associated increase in the frequency of α-cells ($p<0.05$, Fig. 8.3A).

In order to identify the cause of this absolute reduction in the number of β-cells, we analyzed tissue sections from pre-isolation pancreatic biopsies and from isolated islets for the frequency of β-cell apoptosis. We observed a significant increase in β-cell apoptosis in T2DM islets, both *in situ* ($p<0.05$, Fig. 8.3B) and post-isolation ($p<0.001$, Fig. 8.3C). Interestingly, the ratio of extra- to intra-pancreatic β-cell apoptosis was the same between T2DM and non-diabetic donors, suggesting that the isolation procedure affects both T2DM and non-diabetic tissue to the same degree.

While GSIS is typically reduced in isolated islets from T2DM donors [648-651], another common contributor to β-cell dysfunction is decreased insulin content.[648, 650, 651] We compared insulin content between T2DM and non-diabetic islet preparations, and found it to be decreased 2-fold in islets from donors with T2DM ($p<0.05$, Fig. 8.3D).

Although we observed a significant decrease in the frequency of β-cells, this difference was not sufficient to account for the observed decrease in insulin

content. Thus, it appears that insulin content per β-cell was also reduced, suggestive of β-cell dysfunction. To further examine this possibility, we assessed gene expression levels in T2DM and non-diabetic islet samples by qRT-PCR. Indeed, *insulin* transcript levels were reduced 4-fold in T2DM islets ($p<0.05$, Fig. 8.4A). *Pdx1* and *glut2* levels were also correspondingly decreased 2- to 4-fold ($p<0.05$, Fig. 8.4A), suggesting a loss of β-cell phenotype. However, *glucokinase*, *sur1* and *neuroD* expression were unchanged between groups, indicating that not all markers of β-cell function were affected. Moreover, expression of *arnt*, the proposed regulator of β-cell dysfunction in T2DM,[652] was unchanged between non-diabetic and T2DM islets (Fig. 4A). It is noteworthy that *somatostatin* expression was significantly reduced, while *pp* transcript was upregulated in T2DM islets ($p<0.05$, Fig. 8.4A).

We have previously reported on the ability of cultured human islets to dedifferentiate, that is lose the expression of functional markers of adult endocrine cells and initiate expression of primitive or precursor markers, including CKs and/or nestin.[394] To determine whether excessive β-cell dedifferentiation was present *in situ* as a further explanation of T2DM-associated β-cell failure, we compared *nestin* and *vimentin* expression levels between freshly isolated non-diabetic and T2DM islet preparations. Indeed, expression of both *nestin* and *vimentin* was upregulated in T2DM islets ($p<0.05$), although expression of *jagged1* (a Notch ligand), *notch2*, *hes1* (a marker of Notch activation[661]) and *ngn3* were unaffected (Fig. 8.4B). Thus, it appears that *nestin* and *vimentin* expression may represent earlier markers in the dedifferentiation process, while Notch signaling is associated with a more complete loss of β-cell phenotype.[659]

8.4.3 Islet-Derived Duct-Like Structures

We were amongst the first groups to report on the derivation of proliferative precursor-type cells from cultured human islets.[245, 296, 448-450] In our model, isolated islets are embedded in type I rat tail collagen and cultured in a defined medium, leading to the formation of DLS[452] via the dedifferentiation of β-cells into

nestin$^+$ mesenchymal-type cells and α-, δ- and PP-cells into CK$^+$ epithelial-type cells.[660] This process is reversible, however, and ILS can be regenerated.[394] Thus, we sought to determine whether islets from T2DM donor organs retained this morphogenetic plasticity.

Following embedding in collagen and 8 days of culture, T2DM donor islets formed DLS to the same extent as islets from non-diabetic donors (Fig. 8.5A). After switching to a redifferentiation-permissive medium, budding of dithizone$^+$ ILS was observed in cultures from non-diabetic donors ($p<0.05$, Fig. 8.5B). This redifferentiation effect was potentiated by the addition of 667nM INGAP peptide ($p<0.001$ vs. control, $p<0.05$ vs. –INGAP peptide, Fig. 8.5B). However, cultures derived from T2DM donors did not respond to the redifferentiation-permissive medium, nor to the addition of INGAP peptide (Fig. 8.5B). Thus, it appears that T2DM donor islets are capable of dedifferentiation, but do not retain a redifferentiation capacity, at least in this model.

We have previously established that nestin$^+$ cells derive preferentially from β-cells, and CK$^+$ cells derive principally from α-, δ- and PP-cells.[660] While the cellular contributors to islet regeneration in this model are unknown, if β-cell-derived nestin$^+$ cells do contribute, then the β-cell shortage in T2DM islets may explain the observed lack of regenerative capacity. To test this theory, we compared the frequencies of CK$^+$ and nestin$^+$ cells in DLS from T2DM and non-diabetic donor tissue. No difference in individual or overall cell type frequencies was observed between groups (Fig. 8.5C), suggesting that the regenerative defect cannot be attributed to a missing cell type.

We next asked whether differences at the level of gene expression might better explain the apparent defect in redifferentiation capacity of T2DM islet-derived tissue. First, we examined expression of markers of the two primary types of dedifferentiated cells. While *ck19* expression did not differ between T2DM and non-diabetic islet-derived cultures, *nestin* and *vimentin* expression levels were higher in T2DM cultures ($p<0.05$, Fig. 8.6A), compatible with a more complete dedifferentiation of β-cells. General markers of islet cell differentiation, such as *pdx1*, *nkx2.2* and *neuroD*, did not differ between groups (Fig. 6A). Given

the established importance of the Notch signaling pathway in controlling endocrine differentiation[108] and dedifferentiation,[567, 657-659] we also examined the expression of members of this pathway and their downstream targets. Both the *jagged1* ligand ($p<0.05$) and *notch2* receptor ($p<0.01$) were overexpressed at least 2-fold in T2DM islet-derived cultures (Fig. 8.6A). Furthermore, *hes1* expression was also increased more than 2-fold in T2DM cultures ($p<0.05$), with a concomitant 4-fold decrease in *ngn3* expression, although this did not reach statistical significance (Fig. 8.6A).

To confirm the role of Notch signaling in this model, we compared expression levels of ngn3 and hes1 over the time course of islet-to-DLS conversion. In non-diabetic cultures, DLS formation was associated with a 3-fold decrease in *pdx1* expression and a 4-fold increase in *ngn3* expression ($p<0.05$), while *hes1* expression was unchanged (Fig. 8.6B). Conversely, in T2DM cultures, DLS formation was also associated with a 2-fold decrease in *pdx1* expression ($p<0.05$), but there was a 2-fold increase in *hes1* expression ($p<0.01$), while *ngn3* expression remained unchanged (Fig. 8.6B). Taken together, these data are consistent with the reversible nature of islet dedifferentiation in non-diabetic cultures. Moreover, these results suggest that the defect in T2DM cultures exists at the level of Notch overactivation, which serves to hold any precursor-type cells in the dedifferentiated state,[662] thereby preventing endocrine redifferentiation.

Table 8.1: Donor and Isolation Characteristics

Marker	Non-Diabetic	T2DM	p
n	14	8	
Duration of T2DM (years)		3.3 ± 1.4	
T2DM treatment		5/8 Rx, 1/8 insulin	
Age (years)	59.6 ± 1.6	60.1 ± 2.3	n.s.
Sex	10M/4F	5M/3F	n.s.
BMI (kg/m^2)	28.8 ± 1.2	29.3 ± 1.9	n.s.
Glucose (mM)	9.4 ± 0.8	10.7 ± 0.9	n.s.
Cold ischemia duration (hrs)	7.6 ± 1.3	8.9 ± 2.8	n.s.
Pancreatic weight (g)	96.9 ± 7.0	122.4 ± 17.2	0.054

Table 8.2: RT-PCR Primers and Conditions

Gene	Primers	Annealing (°C)	Amplicon (bp)
arnt	5'-CTGGACAGACTGCAGGACAA-3' 5'-TCTGATCTCCCAGCATGGAC-3'	58	186
ck19	5'-CTGGAGATGCAGATCGAAGG-3' 5'-CGGTTCAATTCTTCAGTCCG-3'	58	241
gapdh	5'-TGAGAACGGGAAGCTTGTCA-3' 5'-TCACACCCATGACGAACATG-3'	58	218
glucagon	5'-ACAAGGCAGCTGGCAACGTTCCCT-3' 5'-CCTTCCTCCGCCTTTCACCAGCCA-3'	58	343
glucokinase	5'-GCTGAGATGCTCTTCGACTAC-3' 5'-CTTGGTCCAGTTGAGAAGGATG-3'	58	153
glut2	5'-GTGCTGCTGAATAAGTTCTCTTGG-3' 5'-GTCCACAGAAGTCCGCAATGTA-3'	58	226
hes1	5'-AGAAAGATAGCTCGCGGC-3' 5'-CGTTCATGCACTCGCTGAAG-3'	58	163
Insulin	5'-CCATCAAGCAGATCACTGTCCT-3' 5'-GTGTAGAAGAAGCCTCGTTCC-3'	58	180
jagged1	5'-GATGTCACCAGGTCTTACTACG-3' 5'-GTCAGTGATTTCCTTGATCGGG-3'	58	192
nestin	5'-CTTTCAGGACCCCAAGCTG-3' 5'-TAAGAAAGGCTGGCACAGGTG-3'	58	142
neuroD	5'-GGACCTACTAACAACAAAGGAAATCG-3' 5'-TGAGTCCTCCTCTGCGTTCAT-3'	58	186
ngn3	5'-CGGTAGAAAGGATGACGCCT-3' 5'-TGCTTGCTCAGTGCCAACTC-3'	58	249
nkx2.2	5'-TTCAGTACTCCCTGCACGGT-3' 5'-ACTCGCCGCTTTCGCTTCTT-3'	58	151
notch2	5'-TGTGCCACAGGTTTCACTGG-3' 5'-TTGACCAGGTCAATGCAGCG-3'	58	208
pdx1	5'-ATGGATGAAGTCTACCAAAGCTCAC-3' 5'-TCAAGTTCAACATGACAGCCAGC-3'	58	197
pp	5'-CCACCTGCGTGGCTCTGTTA-3' 5'-AGAAGGCCAGCGTGTCCTC-3'	58	189
somatostatin	5'-CCAGACTCCGTCAGTTTCTG-3' 5'-GCCATAGCCGGGTTTGAGTTA-3'	58	204
sur1	5'-GGAGCACACTGGAGTATTC-3' 5'-GGAGATGGAGGTATAGATGGCAA-3'	58	173
vimentin	5'-CCACGAAGAGGAAATCCAGG-3' 5'-CAGAGAGGTCAGCAAACTTGG-3'	58	188

Figure 8.1: Reduced β-Cell Volume in T2DM
A. Comparison of pancreatic β-cell volume between non-diabetic (□) and T2DM (■) donor organs.
B. Comparison of isolation yield between non-diabetic (□) and T2DM (■) donor organs.
C. Comparison of isolation yield, normalized to organ weight, between non-diabetic (□) and T2DM (■) donor organs. * $p<0.05$, ¶ $p<0.01$, § $p<0.001$ vs. non-diabetic.

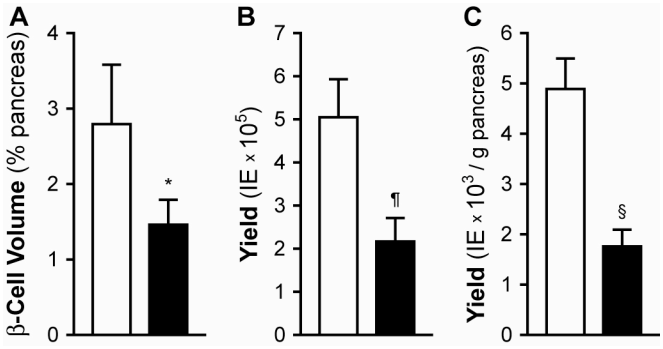

A
β-Cell Volume (% pancreas)
0
1
2
3
4
*
B
Yield (IE x 10^5)
0
1
2
3
4
5
6
7
¶
C
Yield (IE x 10^3 / g pancreas)
0
1
2
3
4
5
6
§

Figure 8.2: Decreased Islet Size in T2DM
A. Contribution of islets with diameter <150μm to isolation yield, in non-diabetic (□) and T2DM (■) islet isolations.
B. Mean *in situ* insulin^{+} cross-sectional area in non-diabetic (□) and T2DM (■) donor organs.
C. Mean *in situ* individual β-cell cross-sectional area in non-diabetic (□) and T2DM (■) donor organs. * $p<0.05$, ¶ $p<0.01$ vs. non-diabetic.

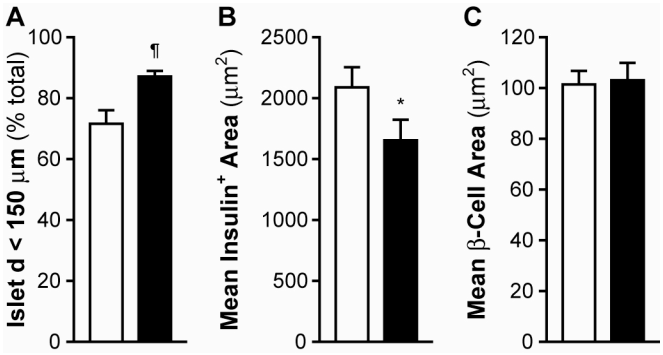

A
Islet d < 150 μm (% total)
100
80
60
40
20
0
¶
B
Mean Insulin+ Area (μm2)
2500
2000
1500
1000
500
0
*
C
Mean β-Cell Area (μm2)
120
100
80
60
40
20
0

Figure 8.3: Decreased β-Cell Frequency in T2DM
A. Immunofluorescent assessment of hormone^{+}, INS^{+}, GCG^{+}, SST^{+} and PP^{+} cell frequencies in isolated non-diabetic (□) and T2DM (■) islet isolations (inset: representative photomicrographs).
B. Comparison of *in situ* β-cell apoptotic indices between non-diabetic (□) and T2DM (■) donor organs.
C. Comparison of β-cell apoptotic indices between isolated non-diabetic (□) and T2DM (■) islets.
D. Assessment of insulin content in isolated islets from non-diabetic (□) and T2DM (■) donor organs. * $p<0.05$, § $p<0.001$ vs. non-diabetic.

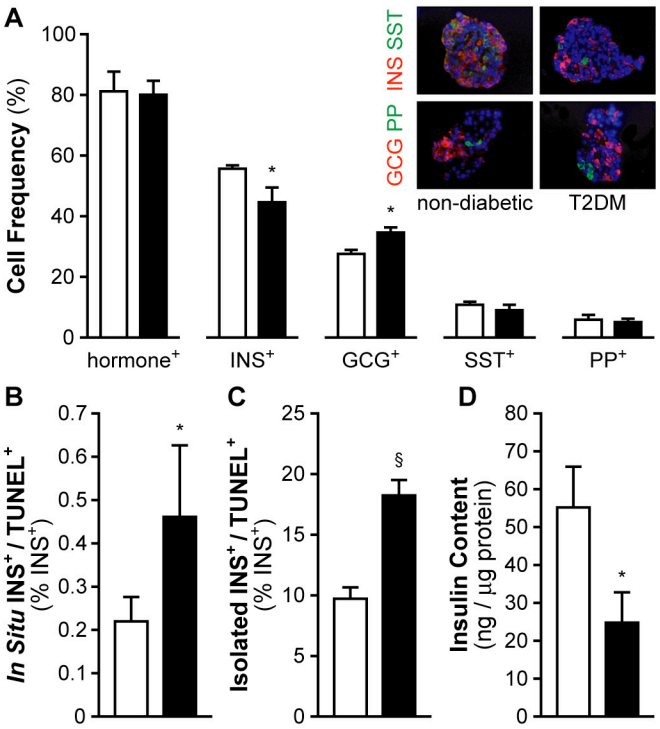

A
Cell Frequency (%)
100
80
60
40
20
0
hormone+
INS+
GCG+
SST+
PP+
*
*
INS SST
GCG PP
non-diabetic
T2DM
B
In Situ INS+ / TUNEL+
(% INS+)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
C
Isolated INS+ / TUNEL+
(% INS+)
25
20
15
10
5
0
§
D
Insulin Content
(ng / µg protein)
80
70
60
50
40
30
20
10
0
*

Figure 8.4: Decreased β-Cell Phenotype in T2DM
Comparison of gene expression levels between isolated islets from non-diabetic (□) or T2DM (■) donor organs. * $p<0.05$ vs. non-diabetic.

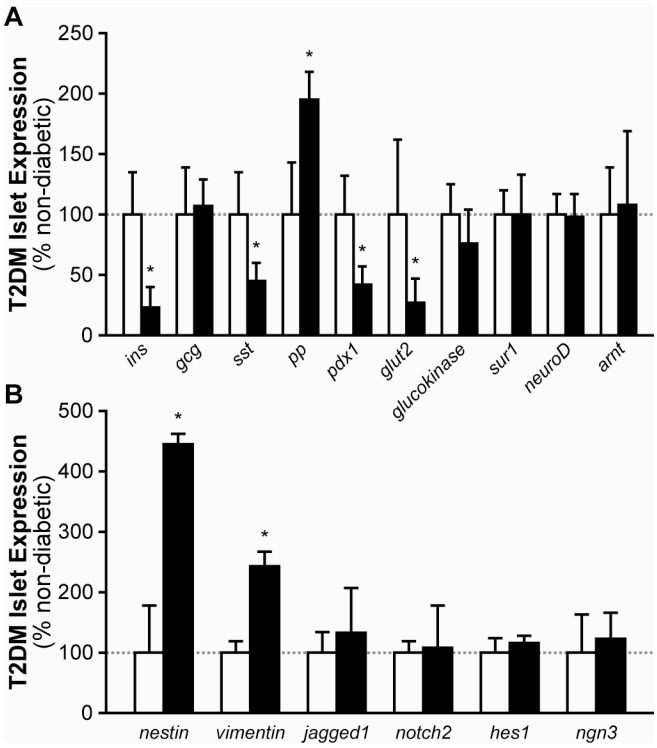

A
T2DM Islet Expression
(% non-diabetic)
250
200
150
100
50
0
ins
gcg
sst
pp
pdx1
glut2
glucokinase
sur1
neuroD
arnt
B
T2DM Islet Expression
(% non-diabetic)
500
400
300
200
100
0
nestin
vimentin
jagged1
notch2
hes1
ngn3

Figure 8.5: Decreased Redifferentiation Capacity in T2DM Cultures
A. Evaluation of DLS formation rates in non-diabetic (□) and T2DM (■) islet cultures.
B. Assessment of islet regeneration (dithizone^{+} structure frequency relative to pre-treatment) in non-diabetic (□) and T2DM (■) DLS cultures.
C. Quantification of DLS cell types, including CK^{+}, CK^{+}/nestin^{+} and nestin^{+}, in non-diabetic (□) and T2DM (■) DLS cultures (inset: representative photomicrographs). * $p<0.05$, ¶ $p<0.01$, § $p<0.001$ vs. control, ‡ $p<0.05$ vs. same group -INGAP peptide.

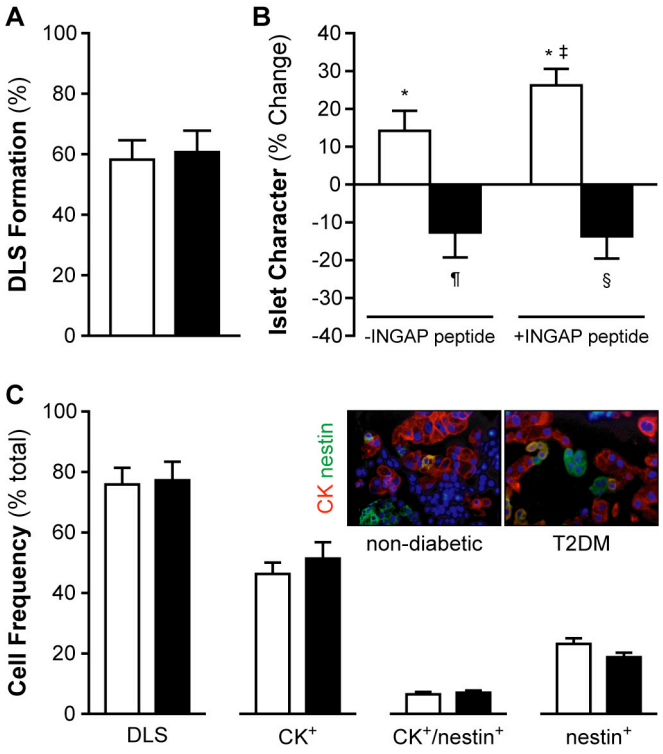
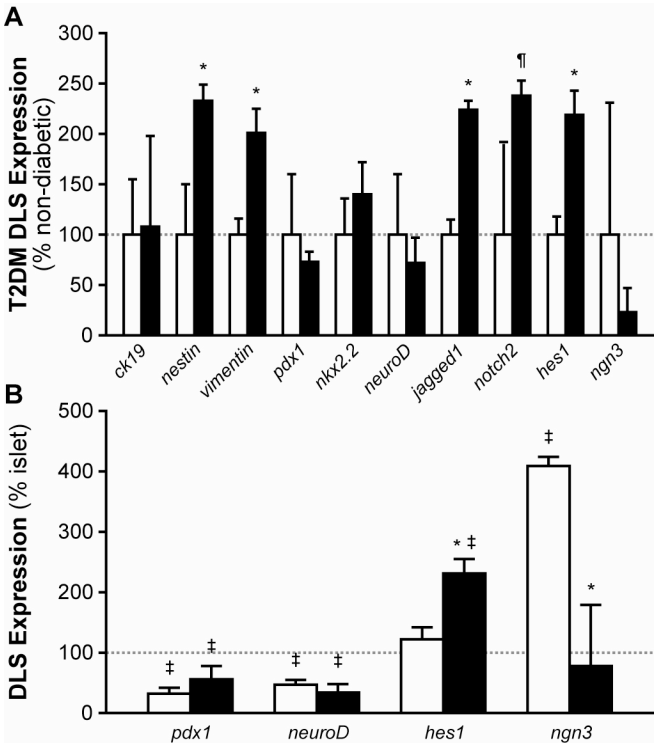

A
DLS Formation (%)
100
80
60
40
20
0
B
Islet Character (% Change)
40
30
20
10
0
-10
-20
-30
-40
*
¶
* ‡
§
-INGAP peptide
+INGAP peptide
C
Cell Frequency (% total)
100
80
60
40
20
0
CK nestin
non-diabetic
T2DM
DLS
CK+
CK+/nestin+
nestin+

Figure 8.6: Increased Notch Signaling in T2DM Cultures
A. Comparison of gene expression levels between DLS cultures from non-diabetic (□) or T2DM (■) donor organs.
B. Comparison of gene expression levels between DLS cultures and islets, for non-diabetic (□) and T2DM (■) donor organs. * $p<0.05$, ¶ $p<0.01$ vs. non-diabetic, ‡ $p<0.05$ vs. same group islet.


A
T2DM DLS Expression
(% non-diabetic)
300
250
200
150
100
50
0
ck19
nestin
vimentin
pdx1
nkx2.2
neuroD
jagged1
notch2
hes1
ngn3
B
DLS Expression (% islet)
500
400
300
200
100
0
pdx1
neuroD
hes1
ngn3

8.5 Discussion

T2DM is now a disease of epidemic proportions, however its etiology remains somewhat uncertain. While specific pathophysiologic conditions, such as insulin resistance and β-cell dysfunction,[191] are attributable to T2DM directly, an absolute deficit in β-cell mass has more recently been associated with the disease.[22] The causal relationship, though, between onset of disease and β-cell deficit is less clear.[135, 136]

In the present study, we reaffirm the decrease in β-cell mass associated with T2DM, both with respect to *in situ* β-cell volume and islet yield on isolation.[22, 649] However, this is the first report of a direct correlation between these two measures, suggesting that the islet isolation procedure is equally efficient in T2DM donor organs. We have previously characterized a similar relationship in non-diabetic donor organs.[663] In keeping with previous reports, we have also found a decrease in islet size, β-cell frequency, β-cell-specific gene expression and insulin content in the islets of T2DM donors;[648-651] findings explained in part by an increase in β-cell apoptosis.[22, 648] In addition, we have identified evidence for *in situ* β-cell dedifferentiation, a phenomenon that has been proposed as an end result of β-cell decompensation[135, 136] but which has not, to our knowledge, been reported previously in human diabetes. What remains to be determined, however, is whether β-cell dedifferentiation represents a late phase of β-cell dysfunction immediately prior to cell death, or whether such cellular dedifferentiation is a part of a compensatory mechanism.

Accordingly, recent studies suggest that isolated adult human islets possess significant morphogenetic plasticity, reflected in the ability to dedifferentiate into primitive cells/structures, proliferate and redifferentiate into functional ILS.[245, 296, 394, 449, 450] While the contribution of such a mechanism to *in situ* β-cell mass dynamics is unknown, it is not unreasonable to ask whether a defect in tissue plasticity contributes to the pathophysiology of T2DM, especially when it is considered that diabetes-associated insults, namely hyperglycemia[664] or cytokine administration,[657-659] induce the loss of β-cell phenotype in animal models.

Given the apparent propensity to dedifferentiate, even *in vivo*, it is not surprising that islets from T2DM organ donors were as capable of dedifferentiating as those from non-diabetic organs. Thus, it appears that cell dedifferentiation may represent a default pathway[165] that is unaffected by the diabetic state. However, an important effect of T2DM seems to be on the inhibition of endocrine redifferentiation. In this regard it is noteworthy that while formation of duct-like epithelial structures from non-diabetic islets is normally associated with an increase in ngn3 expression, no such increase was observed with T2DM islets. In fact, DLS formation in T2DM cultures was associated with a sharp increase in hes1 expression, a marker of Notch signaling. Thus, it appears that while dedifferentiated islet cells are normally primed to undergo subsequent islet redifferentiation, the opposite appears to be the case for dedifferentiated cells derived from T2DM islets. That is to say, while the dedifferentiation process appears to be transient in normal tissue, increased Notch activation freezes such putative precursor-type cells in a dedifferentiated state in T2DM, blocking subsequent islet redifferentiation.

It should be noted that reduced expression of *arnt*, a transcriptional regulator of many β-cell functional markers, has been reported in T2DM islets.[652] We did not observe any difference in *arnt* expression between islets isolated from non-diabetic and T2DM donor organs. While donor age and BMI were comparable between studies, T2DM donors had a shorter duration of disease in our study (3.3±1.4 vs. 5.8±1.1yrs). Accordingly, defective *arnt* expression may mark a later stage in the progression of T2DM, especially given that *arnt* was not the only difference in β-cell marker expression between the two studies.

While, this is not the first observation of loss of β-cell phenotype associated with Notch activation,[657-659] this is, to our knowledge, the first such correlation in non-neoplastic human tissue, especially in the setting of T2DM. Moreover, this is also the first report of *ngn3* re-expression in dedifferentiated islet cells. Based on these observations, it appears that that *in vitro* islet redifferentiation mimics the process of endocrine differentiation during pancreatic development.

Moreover, while we do not provide evidence that the process of islet cell dedifferentiation, proliferation and redifferentiation necessarily occurs in the native pancreas, our studies comparing freshly isolated human islets from T2DM and non-diabetic donor organs suggest that β-cell dedifferentiation is more common in T2DM tissue. Coupled with the finding that cultured islets from T2DM donor organs exhibit overactivation of Notch signaling, these data provide an interesting argument to implicate defective morphogenetic plasticity as a factor compounding the deficit in β-cell mass observed in T2DM.[135, 136]

Studies to identify the role of tissue plasticity in adult human pancreatic disease and regeneration are difficult to undertake outside of defined culture conditions, as autopsy specimens and donor organs represent static time points in the course of dynamic processes. However, recent studies using animal models suggest that rodents may not accurately represent the regenerative mechanisms in play in humans, as it appears that the primary mechanism of β-cell regeneration in rodents is replication,[249] whereas β-cell neogenesis appears to predominate in humans.[22] The observations presented in this paper suggest a role for islet plasticity in the maintenance of β-cell mass, and indicate a defect in islet regeneration associated with T2DM, although a causal relationship is difficult to establish.

8.6 Acknowledgements

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9 Discussion and Concluding Statement

9.1 Islet Isolation

The first objective of this thesis was to establish the quality of the islets being isolated by our facility, in order to validate their use in the forthcoming clinical islet transplantation program at McGill University, but also to confirm the applicability of any results reported using these islets. With this in mind, we undertook a review of islet isolations carried out at our facility over the course of the studies presented in this thesis.

The results of this review, presented in chapter 2,[663] indicate that isolation yield, purity and function were consistent with those reported by other leading facilities.[240, 491] We also identified donor- and isolation-specific variables that correlate with isolation yield and success, to the extent that we were able to establish a simple model that predicted isolation success with an accuracy of 72.3% (Table 2.4).

Given the severe shortage of human organs for clinical islet isolation, we sought to improve islet yield specifically in organs procured from young donors, as these islets tend to be the most functional.[480] To this effect, we identified a slight technical modification to the islet isolation protocol that significantly improved isolation yield when applied to organs from young donors (Table 2.5).

Finally, and perhaps most importantly, we examined the correlation between isolation yield and endogenous β-cell volume within the donor organ. We confirmed that β-cell volume correlated significantly with isolation yield (Table 2.1) and isolation success (Table 2.2), and suggested that research focus on identifying predictors of pancreatic β-cell mass, as these may in fact serve as better predictors of the overall success of islet isolation, as opposed to variables related to the islet isolation procedure.

While these results are immediately applicable to the clinical islet isolation protocol, this application may become outdated as the more recent shortcomings of islet transplantation[498] cause many to question its continued clinical use.[665] Thus, it is possible that a predominant use of islet isolation will be to provide

tissue for *in vitro* experimentation designed to understand the regenerative capacity of human pancreatic tissue, as the focus of research shifts towards the expansion of pancreatic β-cells, first *in vitro*, with the eventual goal of *in situ* β-cell expansion.[666] Recent studies have demonstrated the differences in regenerative mechanisms between the human and murine endocrine pancreas,[22, 245, 249, 296, 394, 449, 450, 462-465, 467] highlighting the need for experiments using human tissue, as opposed to animal models. It is therefore with this notion in mind that we sought to understand how the isolated human islet responds to the isolation protocol with respect to autocrine control of islet survival.

9.2 Autocrine Response to Islet Isolation

As discussed earlier, the proper functioning and survival of adult human islets requires an appropriate microenvironment. This microenvironment includes trophic support provided by cell-cell and cell-ECM interactions, both of which are lost during the isolation procedure.[410-412] Moreover, the isolation process can also induce expression of autocrine factors within the islet, which can act either as pro-survival or pro-apoptotic signals. The balance between survival and death can be tipped by any of these factors, including TNFα, the expression of which we identified as being induced by islet isolation and leading to post-isolation cell death (chapter 3),[495] as well as insulin, which serves not only an endocrine function *in vivo*, but also appears to act locally on isolated islets to protect these cells from post-isolation apoptosis (chapter 4).[494]

The timing of expression of these factors would appear to dictate the predominant signal; TNFα expression was highest in the first 3 days following isolation (Fig. 3.1), leading to a gradual increase in islet cell death over the course of one week of culture (Fig. 3.4). While islets consistently expressed insulin, even immediately post-isolation (Fig. 4.1), it appears that islets require a certain recovery period post-isolation before the endogenous insulin can act to improve islet cell survival via established signaling pathways (Fig. 4.5).

These two observations do seem to be somewhat at odds, however, as it would appear that both pro-survival and pro-apoptotic pathways are being

activated over the same period. This does appear to be the case, and thus it seems that it is the dominance of one signal over the other, and not the exclusive activation of one, that dictates cell survival. Moreover, previous work in our laboratory has confirmed that the commitment to cell death appears to be final, as inhibition of apoptotic pathways will not prevent cell death, but rather convert the signal to a necrotic pathway, with a common result.[402] Thus, the utility of these results resides in therapeutic interventions aimed at preventing the activation of cell death pathways, and the induction of survival pathways very early on in the post-isolation time course.

However, the effects of specific autocrine factors and downstream signaling pathways are not always easy to dissect. For example, our laboratory has previously reported that the increase in cell death that occurs post-isolation correlates with increased JNK phosphorylation,[547] while AKT phosphorylation favors islet survival.[511] In fact, it appears that one of the initial impacts of increased AKT phosphorylation is a downregulation of JNK phosphorylation, indicating a counterbalance between these two important signaling pathways.[501] However, we also demonstrated that even though exogenously administered insulin can increase AKT phosphorylation in the first day post-isolation, this signal does not correlate with increased islet cell survival (Fig. 4.5).[494] Thus, care must be taken to correlate the observed effects on signal transduction pathways with the end results of these same pathways.

Our study of post-transcriptional inhibition of TNFα production using RDP58 is of interest in that our results suggest that much of the cell death that occurs post-isolation may in fact be a result of a factor produced by the islets themselves, and not necessarily via a lack of trophic stimulation.[560, 561] Cytokine production may represent a response to insults associated with the isolation procedure, namely hypoxia.[519-521] Of note, while studies using pharmacological inhibitors have highlighted the importance of generic signaling pathways in determining islet cell fate,[501, 502, 523] our results may be more informative in that the production of a specific cytokine was inhibited (Fig. 3.3). As was eluded to previously, activation of the same signaling pathway in the same cell type, but in a different

cellular context, may lead to very different outcomes,[474] while results gleaned from inhibition of a specific cytokine signal are more easily generalized.

It is an interesting, although quite predictable, observation that the main secretory product of adult human islets can also act as an autocrine survival factor. Moreover, this effect is observed at physiologic concentrations and does not require its exogenous administration.[494] In fact, the administration of exogenous insulin, while having the desired effect on AKT phosphorylation, did not improve islet viability in the early post-isolation period (Fig. 4.5). This observation suggests that competing signals may be acting downstream of this signaling effect to modulate cell survival. Moreover, neither an increased glucose concentration nor addition of IBMX, both classic insulin secretagogues, had any effect on AKT phosphorylation. While we did not specifically assess insulin secretion in this set of experiments, standard testing of islets isolated at our facility confirms a glucose- and IBMX-sensitive insulin secretion.[663] These observations again suggest that context is likely more important than generic signaling event in determining the eventual outcome, although these conclusions cannot be extrapolated beyond the context of isolated human islets.

9.3 Autocrine Response to Islet Culture

As indicated above, the notion of islet transplantation as a definitive therapy for diabetes has fallen out of favour, while some are even questioning the clinical utility of this technique in its current state.[665] A recent shift in the research community has now identified the expansion and/or generation of pancreatic β-cell mass has a more logical therapeutic goal. Thus, studies have focused on two models; that of *in vivo* β-cell mass expansion using animal models,[250, 258, 259, 286, 288, 309, 326, 327, 331-337, 343-345] while others have focused on the culture of isolated human tissue to identify the source tissue and regenerative factors that may allow for subsequent expansion.[245, 296, 394, 448-450] While *in vivo* studies yielded earlier promising results, recent studies have indicated that the dominant mechanisms of *in vivo* β-cell mass dynamics may differ between humans and rodents.[22, 249] Likewise, even more recent studies have questioned the utility of *in*

vitro studies of animal tissue,[467] highlighting the importance of human islet isolation as a source of starting tissue for subsequent experimentation. Surely, however, the end goal remains not to isolate islets for subsequent *in vitro* culture and expansion prior to transplantation, but rather to identify the microenvironment and regenerative factors that would allow for the *in situ* restoration of β-cell mass within the diabetic pancreas.

While certainly a lofty goal, significant inroads have been made into understanding the plasticity that appears to be inherent to certain adult human pancreatic cell types. While precursor-type cells have been reported from virtually every cell population found in the adult pancreas, research has focused on the identification of progenitor-type cells residing either within the pancreatic ducts,[294-296] or even within the islet itself.[245, 296, 447-452]

Our laboratory has developed a model whereby adult human islets can be embedded in a collagen matrix and cultured to yield DLS containing cells expressing markers and a cyto-architecture highly similar to differentiated pancreatic ductal epithelium.[394, 452, 454, 455] These structures can then be induced to redifferentiate into ILS possessing a degree of islet-like form and function, including expression of important endocrine markers, as well as GSIS.[394] While initial experiments suggested a degree of cellular transdifferentiation in this process, the cellular origins of the cells of the DLS remained to be clearly determined, as were the mechanisms by which these adult human islets appeared to regulated their own dedifferentiation into a more primitive structure.

Thus, the next goal of this thesis was to identify autocrine factors that may be acting to modulate this transformation from adult human islet to primitive DLS. Our laboratory had previously established the importance of specific culture conditions for this process to occur, including the presence of a solid collagen matrix and increased intracellular cAMP levels.[454] Studies undertaken as part of this thesis identified EGF (chapter 5) and TGFβ (chapter 6)[474] as autocrine factors produced by the cultured islets, to modulate their response to the aforementioned culture conditions.

Specifically, we confirmed that the process of DLS formation from isolated islets requires the activation of EGF-mediated signaling pathways (Fig. 5.1). Moreover, based on pharmacologic inhibition of EGFR activity, it would appear that islets also act in an autocrine manner to control this process, as islets produce local EGF ligands (Fig. 5.2).

However, it also seems likely that the expression of EGF ligands is not a direct response to the culture conditions that induce DLS formation, as EGF ligand expression was highest following isolation, and was not significantly affected by the act of collagen embedding and culturing under the aforementioned conditions (Fig. 5.2). Thus, it may be that the expression of EGF ligands in cultured islets represents a so-called normal response to islet isolation, and it is this signal combined with the unique culture conditions that leads to islet dedifferentiation. It is not clear to what extent EGF ligands are normally expressed by pancreatic islets, although a recent study certainly suggests that active EGF signaling is required to maintain islet phenotype and function.[76]

With this in mind, it is interesting to note that insulin and EGF share common generic signaling pathways, including AKT phosphorylation. Thus, insulin-mediated AKT phosphorylation in isolated islets cultured in suspension can act as a survival signal,[494] while EGF-mediated AKT phosphorylation in embedded islets acts to facilitate dedifferentiation (chapter 5). It remains to be seen whether EGF would elicit the same result as insulin in freshly isolated islets, and vice versa.

One clue to the role of similar signaling pathways in different cellular responses is the timing of activation of said pathways. AKT phosphorylation appears to act within 24 hours to improve islet survival,[501, 511] while EGF administration, although observed to lead to an acute spike in AKT and ERK phosphorylation (Fig. 5.3), only affected the general phosphorylation state of cultures by day 6 (Fig. 5.2), after the process of dedifferentiation was essentially complete, and the DLS were in a growth phase.[474] Thus, it may be that insulin-mediated signaling plays a more active role in determining cellular fate, while

EGF-mediated signaling plays a more permissive role, and is more involved in cell cycle control.[667]

While the role of EGF in the context of islet phenotypic plasticity may be difficult to elucidate, the role of TGFβ in preserving adult islet phenotype is more readily accepted. Specifically, studies have demonstrated a role for TGFβ ligands in favoring endocrine cell differentiation during development,[86] as well as a need for TGFβ signaling in the adult organ to maintain islet function and phenotype.[88] In fact, loss of TGFβ signaling is commonly associated with pancreatic adenocarcinoma,[178] which possesses certain cellular characteristics not unlike the cells of the DLS.

Thus, it is not surprising that we noted that administration of TGFβ had a deleterious effect on DLS formation, and acted to preserve isolated islets in a more differentiated state (chapter 6).[474] This preservation of islet phenotype was reflected not only in the frequency of islets as opposed to DLS (Fig. 6.2A), but also in insulin content (Fig. 6.2B) and the frequency of insulin$^+$ cells (Fig. 6.3C), mediated at least partially by a decrease in the incidence of β-cell apoptosis (Fig. 6.3D).

However, the preservation of islet phenotype induced by TGFβ administration was not matched by a preservation of GSIS. If anything, TGFβ appeared to act to invert the dynamics of insulin secretion, such that a stimulation index below unity was observed, indicating that greater amounts of insulin were secreted in response to lower glucose levels (Fig. 6.4). While somewhat at odds with the aforementioned studies describing an essential role for TGFβ in maintaining endocrine function in the pancreas, there do exist other reports of an inversion of insulin secretion dynamics in isolated islets.[619]

Again, having established the key signaling pathways relevant to islet-to-DLS conversion,[455] we hypothesized that TGFβ would elicit its effects in this model by modulating the signaling of one of these predefined pathways, namely by inhibiting JNK-mediated signaling early in the differentiation process, or by inhibiting ERK phosphorylation later on. Examination of these signaling patterns at days 1 and 6 of the process indicated a TGFβ-mediated decrease in JNK

phosphorylation and an increase in ERK phosphorylation, although the most pronounced effect was an increase in p38 phosphorylation (Fig. 6.6). Inhibitor-based studies indicated that the other effects were likely secondary to the direct effect of TGFβ on p38 phosphorylation, with this pathway acting as the key transducer of the TGFβ signal (Fig. 6.8). The role of p38 signaling in isolated islet survival and function is not at all clear, as varying reports (including some from our laboratory) have described p38 as being both pro- and anti-apoptotic.[502, 547, 549, 620] Thus, once again, the message garnered from these experiments seems to be that context trumps generic signaling pathways with respect to understanding and predicting the islet response to its environment.

9.4 Cell Origins of Islet Plasticity

Having begun to identify the molecular signals controlling the differentiated and functional state of cells of the adult human islet, the next goal of this thesis was to better understand the origins of the constituent cells of the DLS. Specifically, while it has been shown that cells derived from isolated adult human islets can be cultured, expanded, and induced to undergo redifferentiation into somewhat functional ILS, the identification of the specific cellular source of these redifferentiated structures would allow a better understanding of the mechanisms and requirements for the process. Eventually, the goal would be to determine whether such cells could be induced within the native pancreas to cause an increase in β-cell mass sufficient to restore normoglycemia in a diabetic patient. The first step, however, is the identification of the cells of origin.

To this end, we had previously presented four separate hypothetical mechanisms as to the formation of the DLS, each of which involved either the dedifferentiation of adult endocrine cells, or the persistence of an unidentified precursor cell (Fig. 1.5).[394] While no putative marker(s) have yet been proposed that can reliably identify these hypothetical precursor cells, several reports have presented results of ultrastructural,[394] immunofluorescence[245, 394, 450] and pulse-chase[448] experiments indicating that, at the very least, endocrine cell dedifferentiation is occurring. While more definitive cell labelling techniques in

murine islets have not clarified the debate,[462-465] both our work (chapter 7)[668] and a recently published report from another group[467] present strong evidence that β-cell dedifferentiation is a major contributor to the cell population from whence redifferentiation arises. Moreover, our study indicates that β-cells and non-β endocrine cells beget relatively distinct populations of cells within the subsequent culture (Fig. 7.2, Table 7.6). While previous studies have presented the possibility that it is not the β-cell-derived cells that go on to redifferentiate,[394] our studies using genetic- (Fig. 7.4) or chemical- (Fig. 7.5) based ablation of specific cell types suggest that β-cells play a necessary role in the redifferentiation process, although whether said role is direct or indirect remains to be determined. What's more, it would appear that the notion of a cellular niche is of utmost importance in permitting the dedifferentiation and redifferentiation processes to occur, at least in the context of our established model of islet plasticity, as ablation of any individual endocrine cell type limited the overall plasticity of the culture (Fig. 7.4).

While by no means definitive, these results point to the β-cell as a key player in islet plasticity, and suggest that controlling the state of differentiation of the β-cell may be the key to regulating β-cell mass dynamics in the pancreas. However, given that these observations are conducted within the context of a very specific culture model, without the ability to derive supporting data from a relevant animal model, it is difficult to ascertain the biological significance and applicability of these observations. The next question, therefore, is whether such a process is normally ongoing, and whether T2DM represents a disruption of such a process.

9.5 Islet Plasticity and T2DM

The notion that islet plasticity contributes to the balance between health and disease is by no means a novel one. Studies have demonstrated that β-cell mass dynamics, representing the net result of β-cell formation (replication and neogenesis) and loss (cell death),[246] contribute to the increased β-cell mass observed in certain conditions,[22, 188, 206, 248] and the deficit in β-cell mass observed

in other situations.[22, 189] While this model of β-cell mass dynamics has helped explain many cases of variable β-cell mass, it nevertheless neglects the possibility of the β-cell playing an active role in scenarios of decreased β-cell mass. Thus, just as β-cells may be formed from non-β-cell precursors through the process of neogenesis,[256-260] so too can β-cells undergo dedifferentiation into a non-functional state. While this β-cell dedifferentiation may be either transient or permanent, the fact remains that such an occurrence would reduce the functional β-cell mass, just as β-cell apoptosis would. Moreover, while certain noxious stimuli may not induce β-cell death, it stands to reason that such stimuli could induce a loss of β-cell phenotype. Hence, the notion of β-cell decompensation has been advanced to explain the progressive loss of β-cell phenotype during the progression of T2DM,[135, 136] just as dedifferentiated β-cells have been proposed as the cell of origin of pancreatic adenocarcinomas.[165] However, due to the dynamic nature of these diseases, it has been difficult to establish whether such a mechanism is at play in humans, although animal models do hint at a progressive decompensation in β-cell phenotype and function during diabetogenesis.[136] Having already established what appears to be one of the default pathways of islet dedifferentiation, the next goal of the thesis was to investigate whether such a mechanism was at play in islets.

Our study of freshly isolated human islets (chapter 8) confirmed previous observations of decreased β-cell mass and islet yield in pancreata from T2DM donors (Fig. 8.1).[22, 649] Moreover, we also confirmed a decreased islet size (Fig. 8.2), β-cell frequency (Fig. 8.3) and β-cell-specific gene expression (Fig. 8.4) in islets from T2DM donor organs,[648-651] mediated at least partially by an increase in β-cell apoptosis (Fig. 8.3).[22, 648] Most interestingly, however, we also observed evidence for *in situ* β-cell dedifferentiation in the form of increased islet expression of *nestin* and *vimentin* (Fig. 8.4), both of which have been reported to be expressed by dedifferentiated β-cells.[245, 394, 467, 668]

While islets from T2DM donor organs were no more prone to undergo structural dedifferentiation than islets from non-diabetic donor organs (Fig. 8.5),

these structures were resistant to subsequent redifferentiation strategies, and observation that was also linked to overexpression of Notch signaling molecules and underexpression of *ngn3* (Fig. 8.6), a marker of differentiation potential that is re-expressed by adult islets following dedifferentiation.[394, 567]

Based on these results, it is tempting to speculate that the diabetic milieu provides some signal to β-cells, inducing a degree of functional dedifferentiation, as well as setting a block to the subsequent redifferentiation of these same cells. Several insults, present in the diabetic environment, have been proposed, including the combined effects of hyperglycemia and hyperlipidemia,[211, 669] as well as cytokines.[657, 658]

What is far less evident, however, is whether such a process of dedifferentiation, proliferation, and subsequent redifferentiation, as observed under specific culture conditions, is an ongoing natural process in the human pancreas. If such is the case, it follows logically that T2DM may be marked by a disruption of such a process. Complicating the study of such a phenomenon is the fact that any observations of the human pancreas are static, whereas such processes are dynamic, and may in fact involve cells undergoing differentiation processes along the same pathways, but in opposite directions, Thus, the nestin$^+$/insulin$^+$ cells observed in an adult human pancreas may be comprised of both β-cells undergoing dedifferentiation, as well as nestin$^+$ precursor cells undergoing endocrine redifferentiation.[303] While lineage tracing techniques applied to animal models would appear to be logical way of examining this issue,[462-465] the observation of differing mechanisms of β-cell plasticity between humans and rodents limits the applicability of such studies.[22, 249, 467]

Finally, further complicating our understanding of such a process is the fact that β-cell plasticity is not occurring in isolation. Thus, just as a dedifferentiated β-cell undergoing endocrine redifferentiation will result in a net gain of one β-cell, so too will the neogenesis of a β-cell from a duct-associated progenitor cell. In fact, studies of cadaveric human pancreata suggest that such islet neogenesis is a significant contributor to β-cell mass dynamics.[22]

9.6 Pancreatic Plasticity and T2DM

While T2DM is associated with defective β-cell insulin synthesis and secretion, and/or insulin resistance at target tissues,[191] there remains debate as to the contribution of β-cell mass dynamics to the pathophysiology of T2DM.

It has been established that β-cell mass is increased in obesity,[188, 206] primarily via the mechanism of increased β-cell neogenesis.[22] Furthermore, in both lean and obese patients with pre-established T2DM, there is a relative deficit in β-cell mass, due almost exclusively to increased β-cell apoptosis. Interestingly, neither β-cell neogenic nor proliferative rates appear to be significantly affected by T2DM.[22]

While informative, the above report studied autopsy specimens from patients diagnosed posthumously as being diabetic based on one fasting blood glucose level.[22] Thus, no formal diagnosis of T2DM had been established.[183] As a result, duration of disease was unknown. Moreover, due to the tissue source, study patients tended to be older, especially in the lean group (non-diabetic: 78.1±2.9, T2DM: 80.2±1.9 years).[22] Thus, the results reported likely reflected the state of β-cell mass dynamics long after the period of active diabetogenesis. Hence, questions still remain as to the nature of β-cell mass dynamics early in the progression of T2DM.

Thus, a final goal of this thesis was to compare overall β-cell volume and the constituent mechanisms of β-cell mass dynamics early in the progression of T2DM, in lean and obese donor organs. To this end, we examined 8 lean and 11 obese T2DM donor organs processed at our clinical islet isolation facility, as well as 18 lean and 23 obese sex-, age- and BMI-matched non-diabetic control donor organs (Table 9.1).

Briefly, while age was comparable between studies in the obese groups, the age of donors in the lean groups in our cohort was significantly less than in the previous report,[22] and was comparable to that of the obese groups in our study. We ascribe this to the nature of the donor organ source, as exclusion criteria for organ procurement include age above 80 years. Also, while BMI was comparable between studies in the lean groups, BMI in the obese groups in our study were

significantly less than those in the previous report, even though diagnostic criteria for obesity only differed slightly (25 vs. 27kg/m^2). Again, organ donor screening criteria may be responsible for this difference.

Analysis of the effects of metabolic state and T2DM on β-cell volume and islet isolation yield indicated several points of interest. Firstly, as reported by Butler *et al*.,[22] obesity was associated with an increase in β-cell volume and β-cell mass in non-diabetic donors (Fig. 9.1A&B). Likewise, both β-cell volume and β-cell mass were significantly reduced in obese T2DM donors, as compared to obese non-diabetic donors (Fig. 9.1A&B). However, unlike the previous study, we found there to be no difference in β-cell volume or β-cell mass between lean T2DM and non-diabetic donor organs (Fig. 9.1A&B), although our lean donors was approximately twenty years younger at the time of death. Assuming approximately equal ages of diagnosis of T2DM, the marked difference in β-cell volume patterns may be ascribable to the effect of twenty additional years of disease progression.

While we have previously reported that BMI has little to no effect on isolation yield (chapter 2),[663] this result is further confirmed by the results present herein. Specifically, there was no significant difference in isolation yield between non-diabetic lean and obese groups (Fig. 9.1C). However, β-cell volume was significantly increased in obese non-diabetic donor organs, suggesting that the isolation procedure may be less efficient in obese organs. In fact, both obesity and T2DM lead to decreased isolation yield, when normalized to β-cell mass (Fig. 9.1D).

In order to understand the mechanisms of these changes in β-cell volume, we examined surrogate rates of β-cell neogenesis (Fig. 9.2), replication (Fig. 9.3) and apoptosis (Fig. 9.4). Because neogenesis is a dynamic process and we are only able to examine the static nature of the pancreas at the time of death, surrogate measures for neogenesis have been proposed based on the incidence of small insulin$^+$ structures, comprised of three β-cells or less.[260] Traditionally, these structures have also been defined as being duct-associated,[260] but we have previously proposed that acinar-association should not preclude

consideration as a neogenic structure.[250] Therefore, the area of three β-cells (330μm^2), based on a mean β-cell area determined to be 110±4μm^2 (Fig. 9.2B), was set as the threshold for defining neogenic insulin$^+$ structures.[260] Insulin$^+$ structures were scored as duct-associated based on observed physical contact with the cells lining the lumen of a duct, or observed physical location within the connective tissue immediately surrounding a duct. Structures not meeting these criteria were qualified as acinar-associated (Fig. 9.2A).[250]

Using these criteria, the frequency of insulin+ structures less than 330μm^2 in area was used as a surrogate measure of the rate of β-cell neogenesis.[250] While obesity was associated with an increase in acinar-associated neogenic structures in non-diabetic donors, T2DM was associated with an increase in duct-associated neogenic structures in both lean and obese donors (Fig. 9.2C). When the overall frequency of neogenic structures was assessed, it appeared that either obesity or T2DM was sufficient to induce an increase in neogenesis, with a possible additive effect occurring obese T2DM donors (Fig. 9.2C). These findings are somewhat different from those of Butler *et al*.,[22] who reported an increase in neogenesis associated with obesity only. However, the surrogate measure of neogenesis differed significantly between the two studies, as well as the average age of the donors, meaning direct comparison of these studies is difficult.

Next, we examined the incidence of PCNA staining in insulin$^+$ cells, as a marker of β-cell replication.[250] Obesity was associated with an increased proliferative index in non-diabetic donor organs, while T2DM was associated with a decrease in proliferation in obese donors (Fig. 9.3A). Interestingly, T2DM did not appear to have a deleterious effect on lean donors. The changes in proliferation rates were reflected in the mean cross-sectional area of insulin$^+$ structures, in that the average obese T2DM insulin$^+$ area was significantly reduced (Fig. 9.3B). Given that the mean cross-sectional area of a β-cell was the same between non-diabetic and T2DM donor organs (Fig. 9.2B), this difference in islet area likely represents a difference in cell number. However, the decreased islet area in obese T2DM donor organs likely represents the combined effect of the increased neogenesis (more small structures) and

decreased proliferation (less growing structures) observed in this group. These results also differed from previous reports, whereby proliferation was not reported to differ between any of the study groups.[22]

Finally, we examined the role of β-cell apoptosis in the dynamics of β-cell mass. The frequency of β-cell apoptosis was only significantly increased in obese T2DM donor organs, with a rate of apoptosis that was significantly higher than in any other group (Fig. 9.4). This observation could also explain the decreased average area (Fig. 9.3B) in obese T2DM donor organs (more shrinking structures), and appears to be the main contributor to the significant decrease in β-cell volume and β-cell mass observed in obese T2DM donor organs (Fig. 9.1A&B). Moreover, Butler *et al*. also observed β-cell apoptosis to be significantly increased in obese T2DM organs, and proposed this mechanism as the main contributor to the overall effect on β-cell volume.[22] However, Butler *et al*. also observed a similar increase in β-cell apoptosis in lean T2DM organs,[22] whereas we did not, just as we did not observe a significant decrease in overall β-cell volume in these organs (Fig. 9.1A&B). Knowing the significant difference in age between the lean groups in our study and the previous report, a progressive loss of β-cell mass over the course of the disease would explain these differing results.

To examine the effect of disease progression on overall β-cell volume, we plotted β-cell volume against donor age for each of the four groups (lean non-diabetic, lean T2DM, obese diabetic, obese T2DM), from our study and that of Butler *et al*. (Fig. 9.5).[22] Next we drew trend lines to simulate the progression of β-cell volume with time, using data from our study and that of Butler *et al*. Although these trend lines are likely unreliable, given the variability inherent to each study, as well as the unavailability of individual data points, there trends suggested are compelling.

First of all, an examination of the trend lines associated with lean and obese non-diabetic individuals suggests that β-cell volume slowly increases with age, and that this increase is slightly steeper in the obese group. This seems logical, especially in the case of obese non-diabetic individuals, given that BMI was

significantly increased in the Butler study as compared to our study, suggesting an incremental increase in β-cell volume compensating for an increase in the level of obesity.

Likewise for the lean and obese T2DM individuals, there is a progressive decrease in β-cell volume with age and, presumably, progression of the disease. This observation was alluded to earlier by Butler *et al*., who noted an apparent decrease in β-cell neogenic and proliferative capacity with increasing age.[22] Once again, the slope of the obese T2DM trend line is steeper than the lean trend line; it could be argued that the metabolic environment associated with obesity is more deleterious to the islet, and precipitates a sharper decline in β-cell phenotype and function.[211, 669]

Mean age at diagnosis of T2DM was provided from chart reviews of organ donors in our study. Plotting trend lines backwards to the age at diagnosis, an interesting observation can be made; it would appear that β-cell volume at the time of diagnosis of T2DM was at least equal to, if not greater than that of age, BMI- and sex-matched non-diabetic individuals. If such is the case, it stands to reason that deficits in β-cell volume do not contribute to the pathophysiology of T2DM, but rather may be a consequence of an earlier diabetogenic event. Moreover, attempts to restore the lacking β-cell volume, or interventions to prevent the loss of β-cell volume observed during the course of the disease, may serve to alleviate the symptoms of the disease, but may not address the nature of the disease, which has heretofore been assumed to be a deficit in functional β-cell volume. In fact, just as correlations between β-cell volume and glucose tolerance are not direct,[215] suggesting that sufficient insulin-expressing tissue does not necessarily guarantee normoglycemia, it may be that the progressive loss of β-cell volume over the course of the disease simply represents a subsequent step following the earlier decompensation of β-cells.[135, 136]

9.7 Future Directions

The study of pancreatic plasticity and, more specifically, islet plasticity is still in its infancy. In fact, debate remains as to the extent of these processes, while the

utility of these mechanisms in designing cellular therapies and therapeutic interventions is highly uncertain. That being said, an ever-increasing number of reports are lending credence to the notion that the human pancreas, and possibly even the adult human islet, do possess a degree of plasticity that may contribute to both disease and healing processes.

While this thesis describes a limited number of observations from *in situ* human tissue, any experimental observations are garnered from synthetic *in vitro* models of cellular plasticity. The applicability of these models remains debatable, especially with the recent realization that animal models of pancreatic plasticity may not be transferable to the human pancreas.

Thus, it remains to be seen whether the *in vitro* roles of TNFα (chapter 3), insulin (chapter 4), EGF (chapter 5) and TGFβ (chapter 6) described herein are also ongoing in the native pancreas, or represent purely *in vitro* phenomena. More importantly, there is little evidence to support or detract from whether the cellular dedifferentiation and redifferentiation observed *in vitro* (chapter 7) occurs *in vivo*, and whether these processes have any impact on the etiology and/or pathophysiology of human pancreatic diseases (chapter 8). Studies of cadaveric donor organs suggest that these same processes are ongoing in the native pancreas (chapter 9), but are hard to interpret given the directionality inherent to transdifferentiation processes that cannot be ascertained *in vivo* in the human pancreas.

Such is the nature of studies investigating dynamic *in vivo* processes, especially if these processes are limited to humans. All that can be done is to capitalize on any available *in vitro* models, while collecting observational data from *in vivo* sources, all the while interpreting any new information with a firm understanding of the limitations of the methodology.

9.8 Conclusions

Based on the objectives stated at the outset of this thesis, the present findings support the following conclusions:

- The yield of functional adult human islets from the clinical islet isolation procedure is dependent on the β-cell mass found within the pancreas.
- The response of the adult human islet to isolation includes the production of both pro- and anti-apoptotic autocrine signals, including TNFα and insulin, the balance of which determines the degree to which the islets survive the immediate post-isolation period.
- The response of the isolated adult human islet to embedding and culture includes the production of both dedifferentiation-inductive and -protective autocrine signals, including EGF and TGFβ, the balance of which determines the degree to which cultured islets maintain an adult endocrine phenotype and function.
- The transformation of isolated adult human islets into DLS upon embedding and culture is marked by several processes, including apoptosis, predominantly in β-cells, as well as transdifferentiation, with α-, δ- and PP-cells transdifferentiating predominantly into CK^+ epithelial cells, and β-cells transdifferentiating predominantly into $nestin^+$ fibroblast-like cells.
- Adult human islets isolated from organs procured from T2DM cadaveric donors retain the ability to undergo dedifferentiation *in vitro*, but no longer possess the ability to undergo subsequent redifferentiation.
- The dynamics of β-cell mass over the progression of T2DM suggest that a deficit in β-cell mass may not actually be present at the time on diagnosis of the disease.

Table 9.1: Donor Characteristics

	Lean (BMI<27kg/m^2)		**Obese** (BMI≥27kg/m^2)	
	Non-diabetic	**T2DM**	**Non-diabetic**	**T2DM**
n	18	8	23	11
Duration of T2DM (yrs)		4.0±2.0		6.4±3.2
T2DM treatment		6/8 Rx 2/8 insulin		8/11 Rx 1/11 insulin
Age (years)	58.5±1.7	59.0±2.2	59.2±1.8	60.6±2.5
Sex	13M/5F	6M/2F	10M/13F	5M/6F
BMI (kg/m^2)	24.2±0.6	23.7±0.7	31.4±0.9	32.6±1.6
Glucose (mM)	8.8±0.7	8.4±1.2	10.4±1.4	9.7±0.8
Pancreatic weight (g)	93.1±4.8	90.7±15.8	102.7±6.0	118.9±16.1

Figure 9.1: Effect of Obesity and T2DM on β-Cell Volume
β-Cell volume (**A**) and β-cell mass (**B**), determined as described previously,[663] were compared between lean and obese non-diabetic and T2DM donor organs. While no significant differences were observed between lean non-diabetic and T2DM donor organs, both β-cell volume and β-cell mass were significantly increased in obese non-diabetic donor organs as compared to lean non-diabetic donor organs. Conversely, obese T2DM donor organs were marked by a deficit in β-cell volume and β-cell mass relative to obese non-diabetic donor organs.
C. Isolation yield (purity ≥70%) was compared between lean and obese non-diabetic and T2DM donor organs. While no difference in isolation yield was observed between lean and obese non-diabetic donor organs, lean T2DM donor organs yielded significantly less islets than any other group.
D. Isolation yield (purity ≥70%), normalized to pancreatic β-cell mass, was compared between lean and obese non-diabetic and T2DM donor organs. Normalization to β-cell mass highlighted the decrease in isolation yield related to both obesity and T2DM (* $p<0.05$, ¶ $p<0.01$ vs. lean ND; ‡ $p<0.05$ vs. obese ND).

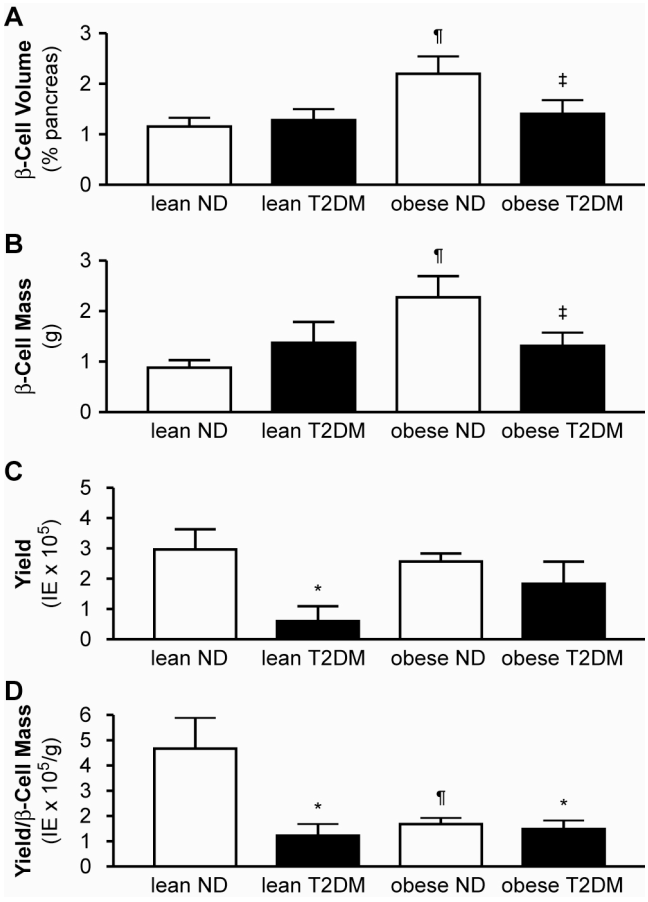

A
β-Cell Volume
(% pancreas)
3
2
1
0
¶
‡
lean ND
lean T2DM
obese ND
obese T2DM
B
β-Cell Mass
(g)
3
2
1
0
¶
‡
lean ND
lean T2DM
obese ND
obese T2DM
C
Yield
(IE x 10^5)
5
4
3
2
1
0
*
lean ND
lean T2DM
obese ND
obese T2DM
D
Yield/β-Cell Mass
(IE x 10^5/g)
6
5
4
3
2
1
0
*
¶
*
lean ND
lean T2DM
obese ND
obese T2DM

Figure 9.2: Effect of Obesity and T2DM on Islet Neogenesis
A. Insulin$^+$ structures were categorized according to size and tissue association. The area of three β-cells (330μm^2, based on a mean β-cell area determined to be 110±4μm^2 (**B**)) was set as the threshold for defining neogenic insulin$^+$ structures, as a surrogate measure of neogenesis.[260] Insulin$^+$ structures were scored as duct-associated based on observed physical contact with the cells lining the lumen of a duct, or observed physical location within the connective tissue immediately surrounding a duct. Structures not meeting these criteria were qualified as acinar-associated.[250]
B. Mean β-cell cross-sectional area did not differ between non-diabetic and T2DM donor organs, indicating that differences in β-cell volume were likely based on differences in cell number, and not cell size.
C. Analysis of the frequency of neogenic insulin$^+$ structures suggests that obesity may be associated with increased acinar-associated neogenesis in non-diabetic individuals. Conversely, duct-associated neogenesis appears to be increased in T2DM, regardless of metabolic state. The overall effect therefore appears to be increased neogenesis associated with both obesity and T2DM, with a possible additive effect in obese T2DM individuals (* $p<0.05$, ¶ $p<0.01$ vs. lean ND).

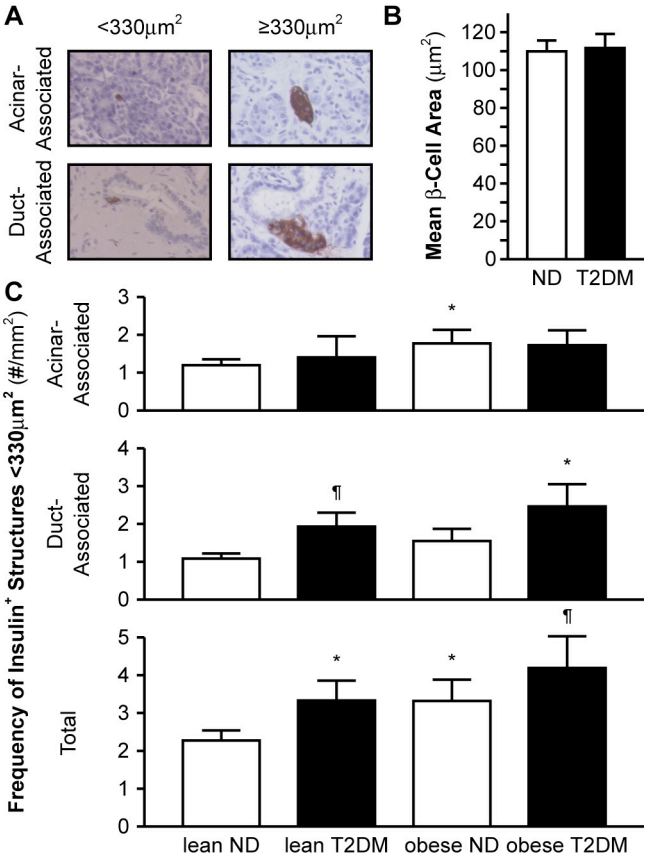

A
<330μm2
≥330μm2
Acinar-Associated
Duct-Associated
B
Mean β-Cell Area (μm2)
120
100
80
60
40
20
0
ND
T2DM
C
Frequency of Insulin+ Structures <330μm2 (#/mm2)
Acinar-Associated
Duct-Associated
Total
3
2
1
0
4
5
*
¶
lean ND
lean T2DM
obese ND
obese T2DM

Figure 9.3: Effect of Obesity and T2DM on β-Cell Replication
A. β-Cell proliferation was assessed by PCNA staining in β-cells.[250] Obesity was associated with an increase in β-cell proliferation in non-diabetic donor organs, while there was significantly less β-cell proliferation in obese T2DM relative to obese non-diabetic donor organs.
B. The mean cross-sectional area of insulin^{+} structures reflected the trend in β-cell proliferation, with significantly decreased mean cross-sectional area in obese T2DM donor organs relative to obese non-diabetic organs (¶ $p<0.01$ vs. lean ND; ‡ $p<0.05$, § $p<0.001$ vs. obese ND).

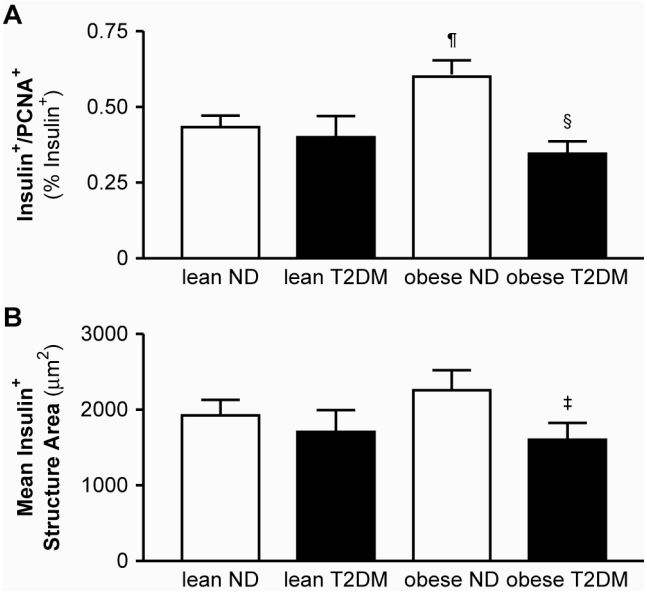

A
0.75
0.50
0.25
0
Insulin+/PCNA+
(% Insulin+)
¶
§
lean ND
lean T2DM
obese ND
obese T2DM
B
3000
2000
1000
0
Mean Insulin+
Structure Area (μm2)
‡
lean ND
lean T2DM
obese ND
obese T2DM

Figure 9.4: Effect of Obesity and T2DM on β-Cell Apoptosis
β-Cell apoptosis was assessed by TUNEL staining in β-cells.[250] Only obese T2DM donor organs were associated with a significant increase in β-cell apoptosis (‡ $p<0.01$ vs. all).

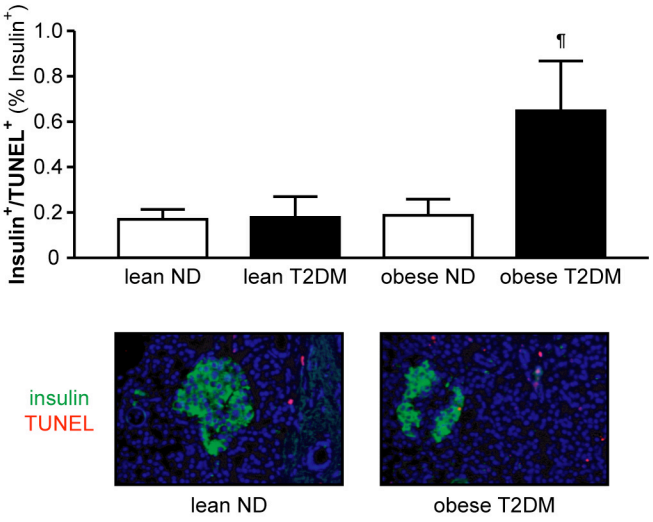
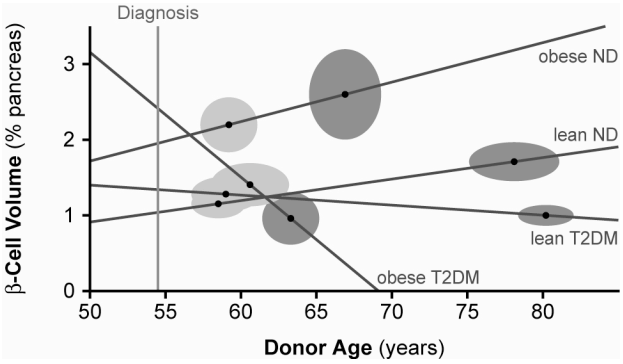

Insulin+/TUNEL+ (% Insulin+)
1.0
0.8
0.6
0.4
0.2
0
¶
lean ND
lean T2DM
obese ND
obese T2DM
insulin
TUNEL
lean ND
obese T2DM

Figure 9.5: Effect of Disease Progression on β-Cell Volume in T2DM
Given that β-cell volume was determined using similar methodology in our and a previous study,[22] but donor age differed significantly, we sought to investigate the effect of T2DM disease progression on β-cell volume. Trend lines were drawn between data points obtained from our (○) and the Butler (●) study to indicate the progression in lean and obese non-diabetic and T2DM β-cell volume. Mean age at diagnosis of T2DM was provided from chart reviews of organ donors in our study, while equivalent data was unavailable from the Butler study given the diagnosis of T2DM was established purely on recorded blood tests values.[22] While lean non-diabetic donors appear to have slowly increasing β-cell volumes with age, lean T2DM donors appear to be progressively loosing β-cell volume. Likewise, while β-cell volume appears to increase with age in obese non-diabetic donors, the decline in β-cell volume with age is quite sharp in obese T2DM donors. Common to both lean and obese groups, however, is the observation that β-cell volume at the time of diagnosis would appear to be equal to, if not greater than, that of non-diabetic controls, suggesting that a loss of β-cell volume does not contribute to the pathophysiology of T2DM at the time of diagnosis, but occurs subsequent to the time of diagnosis, and may contribute to the progression of the disease.


Diagnosis
obese ND
lean ND
lean T2DM
obese T2DM
β-Cell Volume (% pancreas)
0
1
2
3
50
55
60
65
70
75
80
Donor Age (years)

Original Contributions to Knowledge

1. Pancreatic β-cell volume is a predictor of clinical islet isolation yield and success.

2. In young donors (≤25 years), the use of Liberase CI results in increased clinical islet isolation yields than the traditional use of Liberase HI.

3. Post-transcriptional TNFα inhibition improves isolated human islet survival and function.

4. Autocrine insulin improves isolated human islet survival via AKT phosphorylation.

5. Autocrine EGF ligands induce dedifferentiation of cultured human islets via AKT and/or ERK phosphorylation.

6. Autocrine TGFβ inhibits dedifferentiation of cultured human islets via p38 phosphorylation, but also negatively affects GSIS.

7. Islet-to-DLS transformation requires both α-, δ- and PP-cell-to-CK^+ epithelial cell and β-cell-to-$nestin^+$ fibroblast-like cell transdifferentiation.

8. Islet redifferentiation requires the presence of β-derived cells within the DLS.

9. Human islets isolated from T2DM donor organs expressed higher levels of nestin and vimentin, markers of β-cell dedifferentiation. DLS formed from T2DM islets expressed higher levels of Notch signaling molecules, and were not capable of undergoing islet redifferentiation.

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March 14, 2003

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Research Institute of the
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Room C9-133
Montreal General Hospital

RE: REC. 02-003/004 entitled "Pancreatic Beta-cell Expansion & Islet Isolation."

Dear Dr. Rosenberg:

We have received an Application for Continuing Review of the Montreal General Hospital Research Ethics Committee for the research study referenced above and the report was found to be acceptable for ongoing conduct at the McGill University Health Centre. At the MUHC sponsored research activities that require US federal assurance are conducted under Federal Wide Assurance (FWA) 00000840.

The re-approval for the study was provided via expedited review of the Chair until January 2004.

However, the REC must be informed of how many subjects have been involved in your study to date (i.e. how many samples of human pancreas have been obtained).

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We trust this will prove satisfactory to you.

Sincerely,

Denis Cournoyer, M.D.
Chairman
MUHC-Montreal General Hospital
Research Ethics Committee

Cc: REC#02-003/004

1650 Cedar, Montréal (Québec) Canada, H3G 1A4 (514) 937-6011

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Dear Dr. Rosenberg:

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