Pharmacological Induction of Islet Neogenesis and Subsequent Beta-Cell Mass Expansion

by

Mark Andrew Lipsett

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ABSTRACT

Current therapies for diabetes mellitus are insufficient to prevent the devastating complications associated with this disease. A novel approach for the treatment of diabetes is the restoration of an insulin-producing β-cell mass through the stimulation of endogenous progenitor cells. Thus, the aim of this thesis was to determine if pharmacological initiation of islet neogenesis and subsequent β-cell mass expansion will lead to the reversal of hyperglycaemia in a response that is under homeostatic regulation and has efficacy in humans.

A pentadecapeptide fragment of Islet Neogenesis Associated Protein ($INGAP^{104-108}$), was administered to normoglycaemic hamsters and was found to result in an expanded β-cell mass as measured by immunohistochemical morphometric analysis. This expansion was shown to occur through the transformation of duct- and acinar-associated progenitors. In order to determine if this therapeutic approach would be effective in mammals other than hamsters, $INGAP^{104-118}$ was administered to normoglycaemic mice, dogs and monkeys, hyperglycaemic mice, and to human pancreatic tissue cultures.

$INGAP^{104-118}$ administration led to a dose-dependent increase of β-cell mass in mice, with similar trends observed in dogs. Similarly, administration of $INGAP^{104-118}$ to normoglycaemic monkeys for 90 days resulted in profound areas of islet neogenesis. Administration of $INGAP^{104-118}$ to diabetic mice resulted in restoration of euglycaemia and a dramatic increase in β-cell mass. Furthermore, $INGAP^{104-118}$ administration to cultured human acinar tissue, led to the formation of insulin-producing islet-like structures. These results suggest that $INGAP^{104-118}$ therapy has the ability to reverse a diabetic state and could be effective in humans. However, it was necessary to

determine whether the continual stimulation of islet neogenesis through INGAP$^{104-118}$ administration is a safe therapeutic approach.

The β-cell mass dynamics of euglycaemic mice administered INGAP$^{104-118}$ at various doses for 31 or 90 days were determined. β-cell mass was greatly increased at 31 days of therapy, though by 90 days of therapy there was no difference in total β-cell mass between all treatment groups. However, there were marked instances of islet neogenesis in mice treated with INGAP$^{104-118}$ for 90 days. This elevation in islet neogenesis was tempered by decreased β-cell replication and increased β-cell apoptosis, resulting in no overall difference in total β-cell mass. These results suggest that inherent homeostatic regulation persisted to maintain a net β-cell mass that matched the physiological need, even in the setting of continual induction of islet neogenesis.

INGAP$^{104-118}$ therapy has been shown to expand the insulin-producing β-cell mass in a safe homeostatic manner and reverse diabetic hyperglycaemia. These findings suggest that a novel pharmacological agent for the successful stimulation of β-cell mass expansion is within reach, enabling new therapeutic modalities for the treatment of diabetes.

RÉSUMÉ

Les thérapies actuelles ciblant le diabète mellitus sont insuffisantes pour éviter les complications associées à cette maladie. Une nouvelle approche de traitement du diabète serait de stimuler les cellules progénitrices endogènes des cellules b qui produisent l'insuline. Ainsi, le but de cette thèse a été de déterminer si l'initiation par un agent pharmacologique de la néogénèse des îlots de Langerhans, et l'expansion des cellules β qui en découle, peut mener à la suppression de l'hyperglycémie par un effet sous contrôle homéostatique et efficace chez l'humain.

Un fragment d'INGAP (Protéine Associée à la Néogénèse des Ilots) de 15 acides aminés: ($INGAP^{104-118}$) a été administré à des hamsters normoglycémiques et a eu pour effet l'augmentation de la masse de cellules β. Il a été montré que cette expansion se produit par transformation des progéniteurs associés aux canaux et acini. Dans le but de déterminer si l'approche thérapeutique serait efficace chez d'autres mammifères que les hamsters, $INGAP^{104-118}$ a été administré à des souris, des chiens et des singes normoglycémiques, à des souris hyperglycémiques ainsi qu'à des cultures de tissus pancréatiques humains.

L'administration d'$INGAP^{104-118}$ augmente de façon dose-dépendante la masse de cellule β chez les souris. La même tendance a également été observée chez les chiens. De même, l'administration pendant 90 jours d'$INGAP^{104-118}$ aux singes normoglycémiques stimule la néogénèse des îlots. Chez les souris diabétiques, l'administration d'$INGAP^{104-118}$ permet de restaurer l'euglycémie et augmente de façon importante la masse de cellules β. Il a également été observé que les cultures de tissus acinaires humains, traitées avec $INGAP^{104-118}$ forment

des strucutures similaires aux îlots et qui produisent de l'insuline. Ces résultats suggèrent que l'administration d'INGAP$^{104-118}$ a la capacité de supprimer un état diabétique et pourrait être efficace chez l'homme. Cependant, il a été nécessaire de déterminer si la stimulation en continue de la néogénèse des îlots par INGAP$^{104-118}$ est une approche thérapeutique sans danger.

La dynamique de la masse de cellules β a été déterminée chez des souris euglycémiques auxquelles INGAP$^{104-118}$ a été administré à des doses diverses pendant 31 ou 90 jours. La masse de cellules β est considérablement augmentée à 31 jours de traitement. Cependant, aucune différence concernant la masse globale de cellules β ne subsiste entre les différents groupes après 90 jours de traitement. Mais, chez les souris traitées avec INGAP$^{104-118}$, des îlots néoformés ont pu être observés. Cette augmentation de formation de nouveaux îlots est atténuée par une diminution de la réplication des cellules β et par une augmentation de l'apoptose de ces cellules. Ceci explique pourquoi aucune différence dans la masse globale n'a pu etre observée. Ces résultats suggèrent qu'un contrôle homéostatique persiste pour maintenir une masse de cellules β correspondant aux besoins physiologiques, et ceci malgré la stimulation en continue de la néogénèse des îlots.

Ainsi, il a été démontré que l'administration d'INGAP$^{104-118}$ permet d'augmenter la masse de cellules β sans danger et de supprimer l'hyperglycémie diabétique. Ces résultats suggèrent que la réalisation d'un nouvel agent pharmacologique stimulant la croissance de la masse de cellules β est sur le point d'être atteint, permettant ainsi de nouvelles possibilités de traitement du diabète.

To Mum, Dad and Mike,

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LIST OF FIGURES

ABBREVIATIONS

AEC	aminoethyl carbazole
AP	alkaline phosphatase
ARIP	rat pancreatic duct cell line
ATP	adenosine triphosphate
bHLH	basic helix-loop-helix
BrdU	5-bromo-2' deoxyuridine
cAMP	cyclic adenosine monophosphate
CT	cholera toxin
DAB	3,3'-diaminobenzidine tetrahydrochoride
DLS	duct-like structure
DMEM	dulbecco's modified eagle medium
ECM	extracellular matrix
EGF	epidermal growth factor
FBS	fetal bovine serum
GADA	glutamic acid decarboxylase antibody
GIP	glucose-dependent insulinotrophic polypeptide
GLP-1	glucagon-like peptide-1
^{3}H-TdR	tritiated thymidine
HGF	hepatocyte growth factor
HIT-15	human islet tumour cell line
HRP	horseradish peroxidase
IAA	insulin auto-antibody
ICA	islet cell cytoplasm antibody
IAPP	islet amyloid polypeptide
IFNγ	interferon-gamma
INGAP	islet neogenesis associated protein
ILS	islet-like structure
ISL-1	islet-1
LADA	latent autoimmune-diabetes of the adult
NEUROD1	neurogenic differentiation factor
NGN-3	neurogenin-3
Nkx	NK homeobox gene
NOD	non-obese diabetic
PAX	paired-box factor
PCNA	proliferating cell nuclear antigen
PDX-1	pancreatic duodenal homeobox gene-1
REG	regenerating protein
RNA	ribonucleic acid
SCID	severe-combined immuno-deficiency
SNAP	synaptosomal-associated protein
SNARE	soluble N-ethyl maleimide-sensitive fusion protein attachment receptor
TGF	transforming growth factor
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

GUIDELINES FOR THESIS PREPARATION

Manuscript-based thesis

"As an alternative to the traditional thesis format, the dissertation can consist of a collection of paper of which the student is an author or co-author. These papers must have a cohesive, unitary character making them a report of a single program of research. The structure for the manuscript-based thesis must conform to the following: 1. Candidates have the option of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, of the clearly-duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis. (Reprints of published papers can be included in the appendices at the end of the thesis.) 2. The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges preceding and following each manuscript are mandatory. 3. The thesis must conform to all other requirements of the "Guidelines for Thesis Preparation" in addition to the manuscripts. The thesis must include the following: a) a table of contents; b) a brief abstract in both English and French; c) an introduction which clearly states the rational and objective of the research; d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper); e) a final conclusion and summary; f) a thorough bibliography; g) appendix containing an ethics certificate in the case of research involving human or animal subjects, microorganisms, living cells, other biohazards and/or radioactive material."

CONTRIBUTIONS OF AUTHORS

The doctoral candidate performed all human tissue collection and experimental procedures. The candidate performed administration of scrambled INGAP$^{104-118}$ and exendin-4 to diabetic mice (Chapter 2), though others performed all additional animal manipulations. Collection of all data and subsequent analyses were performed by the author with the exception of: islet density, total β-cell mass, blood insulin and total pancreatic insulin content in euglycaemic hamsters (Rennian Wang, Ann Surg. 2004, depicted in Figures 2.2 and 2.3), blood glucose determination of diabetic mice (Ji-Wong Yoon, Ann Surg, 2004, depicted in Figure 2.6), islet density and average islet size in partial duct obstructed hamsters (L Rosenberg, Diabetologia 2006, depicted in Figure 3.2A and B). Rennian Wang generated photomicrographs depicted in Figure 2.1, and Ji-Wong Yoon generated photomicrographs depicted in Figures 2.7 and 2.9. Reid Aikin performed the experiment in Figure 5.2, and Nikolay Tchervenivanov performed the experiment depicted in Figure 5.3. All other co-authors contributed in manuscript preparation and technical expertise in pancreatic digestions and animal manipulations.

PREFACE

The aim of this thesis was to determine the potential of pharmacological induction of β-cell mass expansion to lead to restoration of euglycaemia in individuals with diabetes mellitus. This thesis is composed of 6 chapters including the first in which a general introduction to diabetes, current therapies, and potential new therapeutic avenues are discussed. Chapter 2 examines the potential of INGAP peptide ($INGAP^{104-118}$) to lead to β-cell mass expansion in both normoglycaemic hamsters and mice. As well, the ability of $INGAP^{104-118}$ to induce β-cell neogenesis and reverse hyperglycaemia in a mouse model of type 1 diabetes is assessed. This work is published in *Annals of Surgery* (240: 875-884, 2004). In Chapter 3, evidence for the homeostatic regulation of induced β-cell mass expansion is analysed resulting in the determination that unbridled cellular expansion does not occur in the setting of continual stimulation of islet neogenesis. This work is published in *Diabetologia* (49: 2910-2919, 2006). Chapter 4 explores the potential of $INGAP^{104-118}$ therapy to expand human β-cell mass in a novel model of cellular differentiation. This work is published in *Pancreas* (in press). Chapter 5 examines the current state of therapy for type 1 diabetes as well as future avenues for the successful amelioration of the disease. This work has been published in *International Journal of Biochemistry and Cell Biology* (38: 498-503, 2006). Chapter 6 contains the discussion of results borne from this thesis and how the findings herein expand our understanding of potential therapies for diabetes mellitus.

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CHAPTER 1

INTRODUCTION

1.1 DIABETES MELLITUS

Diabetes mellitus is a metabolic disease in which an insufficient secretion of the pancreatic hormone insulin leads to chronically elevated blood glucose levels (hyperglycaemia) [1]. Diabetes afflicts approximately 3% of the global population with the disease being most prominent in industrialized countries where the national averages reach approximately 8% [2, 3]. Chronic hyperglycaemia associated with diabetes results in damage to many organ systems including the kidneys, eyes, heart, blood vessels and nervous system [4]. Diabetes is the single most common cause for end-stage renal disease with 20 to 30% of patients with diabetes developing evidence of nephropathy [5]. Similarly, more than 60% of individuals with diabetes develop retinopathy during their lifetime [5]. The World Health Organization has recently described diabetes as one of the most devastating global threats to human health, attributing an estimated 3 million deaths per year to this disease [6].

There are two major types of diabetes mellitus: type 1 (formerly referred to as juvenile onset, and accounting for approximately 10% of all cases of diabetes mellitus) and type 2 (adult onset) diabetes. The aetiology of type 1 diabetes has yet to be fully understood, though it appears to involve coordinated interactions of both genetic predisposition and environmental stimuli. Type 1 diabetes results from the autoimmune destruction of the insulin-producing pancreatic β-cells that are located in the islets of Langerhans [7, 8]. Clinically observable type 1 diabetes typically occurs when the normal β-cell mass has decreased by approximately 80% [7]. Virtually all endogenous insulin production is eliminated in individuals

with type 1 diabetes, and as such, these patients must administer exogenous insulin to regulate blood glucose levels.

Type 2 diabetes is characterized by both insufficient insulin secretion and resistance to the action of insulin [1]. Insufficient insulin secretion in type 2 diabetes may be due to reduced β-cell mass and/or impaired β-cell function [7, 9-11]. Overt type 2 diabetes is associated with a 50 to 60% lower β-cell mass as compared to weight matched non-diabetic control subjects [7, 9, 12]. Inappropriate diet and exercise levels have been associated with type 2 diabetes as excessive adipogenesis and lack of sufficient exercise can result in increased insulin resistance [7]. Moreover, elevated blood lipid and glucose levels are believed to stimulate β-cell death through a synergistic mechanism [13]. Decreased peripheral insulin sensitivity leads to increased β-cell demand. Under stress, insulin processing in β-cells is disrupted leading to increased release of the less efficacious pro-insulin, in place of fully-processed insulin [14]. As well, increased stimulation of insulin release from the pancreatic β-cells can result in elevated release of islet amyloid polypeptide (IAPP) [12, 15]. IAPP has fibrilogenic properties that can lead to plaque formation and result in β-cell death. IAPP plaques have been associated with up to 90% of all cases of type 2 diabetes [16, 17].

Both type 1 and type 2 diabetes results from an insufficient level of circulating insulin to meet the body's needs. Even though their aetiologies may be different, the respective reduction in β-cell mass and the lack of an appropriate regenerative response can be seen as the ultimate foundation of this disease.

1.2 ADULT PANCREATIC ANATOMY

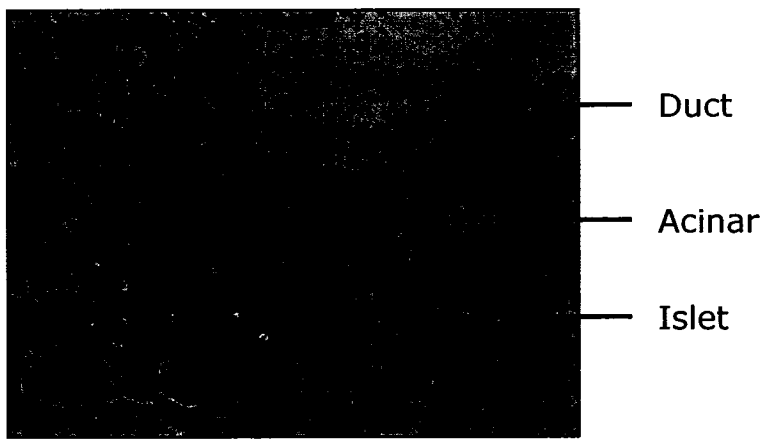
The adult human pancreas is a multilobular organ situated in the retroperitoneal space. It can be divided roughly into three portions; namely the head, body and tail. The head of the pancreas connects to the proximal duodenum via the ampula of Vater, whereas the tail is juxtaposed to the spleen. The pancreas can vary in mass between 50 and 170 grams depending on an individual's size. The organ is comprised of three major cell phenotypes: the endocrine, acinar, and duct cells (Figure 1.1).

1.2.1 The Exocrine Tissue

The acinar and ductal components comprise the exocrine portion of the pancreas and account for more than 95% of the total organ mass. Pancreatic acini, which accounts for approximately 80% of the parenchymal tissue, produce digestive zymogens that are secreted into the ductal network, and subsequently transported to the digestive tract [18]. Centroacinar cells connect the acinar cells to the ductal network. These cells produce large amounts of bicarbonate and other non-digestive components of the pancreatic juice [19, 20]. The remaining 15 to 20% of the exocrine pancreas consists of the ductal network. Pancreatic ducts vary in size from small, intercalated ducts, to the largest main pancreatic duct [21, 22]. Similar to centroacinar cells, duct cells produce bicarbonate and other non-digestive elements of the pancreatic juice.

The ductal components of the exocrine pancreas have long been thought to harbour the regenerative potential of the organ. In support of this notion, Pour et al. have indicated that centroacinar cells can develop into both endocrine and

Figure 1.1: Pancreatic morphology. A photomicrograph of a pancreatic section in which there is a pancreatic duct, acinar tissue (blue cell clusters) and an insulin stained islet of Langerhans (brown). (bar = 25 μm)


Duct
Acinar
Islet

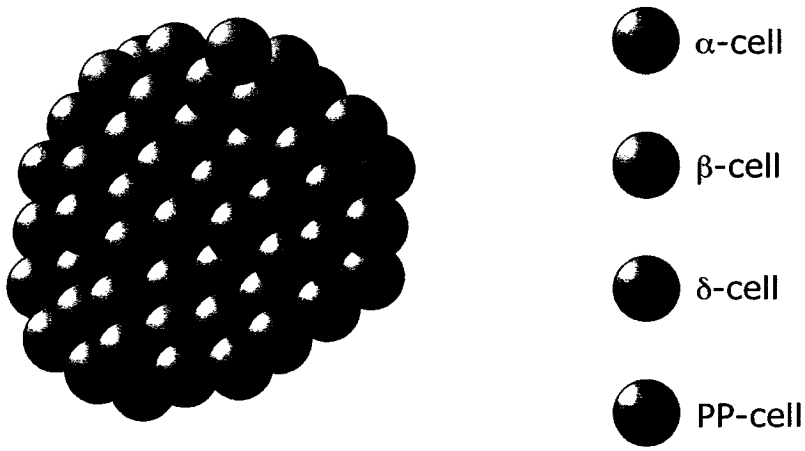
exocrine tissues during pancreatic development [23]. Furthermore, centroacinar cells have elevated expression of the stem cell regulatory factor notch, which is a key component in maintaining progenitor cells in an undifferentiated state during pancreatic ontogeny [19, 20]. As such, these cells are thought to contain regenerative capabilities and may be implicated in the development of pancreatic adenocarcinoma [20, 23-25]. The role of the ductal epithelium in pancreatic regeneration will be described in more detail later.

1.2.2 The Endocrine Tissue

Endocrine cells, which comprise only 1-3% of the pancreas, are generally organized into clusters referred to as islets of Langerhans [26]. An average islet has a diameter of approximately 150 μm and consists of approximately 4000 cells. Four distinct endocrine cells may be contained within an islet including: insulin-producing β-cells (approximately 80% of all endocrine cells), glucagon-producing α-cells (approximately 10%), somatostatin-producing δ-cells (approximately 5%) and pancreatic polypeptide-producing PP-cells (approximately 5%) (Figure 1.2) [26, 27].

Islets are extremely well vascularized, receiving approximately 20% of the total organ blood flow to facilitate their endocrine function [26, 28]. Arterial blood initially flows to the β-cell core and is then directed to the α- and δ-cells, which constitutes the basis for coordinated hormonal release [29-32]. In addition to having intricate circulatory pathways, islets are also heavily innervated.

Figure 1.2: Pancreatic islet. A representative islet of Langerhans indicating the organized cellular structure of a core of insulin-producing β-cells surrounded by a mantle of glucagon-producing α-cells, somatostatin-producing δ-cells and pancreatic polypeptide-producing PP-cells.


α-cell
β-cell
δ-cell
PP-cell

The cellular distribution of pancreatic islets is highly organized in lower mammals with a defined central core of β-cells surrounded by a mantle of the other three endocrine cell types. This organization is less defined in primates and humans [33]. In addition to residing within islets, some endocrine cells are found as single cells or small clusters of cells distributed throughout the acinar tissue or in association with duct structures [34-37].

Insulin and glucagon are the principal endocrine hormones involved in regulating blood glycaemia. Insulin reduces blood glucose levels by stimulating glucose uptake from the blood into target tissues. Insulin binding to its receptor initiates a signalling cascade that ultimately leads to the translocation of intracellular vesicles, containing glucose transporter-4 (GLUT-4), to the cell surface [38]. Glucose then moves down its concentration gradient and into the cells via GLUT-4. Once inside the cell, glucose has one of two possible fates. It can be metabolized through glycolysis and the citric acid cycle, or it can be stored as either glycogen or lipids via glycogen synthase and various lipid synthase enzymes, respectively [39].

The β-cell has a remarkably elegant regulatory system that rapidly reacts to fluctuating blood glucose levels to maintain appropriate levels of circulating insulin. This system is initiated by the introduction of glucose into β-cells via GLUT-2 in rodents and GLUT-1 in humans [40, 41]. Once glucose is inside the β-cell, it is metabolized through the citric acid cycle leading to an increased intracellular adenosine triphosphate (ATP) concentration. The elevation of intracellular ATP causes ATP-sensitive potassium channels to close, resulting in

the depolarization of the cell [42]. Cellular depolarization opens voltage-sensitive calcium channels and the intracellular calcium concentration increases [43, 44]. Elevation of the intracellular calcium concentration activates the vesicle docking proteins, SNAREs, SNAPs and calpains, which leads to the exocytosis of insulin-laden secretory granules [45].

The main product of pancreatic α-cells is glucagon [46]. Glucagon is produced through the processing of pre-pro-glucagon, which can also be cleaved to form glicentin, oxyntomodulin, glucagon-like peptide-1 (GLP-1) or GLP-2 [46, 47]. Within pancreatic α-cells essentially all pre-pro-glucagon is cleaved to form glucagon. Glucagon release is promoted by low circulating blood glucose levels and is suppressed by insulin and somatostatin [48]. The main action of glucagon is to elevate blood glucose levels by stimulating the breakdown of glycogen into free glucose molecules, as well as stimulating gluconeogenesis by the liver.

Somatostatin is the main product of pancreatic δ-cells [49]. Similar to its inhibitory effects in the nervous and gastrointestinal systems, pancreatic somatostatin suppresses both insulin and glucagon release from β- and α-cells, respectively. While gastric tissue secretes large amounts of somatostatin, it is thought that the small amount of somatostatin produced and secreted by pancreatic δ-cells only affects pancreatic endocrine and exocrine function [49].

The PP-cells are responsible for producing pancreatic polypeptide. Very little is known about the function of pancreatic polypeptide though it has been suggested to act as an inhibitor of gastric emptying [50].

1.2.3 The Integrated Organ

Historically, the pancreas has been described as containing two separate organs; the enzyme producing exocrine portion and the metabolic endocrine portion [51, 52]. Histological analyses, as well as in-depth expression assays, have indicated that there is a very structured organization of the pancreas with numerous interactions between the three main cell types. The pancreatic islets are not randomly distributed throughout the organ but are more likely to be near, or juxtaposed to, pancreatic ducts [52]. Furthermore, there are clear distinctions between acini that are in close proximity to islets versus those that are not [53-56]. Peri-insular acini, or acini situated near islets, are larger, contain greater numbers of zymogen granules, and express higher levels of digestive enzyme mRNA than tele-insular acini [55, 57, 58]. The intricate cellular organization of the pancreas suggests that rather than existing as two separate organs, the pancreas functions as a singular intercalated organ with each cell type interacting together to ensure an appropriate balance of exocrine and endocrine function [51, 52].

1.3 CURRENT THERAPEUTIC OPTIONS FOR DIABETES MELLITUS

1.3.1 Discovery of Insulin

A diagnosis of diabetes mellitus was a virtual death sentence prior to the identification and isolation of insulin, credited to Banting, Best, Collip and Macleod in 1922 [59]. Banting and Best initially administered their purified insulin to pancreatectomized dogs resulting in the amelioration of hyperglycaemia, and soon after, recapitulated these results in type 1 diabetics. Of note, Constantin Paulescu used a pancreatic extract to reverse hyperglycaemia in dogs in 1916,

then reconfirmed this and published the results in 1921 [60]. Unfortunately, he was not able to purify this extract sufficiently for human therapy prior to Banting, Best, Collip and Macleod [61]. Once insulin was isolated and administered to diabetic patients, the immediate results were so overwhelmingly positive that it was believed a cure was in hand.

1.3.2 Insulin Augmentation

In addition to insulin administration, conventional therapies for diabetes mellitus include diet modification, exercise and medications that either increase insulin release (sulphonylureas) or enhance insulin sensitivity (thiazoladinediones and biguanides). However, these therapies are not sufficient to prevent the complications associated with diabetes [62, 63]. Two major studies, the Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) demonstrated that intensive diabetes management can slow the progression of diabetic complications, but not affect overall mortality [62, 63]. Intensive insulin therapy performed in the DCCT resulted in a 50% reduced risk of retinopathy and a 69% decrease risk of neuropathy in individuals with type 1 diabetes [62]. In the UKPDS intensive treatment led to a 25% risk reduction in microvascular endpoints for individuals with type 2 diabetes, although there was no significant reduction in diabetes-related mortality [63].

While intensive treatment can reduce the risk of developing complications, it also increases the frequency of hypoglycemic episodes. In both the DCCT and UKPDS, intensive therapies were associated with a 2- to 3-fold increase in the frequency of severe hypoglycemic episodes requiring third party assistance [62,

63]. A potentially superior alternative to insulin augmentation therapy is the restoration of a glucose-sensing, insulin-producing β-cell mass.

1.3.2 Surgical Manipulations

Currently, surgical manipulations are the only successful means for increasing β-cell mass in diabetic individuals [64]. This can be achieved through either whole organ pancreas transplantation or isolated islet transplantation. Furthermore, a surgical duct ligation therapy performed by de Takats in the late 1920s and early 1930s, appears to have resulted in at least partial restoration of a glucose-sensing insulin-producing β-cell mass [65-68].

1.3.2.1 Pancreatic Ligation

Shortly after the discovery and isolation of insulin, de Takats was performing an experimental surgical procedure on patients with type 1 diabetes. His pancreatic ligation procedure resulted in an apparent increase in pancreatic endocrine mass and led to dramatic weight gains and glycaemic tolerance (an indirect way of measuring blood glucose at the time) [66-68]. Unfortunately, the benefits of this surgical manipulation were short-lived and most patients reverted to pre-surgery conditions in approximately 18 months.

1.3.2.2 Pancreas Transplantation

Currently, pancreas transplantation is the only effective means of achieving long-term stringent glycaemic control in diabetic individuals [64]. This therapy has

even been shown to lead to the reversal of pre-existing diabetic complications [64]. Unfortunately, an absolute requirement of this therapy is continual immunosuppression, which can lead to a multitude of secondary side effects. Furthermore, the paucity of organ donors and the invasiveness of the procedure prevent this therapy from reaching a significant number of individuals with diabetes.

1.3.2.3 Islet transplantation

Islet transplantation was pioneered to address the problems associated with whole organ transplantation. It was hypothesized that a pancreas contains more than enough islets to maintain normoglycaemia following the observations that patients who underwent partial pancreatectomy were still euglycaemic and that clinically observed type 1 diabetes occurred only when greater than 50% of islets were destroyed [7]. Therefore, multiple recipients could benefit from the pancreatic islets isolated from a single organ instead of treating only one patient as occurs with whole organ transplant. Furthermore, the suggestion that the exocrine portion of the pancreas was responsible for most of the immune response following whole organ transplantation, led to the initial belief that immunosuppression may not be required or could be drastically reduced, in the setting of isolated islet transplantation.

The initial results of the Edmonton Protocol released in 2000, suggested that islet transplantation could be a viable therapeutic alternative for diabetic individuals [69, 70]. Unfortunately, however, these procedures proved to be more beneficial in theory rather than in practice. In almost all cases, multiple pancreatic

donors and transplantation procedures were required to achieve insulin-independence and patients still required chronic immunosuppression. The procedure, while less invasive than whole organ transplantation, still results in significant physical damage [71, 72]. Furthermore, the liver is a suboptimal transplantation site that exposes islets to very high concentrations of immunosuppressive agents, which are known to be β-cell cytotoxic [73]. The most significant shortcoming of islet transplantation is that the average patient requires exogenous insulin administration after about 15 months post-transplant, versus approximately 10 years for whole organ transplant [64, 72].

Clearly new treatment paradigms are needed to achieve normoglycaemia and prevent complications associated with diabetes mellitus. Restoration of a glucose-responsive islet cell mass through islet and whole pancreas transplantation has allowed people with diabetes to achieve normoglycaemia without an increased frequency of hypoglycaemia [74]. However, lack of adequate transplantable tissue, graft rejection, surgical invasiveness and disease recurrence prevent pancreas and islet transplantation from being a viable therapy for the majority of people with diabetes [72, 74, 75]. A potential therapeutic alternative that may address the insufficiencies inherent to transplantation therapy is the stimulation of endogenous β-cell mass expansion.

1.4 β-CELL MASS DYNAMICS

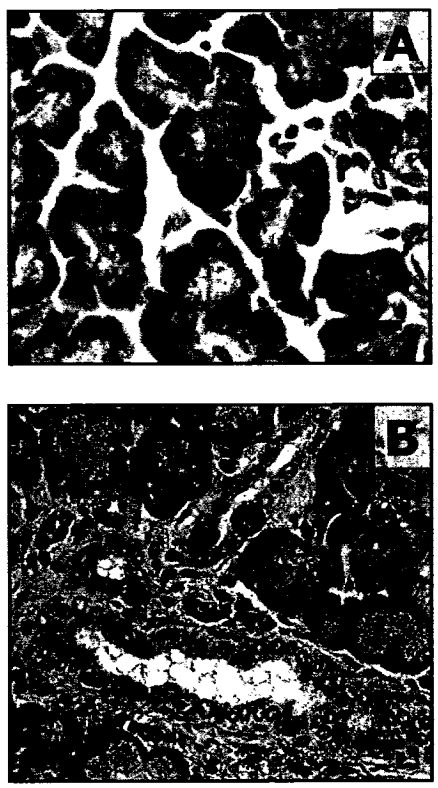
Like virtually all tissues of the body the β-cell mass is dynamic [76-79]. The number of β-cells can be increased by increasing the rate of new β-cell

formation and by decreasing the rate of β-cell loss [79]. New β-cell formation can occur through replication of pre-existing β-cells (hyperplasia) as well as through the formation of new β-cells from non-β-cells (neogenesis). β-cell mass can also be expanded through increased individual β-cell size (hypertrophy). Alternatively, a reduction in β-cell hyperplasia, neogenesis, cellular volume (atrophy) and an increase in β-cell death can lead to a reduction in the number of β-cells and a smaller β-cell mass. In addition, β-cell mass may be reduced by a loss of β-cell phenotypic stability [80, 81].

1.5 DEFINITION OF ISLET NEOGENESIS

Islet neogenesis, or more specifically β-cell neogenesis is the formation of a new insulin-producing β-cell from a non-insulin-producing progenitor. Islet neogenesis occurs when newly formed β-cells cluster together and join with other newly formed endocrine cells. Until very recently it has been impossible to perform *in vivo* cellular lineage tracing experiments to unequivocally determine the mechanism of new β-cell and islet formation within the pancreas [82]. In the past, a histological categorization was developed to identify β-cells thought to be newly forming from non-insulin-producing progenitors [34, 37, 83-88]. β-cells were categorized as being neogenic if they both contained insulin and were situated outside of a fully-formed islet of Langerhans. These extra-islet β-cells are usually adjacent to pancreatic duct cells or located within a pancreatic acinus (Figure 1.3) [37, 85-87, 89, 90]. It is theoretically possible, however unlikely, that these extra-islet β-cells initially formed within a mature islet and subsequently migrated to an

Figure 1.3: Histological representations of islet neogenesis. **(A)** A photomicrograph of a pancreatic section stained for insulin (brown) in which there is an extra-islet acinar-associated β-cell, and **(B)** extra-islet duct-associated β-cells. (bar = 10 μm)


A
B

extra-islet location. Nevertheless, here the term β-cell neogenesis will refer to the formation of these extra-islet β-cells. Advancements in the study of pancreatic ontogeny has facilitated the understanding of the histological classification system and provided insight to the critical elements of new β-cell formation.

1.6 PANCREATIC ONTOGENY

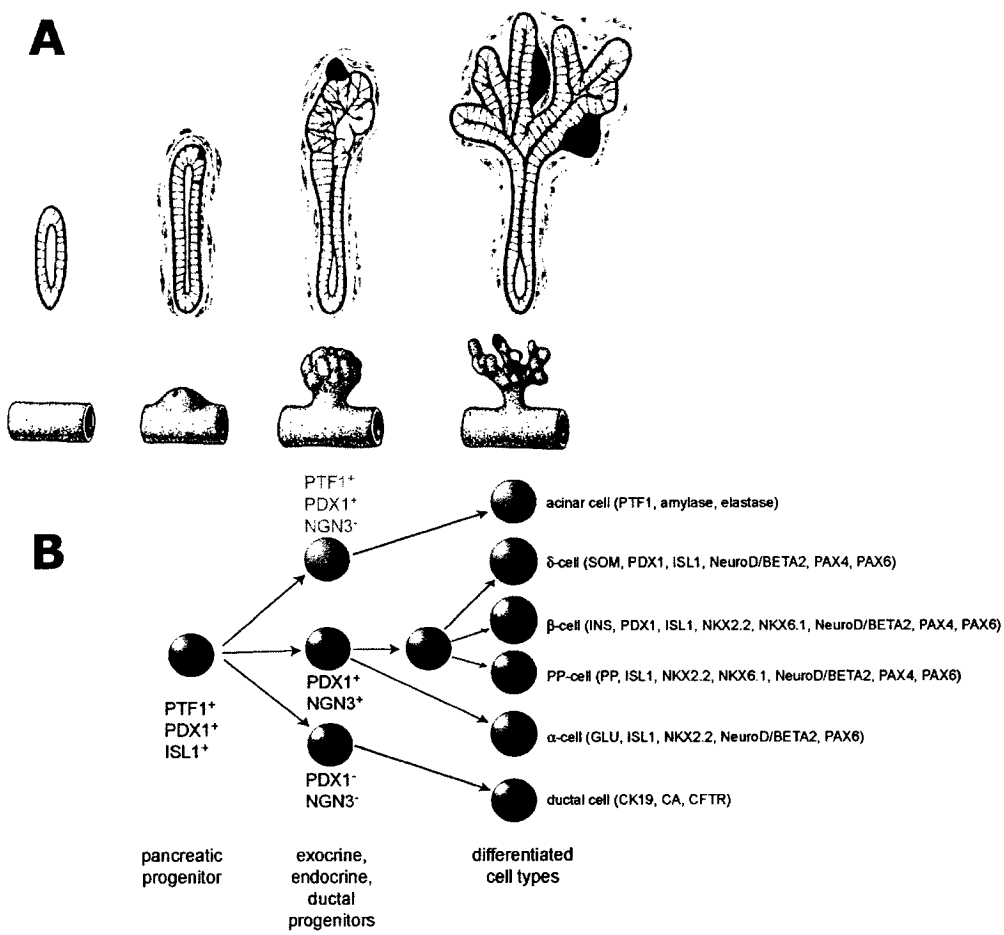
During pancreatic ontogeny, β-cell mass dramatically increases through the mechanisms of cell dynamics described above [88, 91-94]. Critical analyses of pancreatic ontogeny have identified many cellular factors that may be involved in progenitor differentiation and subsequent β-cell mass expansion.

As described previously, the human pancreas is made up of endocrine, acinar, and duct cell types. Pancreatic organogenesis begins with dorsal and ventral protrusions of a region of the primitive gut epithelium (Figure 1.4) [91, 95, 96]. Subsequently, the upper foregut endoderm gives rise to the distinct pancreatic cell types [94, 97, 98]. Early steps controlling the commitment of the gut epithelium towards a pancreatic fate, as well as the mechanisms underlying the development of the different pancreatic cell types, however, are not well understood.

Several homeodomain and basic helix-loop-helix (bHLH) transcription factors, notably Isl1, Nkx2.2, Pax4, Pax6 and NeuroD/BETA2, have been shown to play an important role in the process of pancreatic endocrine cell differentiation [94, 97, 98]. These factors are expressed at early stages of pancreas development, but in their absence, the initial steps of pancreatic development

Figure 1.4: Pancreatic ontogeny. **(A)** Illustration of the budding of primordial gut endothelium and tissue maturation during pancreatic organogenesis. **(B)** Key factors expressed during this morphological transformation.

(A) morphological illustration excerpted from, Pictet RL & Rutter K *Handbook of Physiology*, 1972.


A
B
PTF1+
PDX1+
NGN3-
PDX1+
NGN3+
PDX1-
NGN3-
PTF1+
PDX1+
ISL1+
acinar cell (PTF1, amylase, elastase)
δ-cell (SOM, PDX1, ISL1, NeuroD/BETA2, PAX4, PAX6)
β-cell (INS, PDX1, ISL1, NKX2.2, NKX6.1, NeuroD/BETA2, PAX4, PAX6)
PP-cell (PP, ISL1, NKX2.2, NKX6.1, NeuroD/BETA2, PAX4, PAX6)
α-cell (GLU, ISL1, NKX2.2, NeuroD/BETA2, PAX6)
ductal cell (CK19, CA, CFTR)
pancreatic progenitor
exocrine, endocrine, ductal progenitors
differentiated cell types

proceed normally resulting with later disruption of pancreatic endocrine cell differentiation [94, 97, 98]. In contrast to the above mentioned factors, transcription factor p48, which is required for the generation of exocrine but not endocrine cells [99], and the homeodomain protein PDX-1, are required at an earlier stage in pancreatic development [100-103]. Later stages of pancreatic development, particularly the branching morphogenesis and exocrine differentiation depend on other factors, including Isl1 and NeuroD/BETA2 [104].

While both Isl1 and NeuroD/BETA2 are expressed in all differentiated pancreatic endocrine cells as they develop, neither gene seems to function as a *bona fide* pro-endocrine gene [97], a role that may possibly be attributed to the gene neurogenin-3 (NGN-3) [105]. Evidence suggests that notch signalling controls the choice between differentiated endocrine and exocrine cell fates during development by inhibiting NGN-3 [106-109]. High NGN-3 levels occur when notch signalling is inhibited resulting in endocrine differentiation. As differentiated endocrine cells appear, they separate from the duct-like epithelium and migrate into the adjacent mesenchyme where they cluster to form new islets. Progenitor cells that are not singled out to become endocrine cells will subsequently differentiate into either acinar cells or mature ductal cells.

Analyses of pancreatic ontogeny have led to greater understanding of islet neogenesis and β-cell mass expansion during embryology, but these mechanisms may differ in the adult pancreas. Research into the pathways and animal models of pancreatic regeneration has shed light on the potential mechanisms leading to β-cell mass expansion in the adult.

1.7 PATHWAYS OF PANCREATIC REGENERATION

As previously described, a possible alternative to islet and pancreas transplantation is the induction of new islet cell growth from endogenous progenitors. There are three potential pathways for endogenous β-cell expansion. These pathways are: the proliferation of pre-existing β-cells, the activation and differentiation of stem cells, and the nuclear reprogramming of fully differentiated cells into new cell phenotypes (transdifferentiation).

1.7.1 Replication of Pre-Existing β-cells

Adult human pancreatic β-cells generally do not replicate at a high rate, thereby limiting the usefulness of β-cell hyperplasia as a means of endogenous β-cell mass expansion. Both *in vivo* and *in vitro* studies of β-cell replication have shown that very few β-cells are proliferating at a given point in time [12, 37, 110, 111]. Furthermore, stimulating β-cell hyperplasia *in vitro* results not only in moderate β-cell proliferation but also in decreased insulin content and endocrine function [110]. The balance between endocrine function and proliferative capacity has also been observed *in vivo* in animal models of β-cell stress [89, 112, 113].

In 2004, Dor et al. hypothesized that endogenous pancreatic β-cell regeneration in adults occurred only through β-cell hyperplasia [82]. Unfortunately, the experiments that supported this hypothesis were performed in mice, which have been shown to have a much higher β-cell proliferative potential than humans [11, 78, 114]. Other researchers tried to confirm Dor et al.'s initial findings, but they could not, indicating that alternative mechanisms of β-cell

expansion may contribute to regeneration in this model [115]. Thus, while β-cell replication can lead to an increase in β-cell number in rodents, it appears that this mechanism of expansion may not be the most effective means of endogenous β-cell mass expansion in humans.

1.7.2 Stem Cell Activation and Differentiation

Stem cells are defined as cells that are capable of undergoing self-renewal and multi-lineage differentiation [116]. Stem cells can be classified according to their regenerative potential as being totipotent, pluripotent, multipotent or unipotent. A totipotent stem cell can give rise to a complete offspring including the placental components, whereas a pluripotent stem cell has the capability to form all of the organs of the offspring only. Multipotent stem cells can form more than one cell lineage, however the different possible lineages are usually closely related [43, 117]. Multipotent stem cells have also been categorized as tissue-specific stem cells (TSC).

There is a lot of controversy regarding the existence of pancreatic stem cells [25, 82, 118, 119]. Pancreatic stem cells could be utilized towards regenerating endogenous β-cell mass, though positively identifying them remains a problem. Currently, the most effective means of identifying cell types is through immunohistochemical analysis. Given that stem cells are dynamic cells that change their phenotype as they differentiate, it is difficult to identify pancreatic stem cells *in vivo* based solely on immunohistochemical techniques. Clonal

analyses of rodent pancreatic extracts have indicated that multipotent stem cells reside within the pancreas, though their exact location is unknown [118].

Another strategy for the categorization of progenitors cells is to describe cells in regards to their degree of regenerative-competence. It has become increasingly apparent that cellular differentiation can be stimulated through the orchestration of specific growth factors and extracellular matrix [120]. Depending on the prevailing cellular environment, all cells possess various degrees of regenerative competence [120-124]. By extension, the differentiation potential of cells can drastically change depending on these specific microenvironments, and this can pose problems in experimental reproducibility. If the proper microenvironment can be arranged, it may allow for a mature and terminally differentiated cell to be transformed into a different cell phenotype.

1.7.3 Transdifferentiation

Recent studies have indicated that fully differentiated cells contain a degree of phenotype plasticity [125-127]. Stimulation of mature cells can lead to phenotypic transformation in multiple settings [115, 126-129]. This terminal cell transdifferentiation can occur through two separate pathways [126, 127, 130]. Direct transdifferentiation occurs when a cell transforms into a phenotypically different cell without passage through an intermediate non-specific cell phenotype and without the requirement of replication [80, 127]. The second, indirect pathway, consists of transdifferentiation through a process of cellular dedifferentiation to a more primordial cell phenotype, followed by proliferation, and subsequent redifferentiation into a new mature cellular phenotype [25, 75, 89,

126]. Even though indirect transdifferentiation is more predominant during development than direct transdifferentiation, both of these mechanisms may be active in the adult pancreas and capable of leading to endogenous β-cell expansion [25, 80, 127]. Since transdifferentiation can involve the transformation of mature fully differentiated cells, any of the non-β-cell phenotypes of the adult pancreas can be viewed as a potential source for endogenous β-cell expansion.

1.7.3.1 Duct Transdifferentiation

Several lines of evidence suggest that duct cells are the progenitor cells that transdifferentiate into both endocrine and exocrine pancreatic tissue during fetal development [88, 92, 131, 132]. This assumption is based on morphological observations as well as duct cell replication analyses. Duct cell replication indices are high relative to other tissue types during early fetal development, suggesting that transdifferentiation of duct cells contributes to endocrine and exocrine growth [88, 92]. Further support for the transdifferentiation of duct cells comes from observations of cells with mixed phenotypes during fetal and neonatal development [88, 92]. Early endocrine cells are found to express both insulin and the duct cell specific marker, cytokeratin, when β-cell mass is dramatically increasing and duct cell replication rates are elevated [92]. These findings suggest that these cells co-expressing both duct and endocrine markers are in the process of duct-to-β-cell transdifferentiation [88, 90, 92, 133].

Histological evidence of duct-to-β-cell transdifferentiation has also been observed in the adult pancreas. Single insulin positive cells bordering the duct

lumen have been identified in the adult pancreas, suggestive of duct-to-β-cell transdifferentiation (Figure 1.3B) [37, 86, 133, 134]. Small duct like structures have also been observed to appear from mature ductal elements (main and large pancreatic ducts) raising the possibility that indirect transdifferentiation may also occur from duct cells [135]. In summation, these observations suggest that both direct and indirect duct cell transdifferentiation can lead to new islet formation in the normal and regenerating adult pancreas.

1.7.3.2 Acinar Transdifferentiation

Researchers of exocrine pancreatic disease are well aware that the acinar tissue contains the dramatic potential to transdifferentiate in various pathological settings [20, 128]. More recently, researchers of endocrine pancreatic biology have become interested in the potential for acini to transdifferentiate, especially towards a β-cell fate [89, 115, 128, 136-138].

In cases of chronic pancreatitis and pancreatic adenocarcinoma, patients can develop obstruction of the pancreatic ductal network, which leads to leukocyte infiltration and changes in extracellular matrices and levels of growth factors. These conditions are thought to facilitate the transdifferentiation of pancreatic acini into tubular duct-like structures, a process known as acinoductal metaplasia [128]. The cells of these newly formed tubular complexes are generally more proliferative and primordial in both phenotype and genotype than normal exocrine cells [128]. It is hypothesized that the cells of these tubular complexes give rise to, or potentiate pre-existing, pancreatic adenocarcinoma [128]. Recent *in vitro*

cell lineage studies have indicated that this apparent acinar-to-duct transdifferentiation is indeed a transformation of cell phenotype and not simply the proliferation of pre-existing ductal elements [129]. It has also been hypothesized that the cells of these newly formed tubular complexes contain the potential to transform into insulin-producing β-cells [89, 128, 136, 138]. Thus, a possible avenue for increasing the endogenous β-cell mass is the indirect transdifferentiation of acini that begins with the transformation of adult pancreatic acini into duct-like structures, followed by their redifferentiation to an endocrine phenotype.

In addition to indirect transdifferentiation, direct acinar-to-β-cell transdifferentiation could expand the β-cell. Less is currently known about direct transdifferentiation of acinar cells into endocrine cells, though it has been suggested to occur both *in vivo* and *in vitro* [89, 139-141]. Co-localization analyses of acinar and endocrine factors, similar to that performed in mixed duct and endocrine cell phenotypes have indicated that these cells exist and that they have the potential to increase the endogenous β-cell mass.

1.7.3.3 Endocrine Transdifferentiation

During pancreatic ontogeny cells co-expressing glucagon and insulin are among the first endocrine cells to develop [93, 142]. Recent transgenic studies have questioned whether the co-localization of insulin and glucagon protein in pancreatic progenitor cells is due to a technical anomaly [143]. Herrara et al. indicate that endocrine-to-endocrine transdifferentiation does occur during

pancreatic development, although it is pancreatic polypeptide producing cells and not glucagon expressing cells that co-localize insulin [143]. Furthermore, the transdifferentiation of somatostatin producing δ-cells into insulin-producing β-cells has been observed during islet regeneration in neonatal rats [144]. These observations suggest that direct transdifferentiation of pre-existing endocrine cell phenotypes into insulin-producing β-cells is a possible means for increasing endogenous β-cell mass.

Recently, the morphological plasticity of adult pancreatic islets has also been indicated [81, 125, 145, 146]. Mature adult islets appear to contain the potential to transdifferentiate into duct-like structures, both *in vivo* and *in vitro* [81, 125, 145, 146]. These islet-derived duct-like structures are highly proliferative and have been shown to contain the potential to re-form islet-like structures [125]. The exact mechanism of this transformation is yet to be fully elucidated but *in vitro* studies indicate that changes in integrin as well as other growth factor and cAMP signalling pathways stimulate the initial differentiation into duct-like cells [80, 81, 147-149]. The redifferentiation of these islet-derived duct-like structures into endocrine cells appears to involve duct-to-endocrine differentiation [125]. This indirect transdifferentiation pathway has the potential to increase β-cell mass through the expansion of the primordial duct-like structures prior to their redifferentiation back to islet-like structures.

It is thus apparent that all three major cell types of the adult pancreas have the capability to lead to an increased endogenous β-cell mass through both direct and indirect transdifferentiation processes. A closer look into current *in vivo*

paradigms of pancreatic regeneration and β-cell neogenesis will provide insight to the potential of these various pathways in expanding endogenous β-cell mass in the adult pancreas.

1.8 MODELS OF PANCREATIC PLASTICITY AND REGENERATION

1.8.1 Partial Duct Obstruction

Partial duct obstruction, achieved through the wrapping of the duodenal portion of the pancreas with cellophane tape, is a surgical procedure associated with islet expansion [85, 133, 134, 150, 151]. Partial duct obstruction results in periductal fibrosis without diffuse pancreatitis. This surgical manipulation is associated with both duct cell hyperplasia and islet cell neogenesis [85]. The increase in β-cell mass that follows partial duct obstruction appears to be via the differentiation of duct-associated progenitor cells and not as a result of the replication of pre-existing β-cells [152]. Following the analysis of tritiated thymidine labelling in partially duct obstructed hamsters, it was suggested that duct-to-β-cell transdifferentiation was taking place. Tritiated thymidine administered in a single dose 2 weeks following partial duct obstruction was initially incorporated into ~2.5% of duct cells and 0.4% of endocrine cells. Six weeks later, islet cell labelling was 1.4% suggestive of an initial incorporation of tritiated thymidine into duct cells and subsequent differentiation of these duct cells into endocrine cells [153]. The observation of duct-associated, small endocrine clusters budding from the ductal epithelium supported the authors conclusions of duct-to-β-cell transdifferentiation [154].

Next, Rosenberg et al. sought to determine the underlying factor(s) for this observed islet neogenesis and subsequent β-cell mass expansion. Using an eloquent study entailing parabiosis, it was demonstrated that a paracrine agent produced by the wrapped portion of the pancreas was responsible for this elevated duct-cell proliferation and islet neogenesis [155]. A cytosolic extract, termed ilotropin, was then prepared from partially duct obstructed pancreases and administered to hamsters. Ilotropin administration resulted in increased new β-cell formation through the replication and differentiation of ductal precursors cells [151, 152, 155]. Ilotropin has also been used to reverse streptozotocin (STZ) induced diabetes in hamsters [156].

A Reg3 gene was found to be upregulated in partially duct obstructed pancreases through differential display analysis, and upon further purification of ilotropin, the Reg3 protein was identified [157, 158]. This specific hamster Reg3 protein appeared to contain the growth potentiating effects of ilotropin and was termed islet neogenesis associated protein (INGAP) [158]. When *in situ* hybridization was performed on pancreatic sections from partially duct obstructed hamsters, INGAP was found to be expressed in acinar cells 1 to 2 days following cellophane wrapping. This led to the suggestion that INGAP may be directly associated with the β-cell mass expansion observed in this setting [158]. Antibodies directed against the amino acid sequence in the central core of the protein (comprising amino acids 104-118) abolished the trophic actions of the ilotropin extract suggesting that this pentadecapeptide region contained the biological activity of this protein [158]. These results suggested that the pancreas

contains an inherent system to expand β-cell mass through new islet formation, and that the exogenous administration of this pancreas specific growth factor could lead to islet neogenesis and β-cell mass expansion.

1.8.2 Partial Pancreatectomy

Pancreatic regeneration has been observed following removal of 90 to 95% of the pancreas in normal young (5 to 6 weeks old) rats. The remnant pancreas grows to approximately 30% of the pancreatic mass of non-operated animals [135]. During this regeneration, small ductal structures (tubular complexes) appear approximately 36 hours after surgery and comprise up to 9% of the pancreas volume 48 hours after surgery [135]. By 7 days post-surgery, these tubular complexes are rarely detected [135]. Tubular complexes observed in these animals are thought to be neogenic due to the high replication of the constituent duct cells along with their coincidental appearance and disappearance with the marked growth of the pancreatic remnant [135].

Bonner-Weir et al. suggested that cells of the main pancreatic duct are responsible for the rise of these tubular complexes, and thus the regenerative response, following partial pancreatectomy [135]. It was demonstrated that the 90 to 95% partial pancreatectomy led to successive waves of duct cell proliferation, beginning in larger mature ducts and culminating in the duct cells found in the tubular complexes [135]. Common and main duct cells reached a peak replication index 36 hours after pancreatectomy, then small duct cells at 48 hours, and tubular complex duct cells at 72 hours after pancreatectomy. These data suggest

that proliferation and differentiation of duct cells gives rise to tubular complexes that subsequently differentiate into endocrine and exocrine tissue in this setting of pancreatic regeneration [135].

Regeneration following partial pancreatectomy has been proposed to occur through a recapitulation of embryonic development. The elevated duct cell labelling index followed by an increased β-cell mass is similar to that seen during pancreatic ontogeny. Likewise, tubular complexes observed following partial pancreatectomy appear similar to the morphology of fetal pancreases. Key factors in the embryological development of the endocrine pancreas are upregulated in partially pancreatectomized rodents. PDX-1 is observed to be upregulated in duct cells 2 to 3 days after partial pancreatectomy [159]. Gastrin, a growth factor that influences the expansion of endocrine cells during late fetal development is also believed to be critical during this regenerative period [137, 160]. Other pro-endocrine factors including the incretin GLP-1 and members of the Reg family are associated with regeneration in partially pancreatectomized rats [161, 162]. Together, these studies suggest that the young adult rat pancreas can increase β-cell mass in a manner similar to the β-cell mass expansion that occurs during organogenesis.

1.8.3 Pancreatic Duct Ligation

Complete pancreatic duct ligation is another surgical method used to initiate regeneration of the pancreas. Similar to partial pancreatectomy, tubular complexes form, though in duct ligation normal acinar tissue is replaced with

tubular complexes associated with surrounding lymphocyte infiltration [86, 137, 138, 163]. Neogenesis occurs in pancreatic duct ligated animals as indicated by the appearance of tubular complexes and small duct-associated islets during the rapid expansion of the β-cell mass.

Wang et al. suggested that duct cells are the progenitor cells that give rise to the β-cell mass expansion following ligation. Duct cell replication indices increased by 10-fold to a maximum 18% in the ligated portion of the pancreas 3 days post-surgery [86]. In contrast, the β-cell replication index peaked later, at 7 days post surgery, similar to that observed by Rosenberg et al. [150, 153]. These results suggest that duct to β-cell transdifferentiation contributes to the increased β-cell mass observed in the ligated portion of the pancreas.

The regenerative response observed during pancreatic duct ligation is associated with the upregulation of multiple growth factors. Similar to what occurs following partial pancreatectomy, cytokines and growth factors, including transforming growth factor alpha (TGFα) and gastrin, are upregulated in the ligated portion of the pancreas [163]. TGFα, an epidermal growth factor analog, is produced by macrophages, pancreatic ducts and islet cells [163-165], and can stimulate duct cell proliferation and differentiation [137, 160]. The interaction of TGFα and gastrin is thought to initiate the tubular complex formation and the neogenic response in pancreatic duct ligated animals.

1.8.4 Glucose Infusion

Excessive metabolic supply, specifically through chronic infusion of glucose, results in an increased demand for insulin [166]. Administration of glucose to rats leads to expansion of the β-cell mass through β-cell hypertrophy and hyperplasia [166]. When Finegood et al. performed a mathematically based analysis of the β-cell mass dynamics of rats infused with glucose for up to 6 days, they found that the increased β-cell hyperplasia and hypertrophy could not account for the observed increase in β-cell mass [89, 113]. Thus, it was predicted that β-cell neogenesis must be partially responsible for the expansion of the β-cell mass in this model.

A quantitative analysis of β-cell mass dynamics led to the discovery of increased neogenic β-cell mass with chronic glucose infusion [89]. There was an increase in single extra-islet β-cells associated with acinar tissue and a large number associated with newly formed tubular complexes [89]. An analysis of $CD3^+$ cells, revealed a wave of T-cell infiltration into acinar lobes prior to the development of the tubular complexes. The authors concluded that this T-cell infiltration led to the acinoductal metaplasia, which subsequently resulted in the increase in extra-islet β-cells associated with these structures [89]. These findings suggest that in the setting of chronic glucose infusion either direct acinar-to-β-cell transdifferentiation or indirect acinar-to-duct-to-β-cell transdifferentiation could lead to an expanded endogenous β-cell mass [89].

1.8.5 β-Cell Toxin - Streptozotocin

Streptozotocin (STZ) is a cytotoxic gluconitrosourea that is translocated across the cell membrane by GLUT-2, though negligibly by other glucose transporters and thus, preferentially affects β-cells [167]. Administration of a high dose of streptozotocin (200 mg/kg•bw) to young rats results in the destruction of nearly all β-cells [144]. This initial wave of β-cell death is followed by a ~3-fold increase in PDX-1 expression in somatostatin producing δ-cells [144]. As PDX-1 is a key regulator of the insulin gene, the observation of PDX-1/somatostatin double positive cells is suggestive of the transdifferentiation of δ-cells to a β-cell phenotype. Consistent with this interpretation, Fernandes et al. observed triple positive somatostatin/PDX-1/insulin cells following the increase in double positive somatostatin/PDX-1 cells [144]. This study indicated that, at least in young rats, another source for new β-cell formation is direct endocrine-to-endocrine transdifferentiation.

1.8.6 Transgenic Mice

Transgenic mice, over-expressing interferon gamma (IFNγ) under the control of the human insulin promoter, have also been used to delineate potential regenerative pathways in adult mice. Initially, IFNγ causes an immune response leading to extensive β-cell destruction [168, 169]. In areas of immune cell infiltration, small duct structures form that are similar to previously described tubular complexes [170]. Through analyses of BrdU (a thymidine analog) incorporation into duct and β-cells, Gu et al. suggest that newly formed β-cells

observed in these animals, originate from the transdifferentiation of these dedifferentiated ductal cells. As well, they conclude that over-expression of IFNγ attracts leukocytes leading to the local upregulation of TGFα and epithelial growth factor (EGF). This elevation in growth factors subsequently leads to the differentiation of acinar cells into tubular complexes that give rise to new β-cells, similar to that observed in other instances of pancreatic regeneration [160, 163, 171, 172].

The growth potentiating effects of TGFα and gastrin were further defined through analyses of transgenic mice, which over-expressed TGFα under the control of the metallothionein-1 promoter, and gastrin, under the control of the rat insulin-1 promoter [160]. Over-expressing TGFα in acinar cells leads to a dramatic transformation of the pancreatic acini [160, 173, 174]. A majority of the acini transform into tubular complexes similar to that observed in partial pancreatectomized and duct ligated rats. In addition, there is an increase in duct cell replication in these mice, though there is no increase in β-cell mass [160, 173, 174]. Similarly, mice over-expressing gastrin in β-cells do not have an increased β-cell mass [160]. However, when these two strains of mice are crossed, and both TGFα and gastrin are over-expressed, β-cell mass doubles [160]. These results suggest that indeed β-cell mass is controlled by the interaction of cytokines and growth factors upregulated in the context of pancreatic regeneration.

The aforementioned *in vivo* models of pancreatic regeneration have indicated that two major pathways, direct duct transdifferentiation and indirect acinar transdifferentiation, lead to endogenous β-cell mass expansion in a number

of settings. As well, these studies have indicated a number of factors that appear to be involved in the regeneration of the adult pancreas, and imply that the administration of these factors could lead to endogenous β-cell mass expansion.

1.9 MOLECULAR FACTORS ASSOCIATED WITH PANCREATIC REGENERATION

1.9.1 Regenerating Proteins (Regs)

The Reg family of proteins is composed of members identified in different settings of pancreatic disease and regeneration [158, 175-182]. As such, various nomenclatures arose identifying these proteins [183, 184]. The first factor to be identified was termed pancreatic stone protein (PSP) as it was associated with the formation of pancreatic stones during chronic pancreatitis [185]. PSP was again identified by a different group and termed pancreatic thread protein (PTP) and yet again by another group who designated it lithostatine, as it could be used to block the formation of calcium carbonate crystals [185, 186]. Watanabe et al. also later identified this same factor from the cDNA of regenerating islets and termed it Reg1 [182]. Since these initial observations, researchers have now identified four major types of Reg proteins (Reg1, 2, 3, and 4). Reg1 and Reg4 have been associated with gastric cancers [176, 178], while a Reg member has been found to be upregulated during the early insulitis and regenerative phases in Bio Breeding rats [187, 188]. Additionally, Reg upregulation is observed in multiple models of pancreatic regeneration including partial duct obstruction, partial pancreatectomy and glucose infusion, further indicating that Regs are key factors of pancreatic regeneration in the adult [162, 188, 189].

A multitude of functions have been ascribed to Reg proteins. Reg proteins posses a C-type lectin-like domain motif suggestive of carbohydrate binding activity [184, 190]. This has been confirmed though studies of both human Reg3α and mouse Reg3γ binding lactose and mannose respectively [191, 192]. Furthermore, Reg3α has been indicated to play a role in cell migration and adhesion in hepatocytes, whereas Reg1 has been implicated in the regulation of intestinal bacteria through the promotion of binding and aggregation within the intestine [193, 194]. Regs have also been associated with tissue regeneration, with Reg3 members showing anti-apoptotic activity as well as inhibition of cellular inflammation [195, 196]. This association with tissue regeneration has also been suggested as Reg1 and Reg3 have been shown to stimulate pancreatic duct cell, intestinal cell and Schwann cell replication [158, 197-200].

As described earlier, INGAP, a hamster Reg3 protein, appears to have potential in stimulating endogenous islet neogenesis. The identification of the pentadecapeptide fragment ($INGAP^{104-118}$) that contains similar biological activity as the full length protein, may enable a simpler therapeutic option for the expansion of endogenous β-cell mass. The observations of the *in vivo* effectiveness of ilotropin and the *in vitro* effects of $INGAP^{104-118}$ to stimulate duct cell proliferation, suggest that there is potential in the pharmacological administration of this compound to stimulate new β-cell formation in individuals with diabetes [158].

1.9.2 Combination: EGF and Gastrin

As discussed previously, Wang et al. observed that over-expression of TGFα and gastrin in mice led to significant β-cell mass expansion [160]. In a subsequent study these factors were administered exogenously to hyperglycaemic rats resulting in a significant reduction in blood glucose levels (~20 vs. ~30 mmol/l) [171]. Correspondingly, β-cell mass was significantly increased in mice treated with EGF and gastrin, though only to approximately one tenth that of normoglycaemic controls.

These initial findings have been furthered by Suarez-Pinzon et al. using non-obese diabetic (NOD) mice, which are a rodent model of type 1 diabetes [201]. Administration of EGF and gastrin led to amelioration of hyperglycaemia in 5 of the 6 treated mice and β-cell mass was four-fold greater than at the start of the experiment. Unfortunately, this mouse model is inherently problematic with regards to reproducibility and treatment specific effects. More than 100 various therapies, including some that seem inert, have reversed diabetes in NOD mice [202]. Despite this drawback, the combination EGF-gastrin therapy reported by Suarez-Pinzon et al. led to significant beneficial changes when compared to appropriate experimental controls.

Furthermore, EGF and gastrin combination therapy has been used to greatly expand the insulin content in mixed cultures of human islet and duct tissue [203]. EGF and gastrin therapy led to dramatic increases in CK19 positivity as well as PDX-1 upregulation, along with an approximate doubling of insulin content. When severely combined immunodeficient (scid) NOD mice were transplanted

with human islets, EGF and gastrin therapy was found to enhance the insulin content of these transplanted islets versus saline controls [203]. These results suggest that islet expansion both post-isolation *in vitro* and post-transplantation *in vivo* can be achieved via EGF and gastrin combination therapy. This treatment could dramatically increase the effective yields of current islet isolation/ transplantation therapies.

A potential drawback to this line of therapy is the fact that both EGF and gastrin are carcinogens [128, 204-206]. Recently, this has been partially addressed with the substitution of EGF with the incretin GLP-1, in addition to devising gastrin analogs with potentially less carcinogenic activity (Alex Rabinovitch, personal communication). As well as being less carcinogenic, it appears that this novel gastrin and GLP-1 combination therapy is equally, if not more effective than its predecessor [207].

1.9.3 Incretins

Incretins are proteins produced by the gut in response to nutrient ingestion that serve to help regulate nutrient uptake and metabolism [208]. The existence of incretins was first postulated from the observation that insulin release was much greater following an oral glucose challenge, versus an intravenous glucose challenge [208, 209]. Glucose-dependent insulinotropic polypeptide (GIP) was identified from gastric extracts that showed heightened insulin release when administered to dogs [210]. Brown et al. initially identified GIP in the late 1960s from gastric extracts that led to an inhibition of secretion and thus named the protein gastric inhibitory polypeptide [211]. Later, when its insulinotropic effects

were identified, the name was changed to reflect these findings. GIP potentiates the glucose-dependent secretion of insulin release from pancreatic β-cells. When this protein's actions were blocked, an incretin effect was still observed, suggesting the presence of other incretins. Glucagon-like peptide-1 (GLP-1) was later identified as a second incretin [46, 208].

Whereas GIP is produced by K-cells of the proximal small intestine, GLP-1 is produced by L-cells in the distal small intestine and proximal large intestine [46, 208, 212]. These proteins activate glucose-stimulated insulin release from β-cells through binding to their respective G-protein receptors. This leads to activation of cyclic AMP signalling pathways and ultimately increases the intra-cellular calcium concentration, which in turn potentiates insulin release [44]. Both proteins are rapidly inactivated by dipeptidyl peptidase 4 [46, 208, 212].

Since incretins have the potential to increase glucose-stimulated insulin release, they have become an attractive avenue for diabetic therapies. As well, ncretins delay gastric emptying and enhance insulin release, which both lead to a lower blood glucose profile [46, 208]. These effects can protect pre-existing β-cells from glucolipotoxicity directly following meals, when levels of glucose and lipids are elevated [13]. Furthermore, there is indication that incretins can act as anti-apoptotic agents [47]. Incretins also do not lead to non-glucose stimulated insulin release, and therefore hypoglycaemia may not be an issue with this therapy.

It has been suggested that incretins, especially GLP-1, have *in vivo* islet neogenic effects, but appropriate quantification of neogenic β-cell mass has yet to

be performed in such a model [161, 213, 214]. The known actions of GLP-1 including the potentiating of insulin release, slowing of gastric emptying and therefore decreasing metabolic stress, as well as its potential β-cell anti-apoptotic effects could all aid in supporting endogenous islet neogenesis. Whether incretins are true neogenic agents remains uncertain, though it is clear that they could be very effective adjunct agents in the treatment of diabetes mellitus.

1.9.4 Translation of Factors to Therapy

As noted above, neogenic agents may have carcinogenic attributes. Both EGF and gastrin are well-known epithelial cell carcinogens and the expression of Reg1 and Reg4 has been associated with various gastrointestinal cancers [128, 176, 178, 204-206]. Preliminary evidence suggests that administration of these factors could indeed lead to enhanced endocrine regeneration in the pancreas, though the carcinogenic potential of these compounds cannot be ignored.

1.10 PANCREATIC CANCER

The incidence of adenocarcinoma of the pancreas has risen steadily over the past 4 decades. It currently stands at approximately 29,000 new cases per year in North America, making it the second most common gastrointestinal malignancy and the fifth leading cause of adult deaths from cancer due to its aggressive nature [215, 216]. The diagnosis of pancreatic cancer is usually established at an advanced stage, and a lack of effective therapies leads to an extremely poor prognosis. A meta-analysis of 144 reported studies involving approximately 37,000 patients, found the median survival time to be 3 months

[217]. According to these findings, 65% of patients with pancreatic cancer will die within 6 months from the time of diagnosis, and about 90% will die within 1 year. Surgical resection, if performed early enough, is presently the only effective form of curative therapy [218]. However, fewer than 15% of patients with pancreatic cancer are potential candidates for a curative resection due to metastasis of the cancer to adjacent tissues or beyond [219, 220]. The five year survival rate of pancreatic adenocarcinoma is 1-4% thus making incidence and mortality rates virtually identical [221, 222].

Approximately half of all patients with pancreatic cancer have metastatic disease at the time of diagnosis, while the majority of remaining cases have locally advanced, unresectable disease [223-225]. Metastatic pancreatic cancer is one of the most chemotherapy-resistant tumors, as evidenced by the fact that pancreatic cancer has the lowest five-year survival rate (3%) of any cancer listed in the Surveillance, Epidemiology, and End Results (SEER) data base of the National Cancer Institute (NCI). As such, any diabetes therapy that increases the incidence of pancreatic adenocarcinoma would not be an optimal therapeutic approach.

There are many similarities between the various models of pancreatic regeneration and pancreatic adenocarcinoma. The mechanism of pancreatic regeneration and the initiation of tumor formation have been closely associated with metaplastic acinar transformation and the upregulation of numerous cell cycle and growth factors. If a pharmaceutically induced β-cell mass expansion therapy is to succeed, the potential for inducing unbridled pancreatic cell growth must be addressed.

1.11 REGULATION OF INDUCED β-CELL MASS EXPANSION

Previous studies of endogenous β-cell mass expansion and involution have suggested potential mechanisms that may serve to regulate pharmacologically induced β-cell mass expansion. An intriguing incongruity observed in the immediate post-natal period in the rat pancreas, is a remarkably high level of islet neogenesis despite a relatively stable β-cell mass [226]. To gain insight into the dynamics of the regulation involved, Finegood et al. developed a mathematical model that explained this apparent inconsistency via the simultaneous occurrence of islet neogenesis with increased β-cell apoptosis [79, 226]. Thus, the increase in apoptosis, together with minimal levels of β-cell replication, account for the stable β-cell mass during active β-cell neogenesis [79, 226].

These data support the hypothesis that maintenance of β-cell mass in the post-natal period is an active process in which cell proliferation, differentiation and death are involved in the homeostatic regulation of β-cell mass. Whether there is similar homeostatic regulation of β-cell mass with aging remains to be seen.

Dynamic control of β-cell mass in the adult rodent pancreas is suggested by studies of pregnancy during which the mother's β-cell mass greatly expands as altered hormone levels increase metabolic demands [227, 228]. Shortly after birth, however, the hormonal environment reverts to the non-pregnant state, and the expanded β-cell mass involutes to normal levels through β-cell atrophy and apoptosis and decreased β-cell replication [228]. Similar cell mass regulation has also been reported for lacrimal glands and breast tissue [229-235]. Thus, it appears that common control mechanisms in different tissues may serve to align

cell mass with functional need. It seems likely, therefore, that such mechanisms would also operate in the normal adult pancreas.

Based on a morphometric analysis of single β-cells and small β-cell clusters, Bouwens has suggested that islet neogenesis normally occurs in the adult human pancreas [37], presumably contributing to the ongoing maintenance of β-cell mass. Neogenesis, however, may be more than just a basal activity as Butler et al. have shown that β-cell mass expands without increased β-cell replication in cases of obesity [12, 236]. This finding suggests that neogenesis in the adult pancreas may respond to alterations in the local microenvironment in the setting of obesity. Furthermore, the recent observation that the development of type 2 diabetes is correlated with a lack of a neogenic response in adult humans suggests that a neogenic therapy may ameliorate this disease [236].

1.12 RATIONALE

Diabetes is a devastating disease that affects millions of people. Current therapies are not sufficient to prevent the secondary complications of this disease. New therapies that mimic the stringent glucose regulation that endogenous islets provide are needed in order to better control blood glycaemia levels and prevent diabetic complications. Pancreas and islet transplantation is hindered by the lack of donor organs and the requirement for harsh immunosuppressive drugs, making it an unrealistic therapy for more than 95% of individuals with diabetes. Presently, pharmaceutical expansion of endogenous β-cell mass appears to be the most promising therapy for the successful restoration of euglycaemia.

INGAP$^{104-118}$ is a promising candidate for the pharmacological induction of endogenous β-cell mass expansion. In order to achieve the therapeutic goal of endogenous β-cell mass expansion and restoration of euglycaemia, however, several key issues must be considered. First, it is important to determine if endogenous β-cell mass expansion can be therapeutically induced in animal models other than hamsters, including humans. Furthermore, studies must show whether INGAP$^{104-118}$ can be used to therapeutically reverse diabetic hyperglycaemia. Finally, if INGAP$^{104-118}$ therapy is to be used to clinically treat diabetes mellitus, steps must be taken to prove that this is a safe, as well as efficacious, treatment modality.

1.13 HYPOTHESIS

INGAP$^{104-118}$ administration will initiate islet neogenesis and subsequent β-cell mass expansion, leading to the reversal of hyperglycaemia in a response that is under homeostatic regulation and has efficacy in humans.

1.14 OBJECTIVES

1. Determine if the neogenic agent INGAP$^{104-118}$ can lead to islet neogenesis and β-cell mass expansion in multiple species.
2. Determine the cellular mechanism through which INGAP$^{104-118}$ can reverse diabetic hyperglycaemia.
3. Elucidate the mechanisms involved in the regulation of induced β-cell mass expansion.
4. Develop a model to test the efficacy of INGAP$^{104-118}$ in expanding human β-cell mass

CHAPTER 2

Pharmaceutical Induction of β-cell Mass Expansion In Multiple Animal Species and Reversal of Diabetic Hyperglycaemia

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Doctoral Candidate's Contribution - Manuscript 1

The doctoral candidate performed administration of scrambled INGAP$^{104-118}$ and exendin-4 to diabetic mice as described in this manuscript (Rosenberg et al. Ann Surg 2004), though others performed all additional animal manipulations. The doctoral candidate was responsible for: immunohistochemical staining and analysis of neogenic β-cell mass in euglycaemic hamsters administered saline or INGAP$^{104-118}$; immunohistochemical staining and total β-cell mass determination in INGAP$^{104-118}$-treated CD-1 mice; immunohistochemical staining, β-cell mass distribution and PDX-1 expression in INGAP$^{104-118}$-treated hyperglycaemic C57Bl6/J mice; combination of all data gathered; generation of all figures; all statistical analysis; writing the manuscript. The candidate did not perform the collection of the following raw data sets: islet density, total β-cell mass of euglycaemic hamsters, blood insulin and total pancreatic insulin content in euglycaemic hamsters (Rennian Wang, Ann Surg. 2004, depicted in Figures 2.2 and 2.3), blood glucose determination of diabetic mice (Ji-Wong Yoon, Ann Surg, 2004, depicted in Figure 2.6). Rennian Wang generated photomicrographs depicted in Figure 2.1, and Ji-Wong Yoon generated photomicrographs depicted in Figures 2.7 and 2.9. All other co-authors contributed in manuscript preparation and technical expertise in pancreatic digestions and animal manipulations. The doctoral candidate also performed the immunohistochemical staining and analysis of total β-cell mass depicted in figures 2.10, 2.11 and 2.12.

2.0 ABSTRACT

Objective: To demonstrate that INGAP$^{104-118}$, a pentadecapeptide containing the biologically active portion of native INGAP, increases functional β-cell mass in normal animals and can be used therapeutically to reverse hyperglycaemia in streptozotocin-induced diabetes.

Summary Background Data: Islet Neogenesis Associated Protein (INGAP), a 175 amino acid pancreatic acinar cell protein has been suggested to be implicated in β-cell mass expansion.

Methods: In the first part of this study, normoglycaemic hamsters were administered either 500 μg of INGAP$^{104-118}$ (n=30) or saline (n=20) i.p. daily, and sacrificed following 10 or 30 days of treatment. Blood glucose and insulin levels were measured and a histological and morphometric analysis of the pancreas was performed to determine the effect of INGAP$^{104-118}$ on the endocrine pancreas. In the second part of the study, 6-8 week old C57BL/6J mice (n=8) were administered multiple low doses of the β-cell toxin streptozotocin (STZ) inducing insulitis and hyperglycaemia. The mice were then injected with INGAP$^{104-118}$ (n=4) or saline (n=4) for 39 days and sacrificed at 48 days. Two additional groups of diabetic mice were administered either a peptide composed of a scrambled sequence of amino acids from INGAP$^{104-118}$ (n=5) or exendin-4 (n=5), an incretin that has been associated with amelioration of hyperglycaemia.

Results: Islet cell neogenesis was stimulated in INGAP$^{104-118}$-treated hamsters by 10 days. At 30 days, the foci of new endocrine cells had the appearance of mature islets. There was a 75% increase in islet number, with normal circulating

levels of blood glucose and insulin. Administration of INGAP$^{104-118}$ to diabetic mice reversed the diabetic state in all animals, and this was associated with increased expression of PDX-1 in duct cells and islet cell neogenesis with a reduction of insulitis in the new islets. Diabetic mice treated with exendin-4 or a scrambled INGAP peptide did not revert from hyperglycaemia.

Conclusion: Since there is a deficiency of β-cell mass in both type-1 and type-2 diabetes, INGAP$^{104-118}$ stimulation of fully functional neo-islet differentiation may provide a novel approach for a diabetes therapy.

2.1 INTRODUCTION

β-cell mass is severely reduced in type-1 diabetes, a disease in which a progressive autoimmune reaction results in the selective destruction of insulin-producing β-cells. Even in type-2 diabetes, the β-cell mass appears to be reduced by as much as 60% of normal [9, 12] and declines by 10-20% per year [10] so that β-cell function is dramatically reduced within 5-10 years of diagnosis. The number and size of functioning β-cells in the pancreas is of critical significance for the development, course and outcome of diabetes. Thus the development of diabetes can be understood as a failure of adaptive β-cell growth due to inadequate hyperplasia from either insufficient replication of pre-existing β-cells or neogenesis from adult stem/progenitor cells within the pancreas [25, 237].

In the post-natal period, expansion of the pancreatic endocrine cell mass manifests itself either by a limited proliferation of the existing terminally differentiated islet cells, or by a reiteration of islet neogenesis [75]. In 1983 we addressed the notion that induction of islet neogenesis may be a means whereby diabetes could be ameliorated [154]. Using a surgical model of partial pancreatic duct obstruction (cellophane wrapping of the pancreas), we showed that islet cell differentiation from progenitor cells associated with the ductal epithelium could indeed be induced in the adult pancreas [85, 152, 153] and that the newly formed islets functioned to reverse a diabetic state [151]. Evidence obtained from parabiotic studies, in which a common cross-circulation between animals was surgically established [155], suggested that the induction of cell proliferation and differentiation was mediated by paracrine and/or autocrine mechanism(s) [155].

Using classical protein chemistry techniques, a soluble tissue fraction was isolated from the regenerating pancreas, partially purified and termed ilotropin [157, 238-240]. This crude extract was shown to reverse streptozotocin-induced diabetes in hamsters [156]. Using mRNA differential display [181], we identified, sequenced and cloned a novel gene, whose 16.4 kD protein product INGAP (islet neogenesis associated protein), was found to be a major constituent of the ilotropin extract and appeared to be responsible for the bioactivity of ilotropin [158].

To date, only the *in vivo* effect of the crude pancreatic extract ilotropin, and the *in vitro* activity of INGAP peptide ($INGAP^{104-118}$) have been examined. We demonstrated that the pentadecapeptide is capable of stimulating tritiated thymidine (^{3}H-TdR) incorporation into the DNA of primary hamster duct cells as well as a rat duct tumor cell line (ARIP), but had no effect on the insulinoma cell line HIT-15 [158], suggesting an action on putative stem cells and not on terminally differentiated β-cells.

Various differentiation factors are required to achieve the mature phenotype characteristic of pancreatic β-cells [91]. Perhaps the most important of these is pancreatic duodenal homeobox-1, PDX-1 [91]. In the present study we report that $INGAP^{104-118}$, the putative biologically active portion of native INGAP, induces islet cell neogenesis in both normoglycaemic and diabetic animals associated with an up-regulation of PDX-1 expression in duct cells. The results also indicate that the new islet tissue is hormonally responsive to the prevailing metabolic state, and that $INGAP^{104-118}$ is capable of reversing streptozotocin (STZ) -induced diabetes in mice.

2.2 MATERIALS AND METHODS

2.2.1 Preparation of INGAP$^{104-118}$

A pentadecapeptide (mw 1501.4) corresponding to amino acids 104-118 (Ile-Gly-Leu-His-Asp-Pro-Ser-His-Gly-Thr-Leu-Pro-Asn-Gly-Ser) of INGAP [158] was synthesized at McGill University. This region of the deduced protein was chosen because it has a unique insertion of five amino acids (Ser-His-Gly-Thr-Leu) compared to Reg/PSP, a related family of genes, and it precedes a potential glycosylation site situated at position 126, hence a core of potential biological activity [158]. All peptides were reconstituted in isotonic saline.

2.2.2 Stimulation of Islet Cell Neogenesis In Normoglycaemic Hamsters

All animal studies were performed in accordance with CCAC guidelines. Female Syrian hamsters (Charles River, Quebec), 8 weeks of age, were randomly allocated to receive daily intra-peritoneal injections of INGAP$^{104-118}$ (250 μg b.i.d.) (n=15) or an equivalent volume of saline (n=10) for either 10 or 30 days. This species was selected because all developmental work leading to the discovery of INGAP was performed in hamsters [85, 151, 155, 156, 158, 181, 238, 239]. The INGAP$^{104-118}$ dose was extrapolated by regression analysis from a pilot study in which a recombinant INGAP was demonstrated to reduce blood glucose levels by 2 mmol/l for each log increase in dose in streptozotocin-treated diabetic hamsters [241]. Blood glucose was determined on days 0, 3, 6 and 10 using an AccuChek-EZ glucose monitor (Roche, Indianapolis, Indiana). Plasma and pancreatic levels of insulin were determined on day 10. At the end of the study period, animals

were anesthetized and sacrificed by exsanguination, and the pancreases excised for morphologic and morphometric analysis.

2.2.3 Dose Response Relationship of INGAP$^{104-118}$ and β-cell Mass

To examine the dose response relationship between INGAP$^{104-118}$ and β-cell mass, normoglycaemic CD-1 mice 6-8 weeks of age were administered daily i.p. injections of 0, 50, 500 or 2500 μg INGAP$^{104-118}$ for 34 days (n=10/group). At the end of the study period, animals were sacrificed and the pancreases excised for morphometric analysis to determine β-cell mass.

2.2.4 Treatment of Diabetic C57BL/6J Mice with INGAP$^{104-118}$

C57BL/6J male mice 6-8 weeks of age (Jackson Laboratories, Cold Harbor, ME) were rendered diabetic by intra-peritoneal injection of streptozotocin (STZ) in citrate buffer (40 mg/kg•bw/day for five consecutive days). Following confirmation that all mice were diabetic (blood glucose greater than 16.7 mmol/l) for at least one week, they were randomized to receive daily injections of INGAP$^{104-118}$ (500 μg, n=4) or an equivalent volume of saline (n=4) for 39 days. Blood glucose was determined every 3 days. All animals were sacrificed on day 48 by exsanguination, and the pancreases were excised for morphological analysis.

A second group of animals was rendered diabetic as described above and administered scrambled INGAP peptide (Ser-Ser-Thr-Gly-Gly-Gly-Asp-Ile-Pro-Pro-His-Leu-Leu-His-Asn) (500 μg/day n=5), exendin-4 (Sigma-Aldrich, Oakville,

Canada) (4 μg/day, n=5) [161, 242], or an equivalent volume of saline (n=4) for 39 days. All animals were sacrificed on day 48 by exsanguination, and the pancreases were excised for morphological analysis.

2.2.5 Insulin Measurements

Plasma and pancreatic insulin were measured using a solid-phase radioimmunoassay (Immunocorp, Montreal, Quebec). The collected blood was centrifuged and the plasma frozen at -70°C until assayed for insulin. A portion of each excised pancreas was weighed and then subjected to an overnight acid-ethanol extraction at 4°C. The cell-free extracts were collected, neutralized with 0.4 M Tris base and stored at -70°C until being assayed for insulin. Determinations were performed in triplicate.

2.2.6 Microscopy and Morphometric Analysis

Upon pancreatic excision, each organ was weighed and then fixed in 4% paraformaldehyde. Samples were embedded in paraffin and two sections 4 to 5 μm thick were taken from each pancreas in multiple 100 μm increments and de-waxed in xylene. Endogenous peroxidase activity was blocked with a 3.0% solution of hydrogen peroxide. The sections were washed with PBS and incubated with blocking buffer. Slides were stained with one or more primary antibodies, including insulin (guinea pig anti-porcine, 1:750; Dako, Mississauga, Canada); a cocktail of antibodies to glucagon, somatostatin, and pancreatic polypeptide (rabbit anti-human, 1:750; Dako); and PDX-1 (1:750; a generous gift

from C. Wright, Vanderbilt University). Slides stained for PDX-1 were incubated with citrate buffer for 30 min at 90°C for antigen retrieval before primary antibody application. Slides were incubated with primary antibodies overnight at 4°C, then washed in PBS and incubated with the appropriate secondary antibody for 1 hour at room temperature (1:500; biotinylated goat anti-guinea pig IgG, or biotinylated goat anti-rabbit IgG; Dako). Sections were then washed in PBS, incubated with avidin-biotin complexed with horseradish peroxidase (Dako) for 1 hour, and then developed using either 3,3'-diaminobenzidine (DAB) tetrahydrochoride (Sigma-Aldrich) or aminoethyl carbazole (AEC) (Zymed, San Francisco, USA). All slides were counterstained with Harris' haematoxylin (Sigma-Aldrich) as described previously [87]. A minimum of 2 slides from each animal for each analysis was measured.

Islet number (per mm^2) was determined as previously described [151]. In brief, an average of 12 low power microscopic fields was examined per insulin immunostained tissue section. Three immunostained slides were analysed for each animal. An average of 36 fields was surveyed per pancreas. The number of islets (>3 insulin-positive cells) in each field was determined and a mean (standardized per mm^2 of pancreatic tissue) was then calculated for each pancreas.

To determine β-cell mass (mg/pancreas), each gland was sectioned along its longitudinal axis to avoid any sampling bias due to regional variation in islet distribution and cell composition. Islet cross-sectional areas were traced manually with the aid of an Olympus BX60 microscope connected by video camera to a

computer equipped with Image-Pro Plus software version 4.0 [149]. The total β-cell mass was calculated by a stereological equation, as described previously [87]. Briefly, islets from insulin stained sections were traced and thresholded using the Image-Pro Plus software system to determine stained tissue area. The total pancreatic tissue was also measured and a percent β-cell area was calculated. This percent was then multiplied by the excised organ weight to determine the β-cell mass. An average of 100 islets was examined in each pancreas. Islet size was determined as described by Rosenberg et al. [151].

β-cell neogenesis was analysed as described previously [37, 88-90, 153]. All insulin-positive clusters of one or more cells were loosely traced and the area of the cluster within the trace was determined using the thresholding option. An islet was determined to be duct associated if any portion was juxtaposed to a duct. Extra-islet acinar associated β-cells were defined as insulin positive tissue without any other associated endocrine cells not juxtaposed to a duct. Duct-associated and extra-islet acinar associated β-cell mass were determined along with the percentage of PDX-1 immunopositive duct cells.

2.2.7 Statistical Analyses

Comparison between groups was done using a t-test, 1-way ANOVA, or 2-way ANOVA with post hoc one-tail t-test. Data are presented as mean ± standard error of measurement. Significance was accepted at the 5% level.

2.3 RESULTS

2.3.1 Systemic Effects of INGAP$^{104-118}$ Injection in Normoglycaemic Hamsters

Administration of INGAP$^{104-118}$ (500 μg/day) for either 10 or 30 days did not effect body weight (data not shown). To determine if INGAP$^{104-118}$-treated animals developed disturbed glucose homeostasis, we measured blood glucose levels during the first 10 days of injection, the period when the increase in β-cell mass was expected to occur most quickly (8, 9, 11, 17). No significant difference in blood glucose levels was demonstrated between groups (5.3±0.3 vs. 4.9±0.2 mmol/l). Moreover, plasma insulin levels of the INGAP$^{104-118}$-treated animals at the end of the 10-day period were not significantly different from saline controls (3.3±0.5 vs. 4.3±0.7 ng/ml, $p>0.05$).

2.3.2 β-cell Mass Expansion of INGAP$^{104-118}$-Treated Normoglycaemic Hamsters

The most striking feature of pancreases in INGAP$^{104-118}$-treated animals was areas of neogenesis; small foci of islet-like cells budding from intralobular and terminal ductules (Figure 2.1). Over 90% of the putative new islet cells stained positive for insulin, while a small minority stained positive for glucagon, somatostatin and pancreatic polypeptide. Examination of the mature islet population demonstrated a normal distribution of insulin-, glucagon-, and somatostatin-containing cells. In addition, small ducts were identified with insulin-positive cells in the epithelial wall (Figure 2.1A), and small extra-islet β-cell clusters could also be identified in the tissue adjacent to the ducts, amongst the acinar cells (Figure 2.1B). This process of neogenesis was much less apparent in

the tissue sections from the twenty saline-injected animals, all of which were similar, with a lesser number of mature islets associated with ducts.

To quantify the histological observation of new islet formation and to confirm the visual impression of increased neogenesis in $INGAP^{104-118}$-treated animals, morphometric assessment of the islet cell mass was performed. In $INGAP^{104-118}$-treated animals, the number of islets/mm^2 of pancreatic tissue was 64% greater than in control animals after 10 days (1.8±0.1 vs. 1.1±0.1; $p<0.01$), and 75% greater after 30 days of treatment (2.1±0.1 vs. 1.2±0.1; $p<0.01$) (Figure 2.2A). Ten days after commencing $INGAP^{104-118}$ administration, the median islet size in $INGAP^{104-118}$-treated animals was 53% smaller compared to control animals (5200 vs. 11000 μm^2, $p<0.05$). This shift to smaller islets in $INGAP^{104-118}$-treated animals, reflecting the induction of islet cell neogenesis with new islet formation, is shown in Figure 2.2B.

To further characterize the increase in the amount of islet tissue induced by $INGAP^{104-118}$, quantitative analysis of β-cell mass was performed (Figure 2.3A). Using insulin stained tissue sections, it was determined that the insulin-positive β-cell mass was increased in $INGAP^{104-118}$-treated animals by 44% at 10 days (3.7±0.5 vs. 2.6±0.4 mg; $p<0.01$) and by 31% at 30 days of treatment (4.6±0.4 vs. 3.5±0.3 mg; $p<0.025$) when compared to aged-matched saline-treated animals. To correlate increased β-cell mass and insulin content, we directly determined pancreatic tissue levels of insulin after 10 days of treatment with $INGAP^{104-118}$ (Figure 2.3B). The pancreatic insulin content of $INGAP^{104-118}$-injected animals was increased by 130% in comparison to saline-treated animals (33.5±1.9 vs. 14.6±0.8

μg/g, $p<0.05$). This observation further supports the data obtained by morphometric analysis.

Analyses of β-cell neogenesis were then performed to further characterize the increase in β-cell mass. In comparison to control animals, $INGAP^{104-118}$-treated animals exhibited a 3-fold increase in duct-associated β-cell mass at 10 days (0.73±0.09 vs. 0.24±0.09 mg; $p<0.05$) and more than a 2-fold increase following 30 days (1.67±0.38 vs. 0.74±0.26 mg; $p<0.05$) (Figure 2.4A). Extra-islet acinar-associated β-cell mass was also significantly increased in $INGAP^{104-118}$-treated animals by approximately 3-fold at both 10 and 30 days of treatment (d10: 48±8 vs. 17±4 μg; d30: 33±8 vs. 11±3 μg; $p<0.05$) vs. saline injected controls (Figure 4B).

Having demonstrated the biological activity of $INGAP^{104-118}$ on β-cell mass expansion we sought to study the dose response between $INGAP^{104-118}$ and β-cell mass. β-cell mass increased with escalating $INGAP^{104-118}$ dose by 69%, 114%, and 122%, respectively for 50, 500 and 2500 μg (0.83±0.09, 1.40±0.25, 1.78±0.37, 1.84±0.36 mg, respectively; Figure 2.5).

2.3.3 Reversal of Diabetes After $INGAP^{104-118}$ Treatment

To investigate whether $INGAP^{104-118}$ could reverse hyperglycaemia, diabetic C57BL/6J mice were randomly allocated to treatment with $INGAP^{104-118}$ (500 μg per day i.p.), scrambled INGAP peptide (500 μg per day i.p.), exendin-4 (4μg per day i.p.) or saline (equivalent volume) for a period of 39 days.

At the time of sacrifice, day 48, 4/4 of INGAP$^{104-118}$-treated animals were normoglycaemic (8.1±1.2 mmol/l) (Figure 2.6). In contrast, all animals in the other groups remained hyperglycemic (saline, 23.0±1.5; exendin-4, 27.1±2.6; scrambled INGAP, 27.2±1.9 mmol/l, $p<0.005$).

The pancreatic tissue of INGAP$^{104-118}$-treated animals contained foci of islet cell neogenesis and new islet formation (Figures 2.7A and B). We determined that 23.5±3.2% of pancreatic ducts in INGAP$^{104-118}$-treated mice contained insulin-positive cells versus 1.0±1.0% in control animals ($p<0.001$). Duct-associated β-cell mass in INGAP$^{104-118}$-treated animals was 0.27±0.06 mg, compared to 0.01±0.01 mg for control animals ($p<0.001$). Moreover, the number of islets per mm^2 in INGAP$^{104-118}$-treated mice was 1.9±0.5 compared to 0.2±0.01 in the saline-treated controls ($p<0.01$). INGAP$^{104-118}$-treated animals also demonstrated an increase in PDX-1 immunoreactive duct cells compared to saline-treated mice (19.1±2.2% vs. 0.77±0.0%, $p<0.01$) (Figures 2.8A and B), and this was especially true in areas of islet cell neogenesis (Figure 2.8C). The majority of the cells in the newly formed islets were immunoreactive for insulin, with normal distribution of glucagon and somatostatin (Figures 2.9A and B). In contrast, in saline-treated animals, there was minimal neogenesis and the surviving islets showed evidence of extensive necrosis, along with lymphocytic infiltration (Figure 2.7C). A photomicrograph of an islet from a normal untreated mouse is shown for comparison in Figure 2.7D.

Figure 2.1: Histological features of the pancreas following administration of INGAP$^{104-118}$. **(A)** β-cell neogenesis (arrow) in the wall of a small intralobular duct after 10 days of INGAP$^{104-118}$ treatment. To the left is a normal islet. **(B)** An area of new islet formation outside its putative duct of origin (arrowheads) at 30 days. The red stain identifies insulin protein visualized by immunocytochemistry. (bar = 50 μm)


A
B

Figure 2.2: Change in the islet population induced by INGAP$^{104-118}$. Normoglycaemic Syrian hamsters were treated with saline (□, n=10/time point) or INGAP$^{104-118}$ (■, n=15/time point) for 10 or 30 days. **(A)** Islet number (per mm^2), and **(B)** bins of islet size distribution (μm^2) as determined by computer-assisted morphometric analysis. INGAP$^{104-118}$-treated animals exhibited a significant increase in islet number. This was the result of new islet formation as reflected by the shift in the distribution of islet size to small islets. * $p<0.01$, ** $p<0.05$

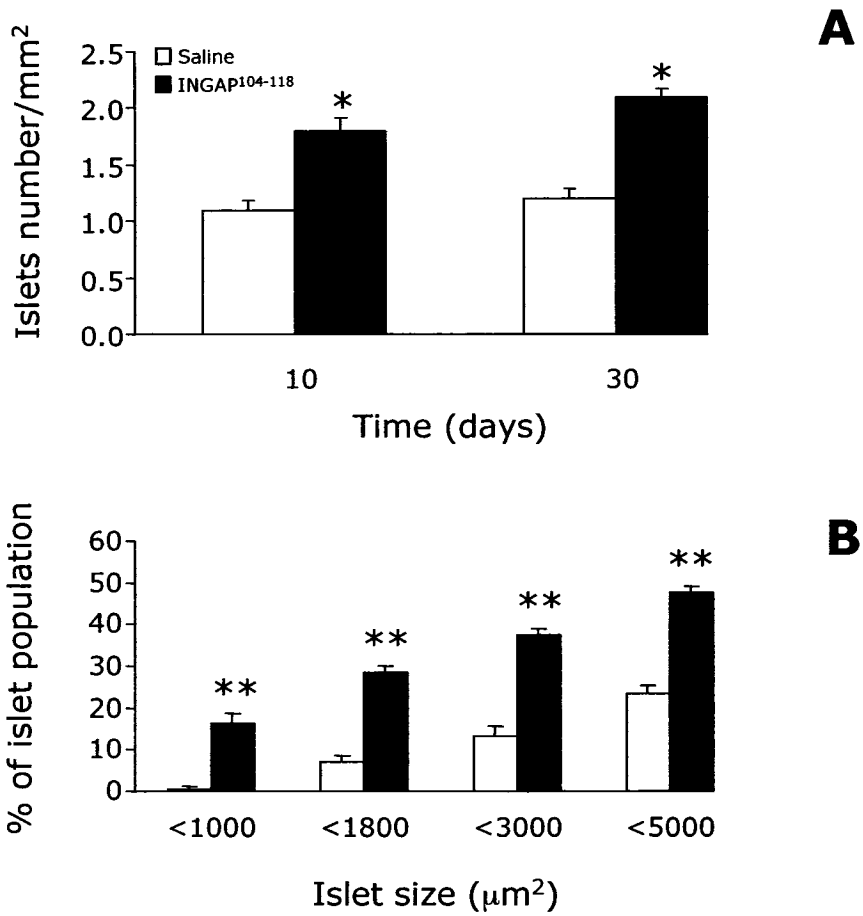
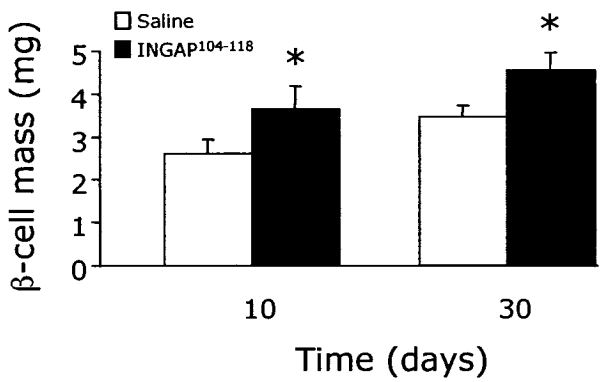
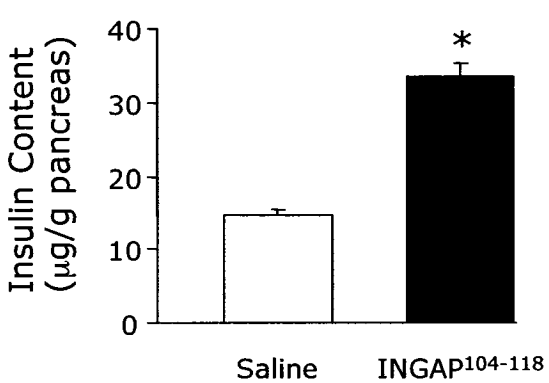

A
Saline
INGAP104-118
Islets number/mm2
2.5
2.0
1.5
1.0
0.5
0.0
*
*
10
30
Time (days)
B
% of islet population
60
50
40
30
20
10
0
**
**
**
**
<1000
<1800
<3000
<5000
Islet size (μm2)

Figure 2.3: (A) β-cell mass of Normal Syrian hamsters treated with saline (□, n=5, 6; for 10 and 30 days, respectively) or INGAP$^{104-118}$ (■, n=5, 11) for either 10 days or 30 days as determined by computer-assisted morphometric analysis. **(B)** Increase in pancreatic insulin content following 10 days of INGAP$^{104-118}$ treatment. * $p<0.025$, ** $p<0.01$.


Saline
INGAP104-118
β-cell mass (mg)
0
1
2
3
4
5
*
*
10
30
Time (days)

A


Insulin Content
(μg/g pancreas)
0
10
20
30
40
*
Saline
INGAP104-118

B

Figure 2.4: Induction of β-cell neogenesis by INGAP$^{104-118}$. Normal Syrian hamsters were treated with saline (□, n=5/time point) or INGAP$^{104-118}$ (■, n=5/time point) for either 10 days or 30 days. **(A)** Duct-associated β-cell mass, and **(B)** extra-islet acinar-associated β-cell mass as determined using a computer-assisted morphometric system. * $p<0.05$.

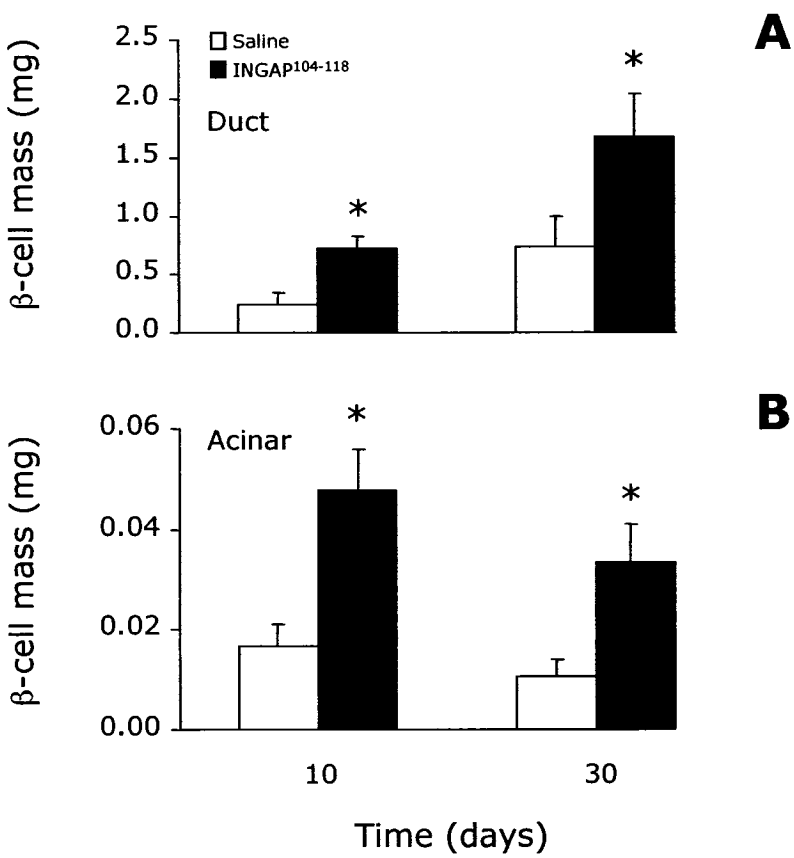

A
B
β-cell mass (mg)
β-cell mass (mg)
Saline
INGAP104-118
Duct
Acinar
2.5
2.0
1.5
1.0
0.5
0.0
0.06
0.04
0.02
0.00
*
*
*
*
10
30
Time (days)

Figure 2.5: Increase in pancreatic endocrine cell mass with escalating INGAP$^{104-118}$ dose. Normal CD-1 mice treated with saline (□, n=10) or INGAP$^{104-118}$ (■, n=10/group) at 50, 500 or 2500 μg/day for 31 days. β-cell mass was determined using a computer-assisted morphometric system. * $p<0.05$ vs. saline.

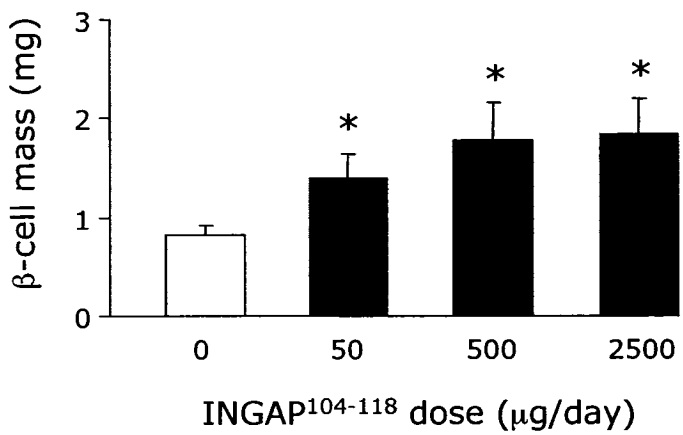

β-cell mass (mg)
3
2
1
0
*
*
*
0
50
500
2500
INGAP104-118 dose (μg/day)

Figure 2.6: Reduction in blood glucose by treatment with INGAP$^{104-118}$. C57BL/6J mice were rendered diabetic using a regimen of multiple injections of low-dose streptozotocin. The animals were then randomized to receive daily treatment with either saline (□, n=4) or 500 μg/day INGAP$^{104-118}$ (■, n=4) for 39 days. * $p<0.0001$

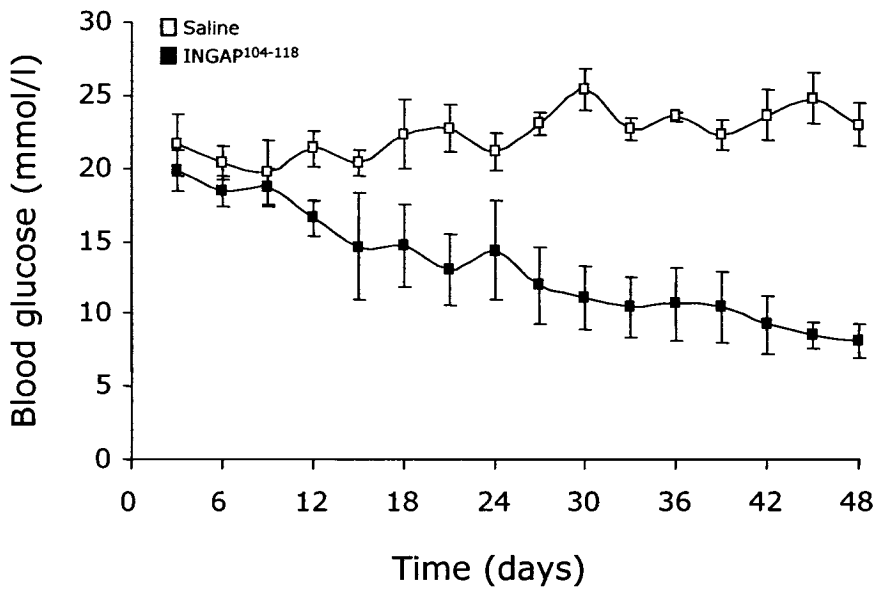

Saline
INGAP104-118
Blood glucose (mmol/l)
30
25
20
15
10
5
0
0
6
12
18
24
30
36
42
48
Time (days)

Figure 2.7: Histological characteristics of the pancreas of streptozotocin-treated C57BL/6J mice with and without $INGAP^{104-118}$ treatment. **(A)** Pancreas of an $INGAP^{104-118}$-treated animal showing an area of islet cell neogenesis, observed as endocrine-like cells budding from an adjacent intralobular ductule. **(B)** A normal appearing islet in a pancreas of an animal treated with $INGAP^{104-118}$. **(C)** The pancreas of a saline-treated animal showing an islet with inflammatory cell infiltration characteristic of insulitis. **(D)** The pancreas of a normal aged-matched non-treated control mouse showing the histological appearance of a normal islet for comparison. (bar = 50 μm)

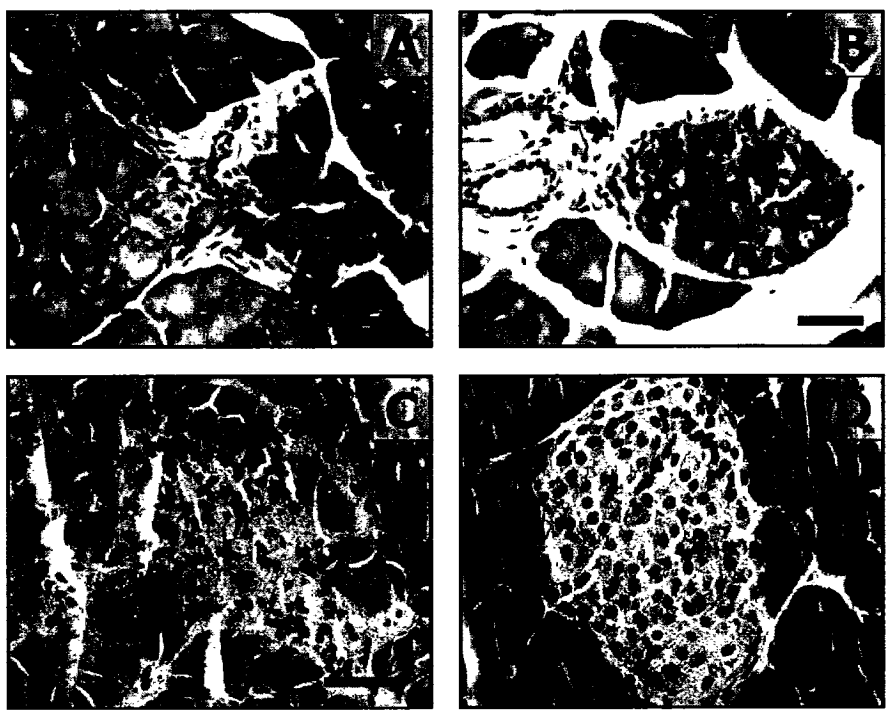
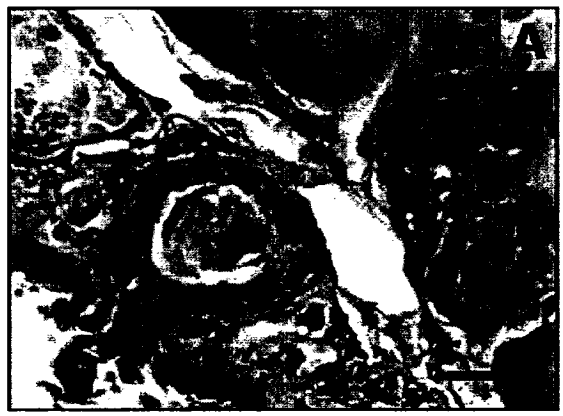
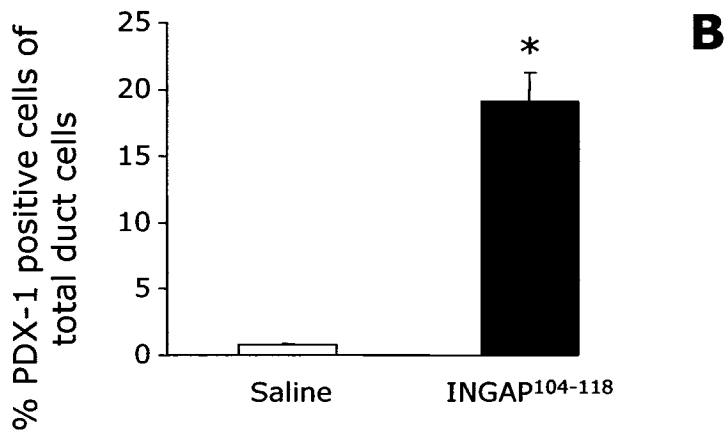

A
B
C
D

Figure 2.8: Expression of PDX-1 and insulin in the pancreas of streptozotocin-treated C57BL/6J mice administered either saline (□, n=4) or INGAP$^{104-118}$ (■, n=4) for 39 days. **(A)** Photomicrograph of a PDX-1 stained pancreatic section from an INGAP$^{104-118}$-treated animal, and **(B)** PDX-1 positivity quantified by computer-assisted morphometric analysis. (**C**) Area of duct-associated islet neogenesis in an INGAP$^{104-118}$-treated animal stained for both PDX-1 (brown) and insulin (red). (bar = 25 μm) * $p<0.01$


A


B
% PDX-1 positive cells of total duct cells
25
20
15
10
5
0
*
Saline
INGAP104-118

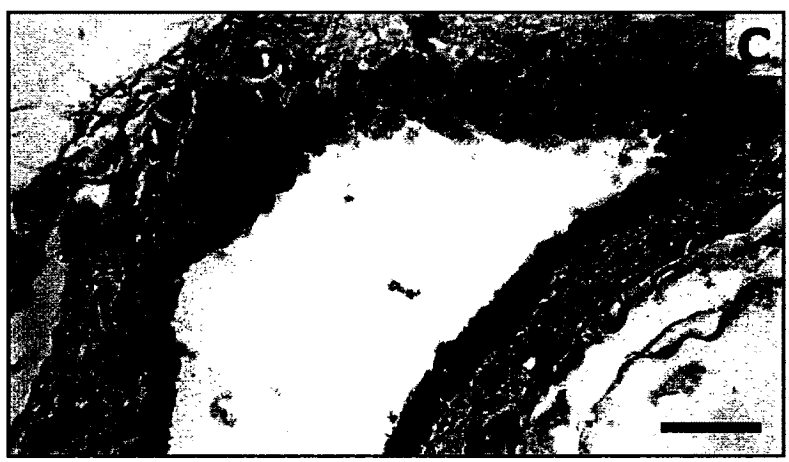
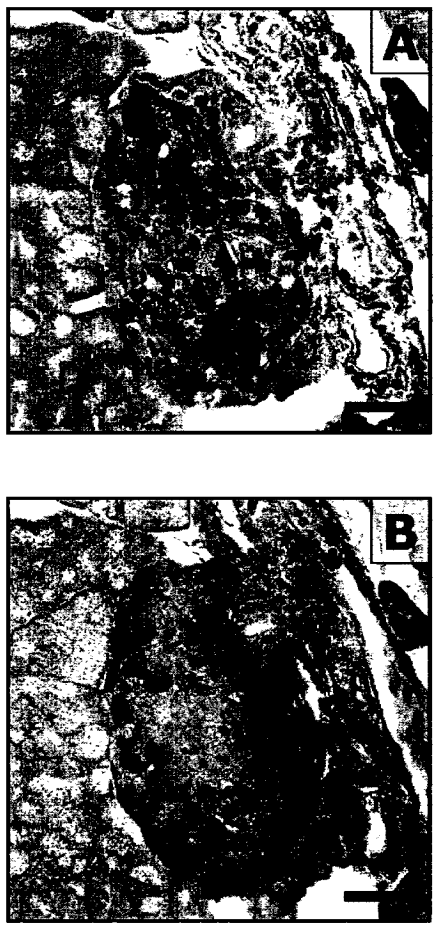

C

Figure 2.9: Immunocytochemistry of islet cell hormones in the pancreas of streptozotocin-treated diabetic C57BL/6J mice administered $INGAP^{104-118}$. **(A)** An islet associated with a segment of duct epithelium (to the right) stained for insulin (black), and **(B)** for both glucagon and somatostatin demonstrating a normal presence and distribution of islet cell hormones. (bar = 30 μm)


A
B

2.4 DISCUSSION

We originally observed that duct-associated islet cell neogenesis, a hallmark of pancreatic organogenesis, occurred in response to partial pancreatic duct obstruction [85]. Subsequently, we reported that the crude tissue extract, ilotropin, prepared from the partially obstructed pancreas [155, 239], reversed hyperglycaemia in up to 60% of hamsters rendered diabetic with high dose STZ used to induce islet necrosis [156]. In this paper we now demonstrate that INGAP$^{104-118}$ is capable of stimulating both duct and acinar-associated islet neogenesis, increasing β-cell mass in a dose dependent manner and reversing STZ diabetes with insulitis in 100% of animals. We also show that the effects of INGAP$^{104-118}$ are associated with induction of PDX-1 expression, an important transcription factor involved in normal islet development [91]. It is evident from these experiments that the adult pancreas contains putative stem/progenitor cells that remain responsive to an appropriate stimulus for differentiation along an islet cell lineage and that INGAP$^{104-118}$ can be this stimulus leading to reversal of a diabetic state.

The greater response in the C57BL/6J animals treated with INGAP$^{104-118}$ in the present study compared to the previous results with the crude tissue extract [156] may be related to the higher dose of INGAP$^{104-118}$ used on a molar basis and/or the lower doses of STZ required to create diabetes in mice. Thus, in comparison to hamsters, higher doses of STZ used in the earlier studies may have been toxic to the putative precursor cells destined for regeneration. We also cannot discard the possibility of greater efficiency of INGAP$^{104-118}$ relative to native INGAP present in the crude ilotropin extract [158].

The results obtained from normal hamsters in this study suggest that induction of an increase in new islet cell mass induced by INGAP$^{104-118}$ reaches an asymptote and is not an unbridled growth. These results also suggest that the pancreas contains resident factors that exercise apparent homeostatic control of β-cell expansion that serve as an effective brake against the development of islet cell tumors due to unbridled cell growth. Furthermore, in none of the INGAP$^{104-118}$-treated animals did we observe the development of hypoglycaemia due to the increased β-cell mass. In fact, despite an elevation of pancreatic tissue levels of insulin, circulating insulin concentrations were virtually identical to those seen in saline-treated controls. This indicates that a mature counter-regulatory mechanism, which was able to adjust the circulating level of insulin to the prevailing level of glucose, was functioning in INGAP$^{104-118}$-treated animals.

In these studies, the histologic and morphometric evidence strongly supports the notion that the increase in β-cell mass was due to islet cell neogenesis. INGAP$^{104-118}$ administration, caused an increase in duct-associated β-cell mass as well as an increase in extra-islet acinar-associated β-cell mass, both of which are indicative of β-cell neogenesis [37, 88-90]. This is in keeping with the report by Bouwens et al. who have suggested that single β-cells or small clusters of β-cells in the parenchyma of the pancreas are indicative of newly formed β-cells [37]. Likewise, the observation of duct-associated β-cells (or β-cell clusters) suggests that these cells were derived from duct-associated precursors by a process of neogenesis as well [85, 88, 90, 152, 153]. Importantly, these observations were extended and confirmed in the second part of this study in

which PDX-1 immunoreactivity was quantified. Thus, it was demonstrated that INGAP$^{104-118}$ is capable of inducing β-cell neogenesis and new islet formation in STZ-diabetic animals in which the original adult islet population was destroyed. Furthermore, the reversal of STZ-induced diabetes, by INGAP$^{104-118}$ administration, indicates the target effect of INGAP$^{104-118}$ on non-β-cells, supporting the notion of neogenesis. Moreover, the failure of a scrambled sequence of amino acids derived from INGAP$^{104-118}$ to reverse hyperglycaemia highlights the specificity of the biological activity of INGAP$^{104-118}$ with respect to induction of islet neogenesis and reversal of hyperglycaemia.

Animals injected with exendin-4, a growth factor reported to stimulate islet neogenesis [161] failed to return to a normal glycaemic state. The failure of exendin-4 to reverse hyperglycaemia may be due to an insufficient dose for the specific animal model used here, even though this effect was reported in other types of animal models at similar doses [161, 242]. All of the previous work describing the glucose lowering and neogenic activity of exendin-4 has been performed in models of type-2 diabetes, and therefore the dose we used may not have been adequate to reverse hyperglycaemia in a model of type-1 diabetes where the β-cell mass is markedly reduced. To date, the benefit of exendin-4 in models of type-1 diabetes remains an unresolved issue in the literature. In the only published report of the effects of exendin-4 in a multi-low dose STZ mouse model of type-1 diabetes, exendin-4 was injected before, during and after STZ administration, producing a modest reduction in blood glucose levels [243]. However, this experimental design is substantially different from the one we used

in our study, in which exendin-4 was only administered after hyperglycaemia was produced.

The administered daily dose of 500 μg of $INGAP^{104-118}$ may appear to be high, especially when compared to the administered dose of exendin-4, yet it has been shown that GLP-1 is most effective at doses between 100 and 1000 μg (23). Likewise, it should be noted that exendin-4 is actually the reptilian homologue of mammalian GLP-1 and has a potency more than 5000-fold greater than an equimolar amount of the latter [161]. $INGAP^{104-118}$ given intravenously or subcutaneously to CD-1 mice at 200 mg/kg•bw produces plasma concentrations of about 1000 ng/ml at early time points (less than 30 minutes) corresponding to 666 nM knowing the molecular weight of $INGAP^{104-118}$ is 1501.4. At times later than 30 minutes, concentrations quickly decrease to less than 10 ng/ml (6.66 nM) (personal communication from J. Joly, Procter & Gamble). Assuming linear pharmacokinetics, the dose used in the current study (approximately 20 mg/kg•bw) would be expected to produce approximately 10-fold lower concentrations at similar times (i.e., resulting in a range from about 1 to 100 ng/ml). These predicted concentrations are less than the concentration (250 ng/ml, 167 nM) known to induce differentiation of human duct epithelial cells to islets *in vitro* [188]. GLP-1 was not used in our study because it has previously been shown to produce only transient reductions in blood glucose [244] due to its very short plasma half-life [245], necessitating a continuous intravenous infusion to obtain acceptable glycaemia in type 2 diabetic patients [244]. These data indicate that the administered dose of $INGAP^{104-118}$ is appropriate. However, in the

future, INGAP analogs might have more potent biological actions, like exendin-4. To rule out a non-specific effect on blood glucose of this dose of $INGAP^{104-118}$, we administered an equimolar dose of a peptide synthesized from a scrambled sequence of the amino acids comprising $INGAP^{104-118}$. This scrambled peptide had no demonstrable effect on blood glucose nor on the induction of islet cell neogenesis.

A number of transcription factors are required to be expressed at appropriate times during the course of islet development in order to achieve the mature phenotype characteristic of pancreatic β-cells [91]. Possibly the most significant of these is PDX-1 [91, 246]. In adult animals, the expression of PDX-1 is repressed in the majority of pancreatic cells, with the exception of the β- and a subset of δ-cells of the islets of Langerhans [247]. In this study the key observation has been made that $INGAP^{104-118}$ is not only a potent inducer of islet cell differentiation, but that new islet formation appears to involve expression of PDX-1. Even more surprising was the extent to which ductal cells became positive for PDX-1 suggesting that many cells have the capacity to transdifferentiate. However, only a fraction of these cells developed into β-cells suggesting that there are other determinants for progression to full hormonal secretory status.

The observation of islet cell neogenesis with new islet formation in this study is in keeping with previous reports [85] of potential islet cell progenitors in the adult pancreas that are capable of islet neogenesis following partial pancreatic duct obstruction. In this regard, it is of interest to note that a trial of partial

obstruction of the pancreas in a group of type-1 diabetic children was conducted over 70 years ago in New York by de Takats [65-68], who reported an "increase in sugar tolerance" following surgery. β-cell neogenesis from pancreatic duct epithelial cells has also been found in other models of islet neogenesis [248] and raises the question of whether there is a futile attempt at new islet formation in patients with type-1 diabetes or that the newly formed islets are destroyed by ongoing immune-mediated mechanisms. The observation that an INGAP immunoreactive protein is found in the pancreases of patients with type-1 diabetes for 50-60 years [249] suggest that there may be factors that mitigate its biologic effects. It will be important to determine these factors to develop a complete understanding of the role of INGAP in islet neogenesis.

The reversal of diabetes in C57BL/6J mice is all the more pertinent to diabetes in humans. Considerable evidence implicates autoimmunity as a primary mechanism underlying β-cell destruction in some forms of insulin dependent diabetes in humans [250]. Male mice of the C57BL/6J inbred strain are especially sensitive to the induction of insulin dependent diabetes by the β-cell cytotoxin STZ and will produce β-cell cytotoxic alloantiserum when immunized with islets [251]. When STZ is administered in a multi-low-dose regimen to male C57BL/6J mice, induction of hyperglycaemia is preceded by a mononuclear cell infiltrate in the pancreas [252, 253], as observed in our STZ-treated animals. The reversal of diabetes in this setting may be unique to INGAP, among the other members of the Reg/PSP family of proteins, which have been demonstrated to be associated with inflammation and autoimmune insulitis, but not to reverse diabetes [177, 180]. It

is of interest to speculate on why, in the presence of insulitis, the new islets were not destroyed as would be anticipated in human type-1 diabetes. There are at least three explanations for this. First, the newly differentiated cells may exhibit an antigenic profile that does not predispose them to recognition and subsequent destruction in this model of diabetes. Secondly, the observed lymphocytic infiltrate may not be as cytotoxic as that which characterizes the human disease. Thirdly, INGAP$^{104-118}$ may exhibit immunomodulatory effects.

Alternative animal models of type-1 diabetes include the NOD mouse and the BB rat, each of which has its own biological, and in particular immunological, idiosyncrasies [254]. We are currently working through many of these issues in the NOD mouse, including the prerequisite for immunosuppression, the need to deal with ongoing islet cell apoptosis, and the necessity for early trophic support of the newly forming islets. Our preliminary efforts suggest that it may indeed be possible to induce islet cell neogenesis using INGAP$^{104-118}$ as part of a multi-factorial approach (data not shown).

Thus, the reversal of hyperglycaemia in an insulitis-like model of diabetes augurs particularly well for the condition in humans. Although, INGAP was identified in the hamster pancreas, a human protein is recognized by an antiserum made to the hamster peptide. Moreover, this protein is highly expressed in the presence of islet neogenesis in the setting of partial obstruction in patients with chronic pancreatitis [249]. This novel β-cell growth factor is likely to play a role in islet cell neogenesis in humans and INGAP is likely conserved between species [91].

The induction of islet cell neogenesis by INGAP$^{104-118}$ leading to new islet formation represents a potentially important approach for endogenous progenitor cell therapy. This approach has the potential to complement the use of fetal, allogeneic or xenogeneic tissue for transplantation of β-cells into insulin-dependent diabetic patients, and may be essential to *in vitro* expansion of putative stem/progenitor cells for subsequent transplantation. While this approach would have to be combined in some fashion with a tolerance induction strategy to prevent recurrence of insulitis in type-1 patients [255], this would not be necessary in patients with non-immune type-2 diabetes who could benefit from an augmentation of their native β-cell mass.

In summary, we have demonstrated that a pentadecapeptide of native Islet Neogenesis Associated Protein (INGAP), a product of the pancreatic acinar cell, can induce *in vivo* islet cell differentiation from a putative progenitor cell(s) associated with the ductal epithelium, resulting in sufficient new islet formation to reverse a diabetic state. The ability of INGAP$^{104-118}$ to promote fully functional and controlled islet cell differentiation and growth *in vivo* may provide a novel approach to the treatment of diabetes, and one that requires neither transplantation, nor the *in vitro* use of controlled differentiation of stem cells. Moreover, the demonstration that this effect can be achieved using a small peptide enhances its potential as a pharmacologic therapy. INGAP$^{104-118}$ has recently successfully completed Phase 1/2a clinical trials.

2.5 ACKNOWLEDGEMENTS

L.R. is a chercheur national (national scientist) of the fond du recherche scientifique du Quebec (FRSQ). M.L. is supported by a fellowship from the CIHR (Canadian Institutes for Health Research) and the Diabetic Children's Foundation. J.W.Y. is a Canada Research Chair in Diabetes. M.P. is a CIHR scientist. This work was supported in part by a grant from the CIHR. The authors would like to thank Julie Lemay for her work with the INGAP$^{104-118}$-dose response analyses and Stephen Hanley, Al-Maleek Jamal, Mauro Castellarin, Nora Malek and Despina Agapitos for their technical expertise.

BRIDGING STATEMENT BETWEEN MANUSCRIPTS

The previous chapter demonstrated that INGAP$^{104-118}$ therapy alone was sufficient to initiate islet neogenesis and result in quantifiable β-cell mass expansion in hamsters and mice. It was also determined that β-cell mass expansion increased in magnitude with the escalation of INGAP$^{104-118}$ dose. Further to this work, INGAP$^{104-118}$ administration to euglycaemic beagle dogs led to similar trends in β-cell mass expansion (n=10/dose, p=0.22; Figure 2.10). When INGAP$^{104-118}$ was administered to normoglycaemic cynomolgus monkeys there was no change in total β-cell mass (n=8/dose, p=0.47; Figure 2.11) though there were indications of profound islet neogenesis (Figure 2.12). These results suggest that INGAP$^{104-118}$ therapy could be effective across multiple species, including humans. Furthermore, when INGAP$^{104-118}$ was administered to diabetic mice, it led to the reversal of their drug-induced hyperglycaemia. These findings, while promising, elicited the question as to whether the continual stimulation of islet neogenesis via INGAP$^{104-118}$ administration is a safe therapeutic approach that would not lead to unregulated β-cell mass expansion and possible carcinogenesis. Therefore, the aim of Chapter 3 was to analyse the homeostatic mechanisms regulating induced β-cell mass expansion.

Figure 2.10: Total pancreatic β-cell mass in euglycaemic dogs with escalating INGAP$^{104-118}$ dose. Beagle dogs treated with saline (□, n=10) or INGAP$^{104-118}$ (■, n=10/dose) at 0.5, 1.5 or 10 mg/kg•bw/day for 34 days. β-cell mass was determined using a computer-assisted morphometric system. p=0.22

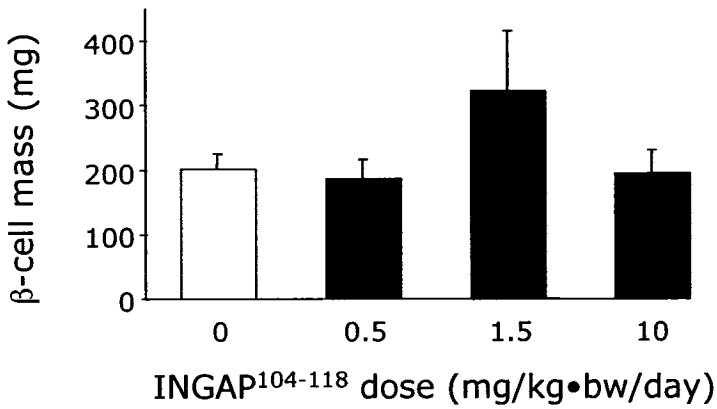

β-cell mass (mg)
400
300
200
100
0
0
0.5
1.5
10
INGAP104-118 dose (mg/kg•bw/day)

Figure 2.11: Total pancreatic β-cell mass in euglycaemic cynomolgus monkeys with escalating INGAP$^{104-118}$ dose. Cynomolgus monkeys treated with either saline (□, n=8) or INGAP$^{104-118}$ (■, n=8/dose) at 10, 100 or 400 mg/kg•bw/day for 90 days. β-cell mass was determined using a computer-assisted morphometric system. p=0.47

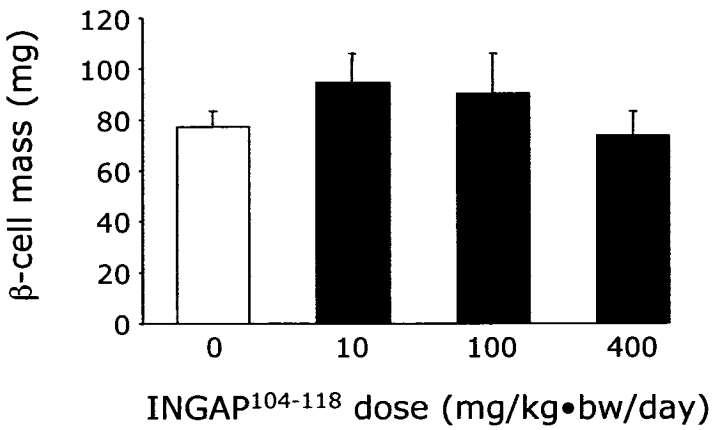
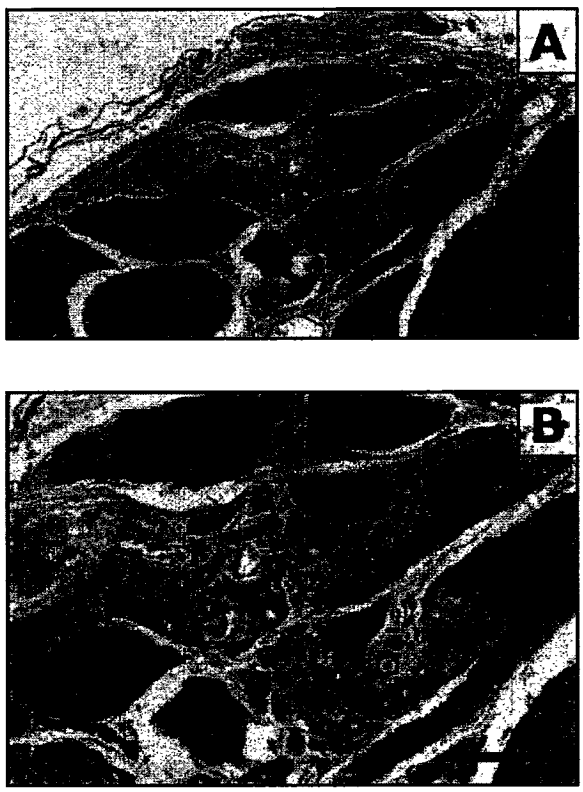

β-cell mass (mg)
120
100
80
60
40
20
0
0
10
100
400
INGAP104-118 dose (mg/kg•bw/day)

Figure 2.12: INGAP$^{104-118}$ administration to cynomolgus monkeys lead to dramatic transformation of pancreatic tissue. **(A)** Photomicrograph of an insulin stained pancreatic section from a normoglycaemic male monkey administered INGAP$^{104-118}$ (400mg/kg•bw/day) for 90 days. **(B)** Note the appearance of acinar metaplasia to duct-like structures and apparent islet neogenesis. (bar = 125 μm)


A
B

CHAPTER 3

Documentation of Homeostatic Regulation of Induced β-cell Mass Expansion

Published as:

Lipsett M, E Austin, M Castellarin, J Lemay, L Rosenberg. Evidence for the homeostatic regulation of induced β-cell mass expansion. Diabetologia Dec;49(12): 2910-2919, 2006.

Doctoral Candidate's Contribution - Manuscript 2

The doctoral candidate did not perform any of the animal manipulations depicted in this manuscript. Collection of all data and subsequent analyses were performed by the candidate with the exception of: total β-cell mass in euglycaemic hamsters (Rennian Wang) and the collection of the raw data for average islet size and density in partial duct obstructed hamsters (Lawrence Rosenberg). The doctoral candidate performed all other data and image collection, figure creation and statistical analysis as well as writing of the manuscript. All other co-authors contributed in manuscript preparation and Julie Lemay and Minh Duong aided the candidate with immunohistochemical staining.

3.0 ABSTRACT

Aims/hypothesis: Diabetes results from an insufficient insulin secreting beta-cell mass. Restoration of beta-cell mass through pharmaceutically induced, endogenous beta-cell mass expansion may revolutionize diabetes therapy. However, it remains to be determined whether the induced beta-cell mass expansion is under homeostatic regulation.

Materials and methods: Beta-cell mass expansion rates were derived from three separate studies of continuous stimulation of islet neogenesis, including the partial duct obstruction (PDO) of euglycaemic Syrian hamsters, administration of the beta-cell neogenic agent INGAP$^{104-118}$ to euglycaemic Syrian hamsters, as well as to euglycaemic CD-1 mice. Incidence of islet neogenesis, average beta-cell size, as well as beta-cell replication and apoptotic rates were determined.

Results: PDO led to a ~2.5-fold increase in endocrine tissue following 56 days ($p<0.05$). From day 0-to-7 the average rate of change of islet area was 12.7%/day, which then decreased to 5.3%/day from day 7-to-42 and further decreased to 2.8%/day from day 42-to-56. INGAP$^{104-118}$ administration to adult hamsters led to a 31% increase in total beta-cell mass at 30 days ($p=0.031$). From day 0-to-10 the average rate of beta-cell mass expansion was 148 μg/day, whereas from day 10-to-30 it decreased to 45 μg/day. INGAP$^{104-118}$ administration to adult CD-1 mice resulted in a ~2-fold increase in beta-cell mass following 31 days ($p=0.021$), however after 90 days there was no significant difference versus age-matched controls ($p=0.30$), though the neogenic beta-cell mass was ~4-fold greater ($p=0.026$). Beta-cell replication was decreased by 56% ($p<0.048$),

whereas beta-cell apoptosis was 4-fold greater ($p<0.003$) in 90-day INGAP$^{104-118}$-treated mice compared to age-matched controls.

Conclusions/interpretation: These data indicate that in the presence of ongoing islet neogenesis, homeostatic regulatory mechanisms intervene to match beta-cell mass to the prevailing metabolic requirements.

3.1 INTRODUCTION

Diabetes, whether type 1 or type 2, is arguably a disease ultimately caused by a loss of β-cell mass [7, 11, 12]. Over the last decade it has been recognized that tight glycaemic control can prevent diabetes-associated complications [62, 63, 256]. To date, the only means of achieving such stringent control has been the restoration of β-cell mass through pancreas or islet transplantation, which results in near perfect control of blood glucose levels without hypoglycaemic episodes that are associated with intensive insulin therapy [69, 70]. Pancreas transplantation is currently far more successful in maintaining long-term reversal of hyperglycaemia than islet transplantation, which is still an emerging technology [64, 71, 72]. However, both approaches are limited by the shortage of available organs and the need for chronic immunosuppression. Thus, novel strategies need to be developed that increase the insulin-producing β-cell mass.

An innovative alternative to islet replacement by transplantation is the induction of islet cell neogenesis, i.e. the growth of new insulin-producing cells from non-insulin-producing progenitors. This approach is appealing since the need for organ donation would be removed as a limiting factor. Moreover, the pharmacological induction of islet neogenesis has been confirmed to lead to β-cell mass expansion and reversal of hyperglycaemic states in various animal models [137, 139, 171, 201]. However, it is possible that the stimulation of cell differentiation and expansion could potentially lead to unrestricted growth and hyperfunction that is characteristic of neoplasia [128]. For example, unbridled islet

neogenesis, termed nesidioblastosis, was initially reported in 1938 [257]. Similar cellular mechanisms associated with islet neogenesis, including phenotypic dedifferentiation and increased cellular proliferation, are implicit in the transformation of cells to a cancerous state [128]. Moreover, growth factors used to stimulate islet neogenesis have also been associated with various cancers [128, 178, 205, 206]. It is therefore of utmost importance to determine if pharmaceutically induced β-cell mass expansion remains under homeostatic control.

Previous work does indeed suggest that the β-cell mass is responsive to changing metabolic demands. In the pregnant rat the β-cell mass initially expands and then involutes following parturition, closely matching β-cell mass to the prevailing metabolic demands [227, 228]. This regulation of β-cell mass may also be operative in humans, as increased islet neogenesis and β-cell mass expansion in the presence of obesity have been reported [12]. Comparable cell mass regulation has also been observed in lacrimal glands and breast tissue [229-235].

Accordingly, we hypothesise that in the setting of continual induction of islet neogenesis, homeostatic cellular mechanisms regulate β-cell mass to meet the prevailing functional needs. In order to test this hypothesis a new analysis of β-cell mass expansion dynamics was performed using data from two previously reported studies [85, 139]. To acquire additional corroborating data, an analysis of chronic pharmacological stimulation of islet cell neogenesis in a third study was performed in which total β-cell mass, neogenic β-cell mass, average β-cell size, replication and apoptosis were determined.

3.2 MATERIALS AND METHODS

Data from three separate animal studies are reported: (1) partial duct obstruction (PDO) of euglycaemic adult hamsters [85]; (2) administration of a pentadecapeptide with the same amino acid sequence as residues 104-118 of islet neogenesis-associated protein (INGAP$^{104-118}$) to euglycaemic hamsters [139]; and (3) administration of INGAP$^{104-118}$ to euglycaemic mice. All animal studies were performed in accordance with Canadian Council of Animal Care and McGill University guidelines.

3.2.1 Animal Models

3.2.1.1 Partial Duct Obstruction

Experiment 1, first described in 1983, involved the partial duct obstruction of adult female Syrian hamsters (Charles River Breeding Laboratories, Syracuse, NY; n=24) by performing a midline laporotomy and wrapping the head of the pancreas with a 2 mm wide piece of cellophane tape or sham operation [85]. After 7, 42 and 56 days hamsters were anesthetized with sodium pentobarbital, and a thoracotomy performed. Tissues were perfused with a solution of 1% gluteraldehyde and 4% formaldehyde and the pancreas was then removed and post-fixed in 10% formalin and processed for immunohistochemical analysis.

3.2.1.2 Administration of INGAP$^{104-118}$ to Euglycaemic Hamsters

Experiment 2, first described in 2004, involved administration of INGAP$^{104-118}$ (a Reg family member) to 8 week old female Syrian hamsters (Charles River Breeding Laboratories, St Constant, QC, Canada; 250 μg twice daily; n=30) or an

equivalent volume of saline (n=20) [139]. After 10 and 30 days hamsters were anesthetized with sodium-pentobarbitol (40 mg/kg body wt, i.p.) and killed by exsanguination. The pancreas was removed, blotted on tissue paper, weighed, fixed in 10% formalin and processed for immunohistochemical analysis.

3.2.1.3 Administration of $INGAP^{104-118}$ to Euglycaemic Mice

Experiment 3 involved the administration of $INGAP^{104-118}$ to 6-8 week old CD-1 mice (Charles River, MA, USA) for 31 days (0, 50, 500, or 2500 μg once daily, i.m.; n=40) or for 90 days (0, 1000, 2500 or 5000 μg once daily, s.c.; n=78). Animals were killed by cervical dislocation, the pancreas excised, blotted, weighed, fixed in 10% formalin and processed for routine immunohistochemistry.

3.2.2 Immunohistochemistry

Pancreatic sections, 4-5 μm thick, were de-waxed in xylene and endogenous peroxidase activity was blocked using a 3% solution of hydrogen peroxide in methanol (for sections to be stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich Canada, Oakville, ON, Canada)). Sections were then washed in PBS and incubated with blocking buffer (Zymed Laboratories, San Francisco, CA, USA) for 15 minutes at room temperature. Slides were stained with one or more primary antibodies, including insulin (guinea pig anti-porcine, 1:1000; Dako Diagnostics Canada, Mississauga, ON, Canada); proliferating cell nuclear antigen (PCNA) (mouse anti-rat, clone PC10, 1:100; Dako Diagnostics Canada) and TUNEL detection kit (Roche, Laval, QC, Canada).

Slides stained for PCNA and TUNEL were incubated with 0.1M citrate buffer for 9 minutes at 90°C for antigen retrieval before primary antibody application. Slides were incubated with primary antibodies overnight at 4°C before being washed in PBS and incubated with the secondary antibodies for 20 minutes (Histostain Plus Kit, Zymed Laboratories, San Francisco, CA, USA) or 1 hour (rhodamine-conjugated anti-guinea 1:200; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at room temperature. After washing in PBS immunofluorescent sections were cover-slipped using Vectashield (Vector Laboratories, Burlingame, CA, USA) and light sections were incubated with either horseradish peroxidase or alkaline phosphatase conjugate (Histostain Plus Kit) for 20 minutes, and developed using DAB or New Fuchsin (Dako), respectively. All light immunohistochemical slides were counterstained with Harris' haematoxylin (Sigma-Aldrich) and cover slipped using Permount (Fisher Scientific, Ottawa, ON, Canada).

3.2.3 β-cell Mass Determination

Percentage β-cell area was determined through analyses of insulin-stained sections as previously described [87, 89]. Histological sections were analysed using an Olympus BX60 microscope and Image-Pro Plus software, version 4·0 (Olympus Canada, Markham, ON, Canada; experiments 1 and 2), or using a Zeiss Axioskop 40 microscope and Northern Eclipse software, version 6·0 (Empix Imaging, Mississauga, ON, Canada; experiment 3). Islets from insulin-stained sections were identified, traced and thresholded using the software system to

determine stained tissue area. The total pancreatic tissue area was also measured and the percent β-cell area calculated. This percentage was then multiplied by the weight of the excised organ to derive the β-cell mass.

Classification of neogenic β-cell mass was performed as previously described [37, 87, 89, 137]. Duct-associated neogenic β-cell structures were categorized as small (<500 μm^2, as determined by the image analysis system) insulin-positive clusters associated with the ductal epithelium (Figure 3.1A). Acinar-associated neogenic β-cell structures were categorized as small (<500 μm^2) acinar-associated structures devoid of any other juxtaposed endocrine cells (Figure 3.1B). Focal areas of neogenesis were categorized as structures characterized by disorganized insulin-positive staining with apparent infiltration of duct-associated cells (Figures. 3.1C and D).

For the analysis of hamster pancreases with partially duct obstruction (experiment 1), percentage islet area was used as a surrogate for β-cell mass. Briefly, the number of islets per mm^2 of pancreatic tissue and the average islet size were determined by manually tracing a minimum of 100 islets per animal. Total islet area was then determined by multiplying the average islet size by the number per mm^2.

3.2.4 Determination of β-cell Size, Replication and Apoptosis

Average β-cell size, replication and apoptotic indices were determined for mice administered saline or $INGAP^{104-118}$ for 31 or 90 days (2500 μg/day) in experiment 3. The total number of β-cell nuclei and the number of PCNA-positive

β-cell nuclei and TUNEL-positive β-cell nuclei were determined using the Northern Eclipse image analysis system (Empix Imaging). Average β-cell size was then determined as total insulin area divided by total number of β-cell nuclei for each animal. β-cell replication indices were determined as the number of β-cell PCNA-positive nuclei divided by total number of β-cell nuclei (average number of nuclei counted/animal: 3670±411; n=5 $INGAP^{104-118}$, n=4 saline for each time point). Apoptotic indices were determined as total number of TUNEL-positive nuclei divided by total number of β-cell nuclei for each animal (average number of nuclei counted/animal: 2858±340; n=5 for each time and treatment).

3.2.5 β-cell Mass Dynamics

The average daily rate of change of islet area (experiment 1) or β-cell mass (experiment 2) was determined by subtracting the initial value from the islet area of the next time point and dividing by the number of days between time points. Curves predicting islet or β-cell mass expansion dynamics were derived from islet area or β-cell mass from each treatment group, expressed as a percentage of age-matched control animals (Fig. 7). The β-cell mass dynamics curve for experiment 3 was constructed using the β-cell mass from mice in the 2500 μg/day group.

3.2.6 Statistical Analyses

All values are reported ± standard error of measurement. Student's t-test or one-way ANOVA were performed where appropriate. Results with a p value of < 0.05 were considered significant.

3.3 RESULTS

Following partial duct obstruction, average islet size initially decreased (day 7) before increasing thereafter by ~25% (day 56, $p<0.05$; Figure 3.2A). Islet density (the number of islets per mm^2), however, was consistently double that of control animals ($p<0.05$; Figure 3.2B), resulting in an approximately 2.5-fold increase in islet area by day 56 ($p<0.05$; Figure 3.2C). From day 0 to 7, the average daily rate of change of percent islet area ($d(\%\text{ islet area})/dt_{day}$) was determined to be 12.7%/day. This rate then declined to 5.3%/day from day 7 to 42 and again to 2.8%/day from day 42 to 56 (Figure 3.2D).

Administration of $INGAP^{104-118}$ to euglycaemic hamsters induced a 2.5-fold increase in total duct-associated β-cell mass ($p=0.004$; Figure 3.3A) and an approximate 3-fold increase in acinar-associated neogenic β-cell mass ($p<0.001$; Figure 3.3B) [139]. Total β-cell mass increased by 44% at day 10 and 31% at day 30 in comparison to saline controls ($p=0.031$; Figure 3.3C) [139]. This increase of 44% corresponds to an initial β-cell mass expansion rate of 148 μg/day (from day 0 to day 10). The average rate of expansion then decreased to 45 μg/day (day 10 to 30; Figure 3.3D) representing a reduction in the rate of β-cell mass expansion of ~70%..

Following the administration of $INGAP^{104-118}$ to euglycaemic CD-1 mice induced, a dose-dependent increase in total β-cell mass at 31 days (mean total mass: 0.83±0.09, 1.40±0.25, 1.78±0.37, 1.84±0.36 mg for 0, 50, 500, 2500 μg/day, respectively; $p=0.021$; Figure 3.4A). At 90 days of treatment there was no difference in total β-cell mass between any of the groups (1.87±0.15, 1.55±0.12,

1.88±0.13, 1.69±0.18 mg for 0, 1000, 2500, 5000 μg/day, respectively; p=0.30; Figure 3.4B). The observation that the β-cell mass was not different from control animals at 90-days, despite the persistence of a neogenic stimulus, led us to investigate the potential mechanisms of β-cell mass expansion and involution in the CD-1 mice.

To investigate whether the degree of β-cell neogenesis differed between control mice (saline) and those administered INGAP$^{104-118}$ for 90 days, neogenic structures were quantified from insulin stained slides. In INGAP$^{104-118}$-treated mice, the number of small duct-associated neogenic structures doubled (p=0.029; Figures 3.1A and 3.4C) and the number of focal areas of neogenesis increased approximately 4-fold (p=0.008; Figures 3.1C, D and 3.4D) versus age-matched control mice. The neogenic β-cell mass increased ~4-fold in INGAP$^{104-118}$-treated mice compared with that in control mice (p=0.026; Figure 3.5A). This result, together with the observation that total β-cell mass of the INGAP$^{104-118}$-treated mice did not differ from control animals (p=0.30; Figure 3.5B) suggests that there is a corresponding increase in β-cell loss to maintain the β-cell mass at control levels (Figure 3.5C).

In order to determine if β-cell atrophy, decreased replication or apoptosis could account for this proposed β-cell loss, analyses of average β-cell size, replication rate and apoptotic index of 31-day and 90-day control and INGAP$^{104-118}$-treated mice were performed. There were no significant difference in average β-cell size (119.1±5.7 vs. 128.4±3.9 μm^2 p=0.18; Figure 3.6A) or replication (0.66±0.12 vs. 0.67±0.16% p=0.94; Figures 3.6B and D) in control versus

INGAP$^{104-118}$-treated mice after 31 days, respectively. However, following 90 days of INGAP$^{104-118}$ treatment there was a significant increase in average β-cell size (119.6±2.5 vs. 141.9±1.2 μm^2, Figure 3.6A; $p<0.001$). Conversely, there was a concomitant decrease of ~50% in the β-cell replication index of INGAP$^{104-118}$-treated mice (0.79±0.17 vs. 0.35±0.05 %; $p=0.048$; Figure 3.6B). When β-cell apoptosis was analysed at 31 days by TUNEL, no significant difference was observed between the control and INGAP$^{104-118}$-treated mice (0.132±0.027 vs. 0.091±0.035 %, $p=0.33$, respectively, Figures 3.6C and E). In contrast, following 90 days of treatment there was an approximate 4-fold increase in TUNEL-positive β-cells in INGAP$^{104-118}$-treated mice (0.079±0.021 vs. 0.313±0.016 %; $p=0.003$; Figure 3.6C).

Figure 3.1: Insulin stained sections (brown) from CD-1 mice administered INGAP$^{104-118}$ (2500 μg/day) for 90 days illustrating small, extra-islet duct-associated β-cell clusters **(A)**, an extra-islet acinar-associated single β-cell **(B)**, and duct-associated focal areas of neogenesis **(C, D)**. (bar = 50 μm)

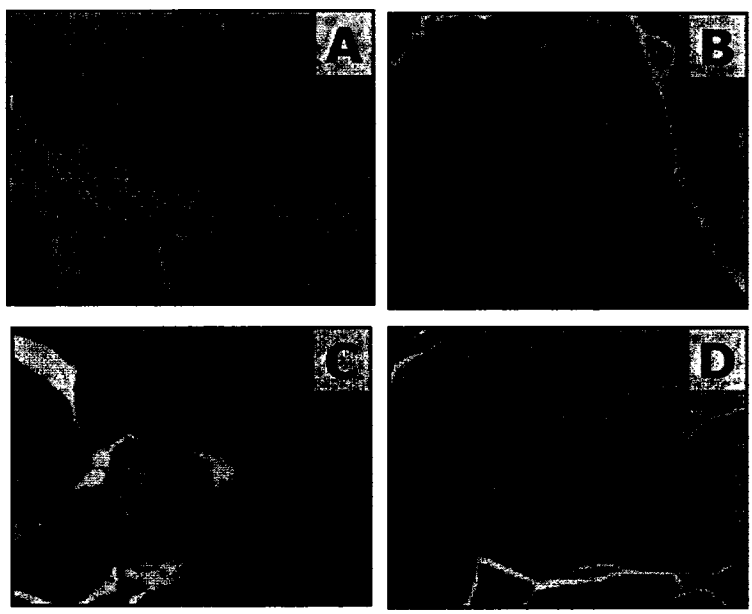

A
B
C
D

Figure 3.2: Average islet size **(A)**, islet density **(B)** and insulin area (percent of total tissue) **(C)** of hamsters following sham surgery (□, n=6 per time point) or partial duct obstruction (■, n=6 per time point). **(D)** Daily rate of change of insulin area in hamsters with partial duct obstruction (n=6 per time point). *$p<0.05$ versus sham control hamsters.

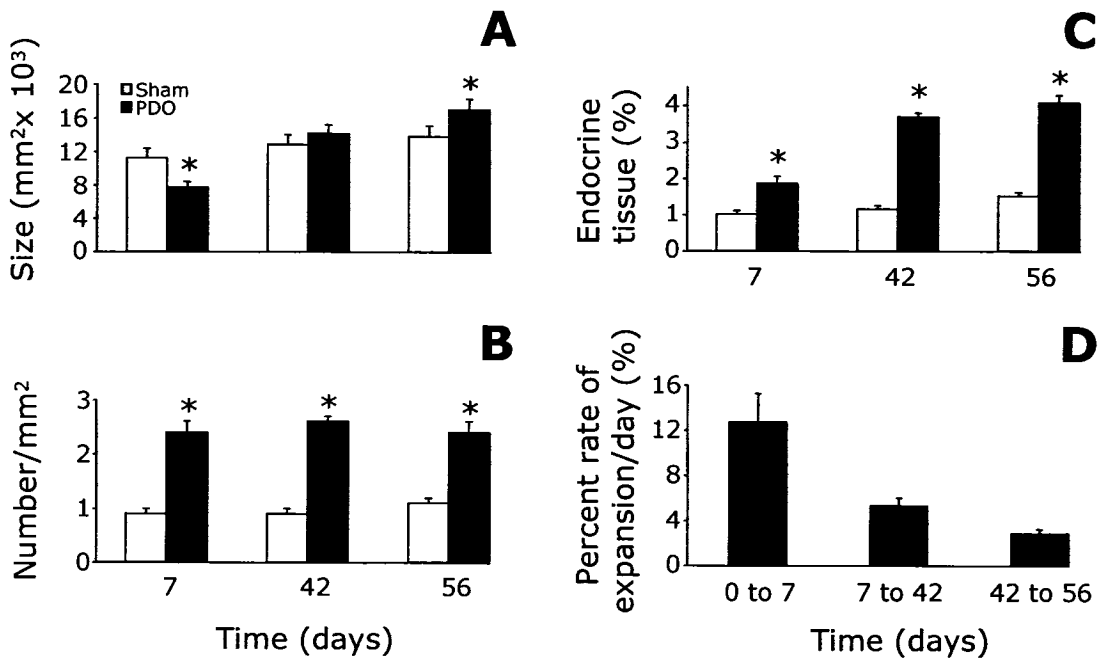

A
Size (mm²x 10³)
Sham
PDO
20
16
12
8
4
0
*
*
C
Endocrine tissue (%)
4
3
2
1
0
*
*
*
7
42
56
B
Number/mm²
3
2
1
0
*
*
*
7
42
56
Time (days)
D
Percent rate of expansion/day (%)
16
12
8
4
0
0 to 7
7 to 42
42 to 56
Time (days)

Figure 3.3: (A) Total duct-associated β-cell mass in hamsters administered saline (□, n=5 per time point) or INGAP$^{104-118}$ (■, n=5 per time point) for 10 or 30 days; **(B)** extra-islet acinar-associated neogenic β-cell mass; **(C)** total β-cell mass; **(D)** daily rate of change of β-cell mass in INGAP$^{104-118}$-treated hamsters (n=5 per time point). *$p<0.05$ versus saline control mice.

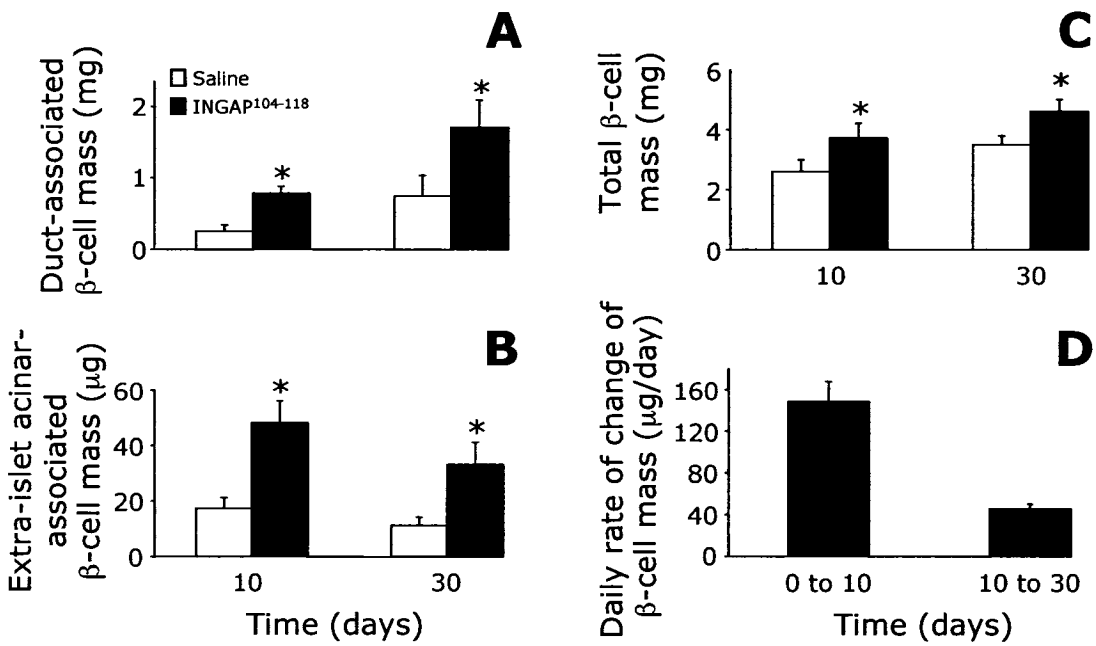

A
Saline
INGAP104-118
Duct-associated β-cell mass (mg)
0
1
2
*
*
B
Extra-islet acinar-associated β-cell mass (μg)
0
20
40
60
*
*
10
30
Time (days)
C
Total β-cell mass (mg)
0
2
4
6
*
*
10
30
D
Daily rate of change of β-cell mass (μg/day)
0
40
80
120
160
0 to 10
10 to 30
Time (days)

Figure 3.4: (A) β-cell mass in CD-1 mice administered saline or $INGAP^{104-118}$ at various doses for 31 days (n=10 per group); **(B)** β-cell mass in mice administered saline or $INGAP^{104-118}$ for 90 days (n=20 per group, except for the 5000 μg/day group, where n=18); **(C)** density of small duct-associated insulin-positive clusters in 90-day treated animals (n=8 per group); **(D)** density of focal areas of neogenesis in 90-day treated animals (n=8 per group). *$p<0.05$ vs. saline control mice.

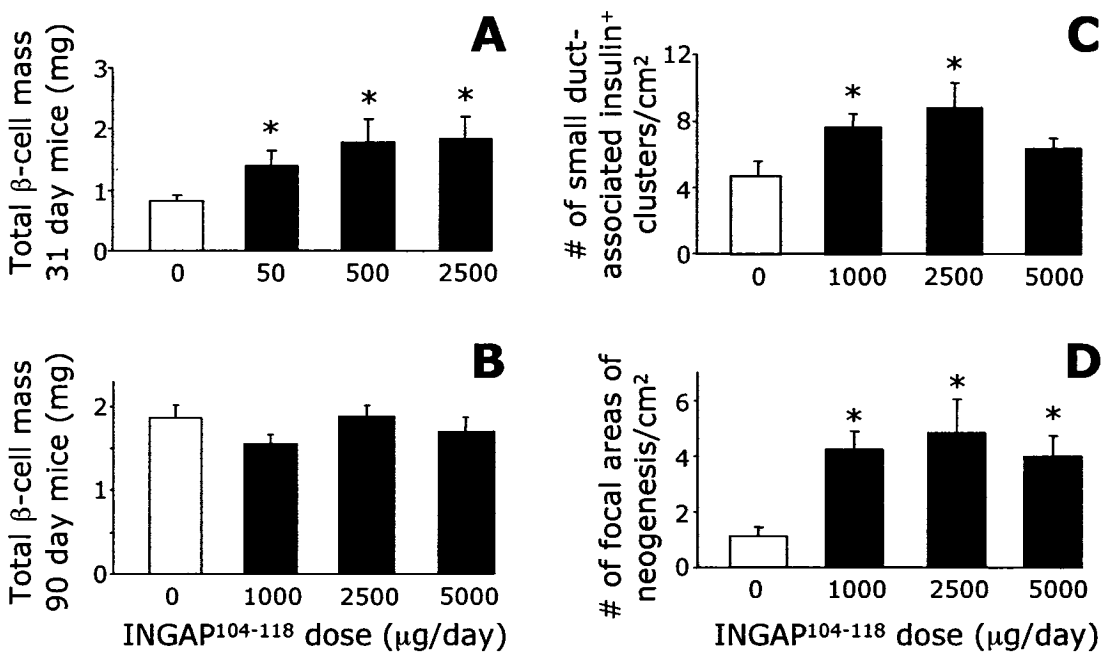

A
Total β-cell mass
31 day mice (mg)
3
2
1
0
*
*
*
0
50
500
2500
B
Total β-cell mass
90 day mice (mg)
2
1
0
0
1000
2500
5000
INGAP104-118 dose (μg/day)
C
# of small duct-
associated insulin+
clusters/cm2
12
8
4
0
*
*
0
1000
2500
5000
D
# of focal areas of
neogenesis/cm2
6
4
2
0
*
*
*
0
1000
2500
5000
INGAP104-118 dose (μg/day)

Figure 3.5: (A) Total neogenic β-cell mass from 90-day saline and INGAP$^{104-118}$-treated mice (n=8 per group); **(B)** total β-cell mass in 90-day saline and INGAP$^{104-118}$-treated mice; **(C)** predicted net β-cell loss in 90-day INGAP$^{104-118}$-treated mice, derived from values in **(A)** and **(B)**. *$p<0.05$ versus saline control mice.

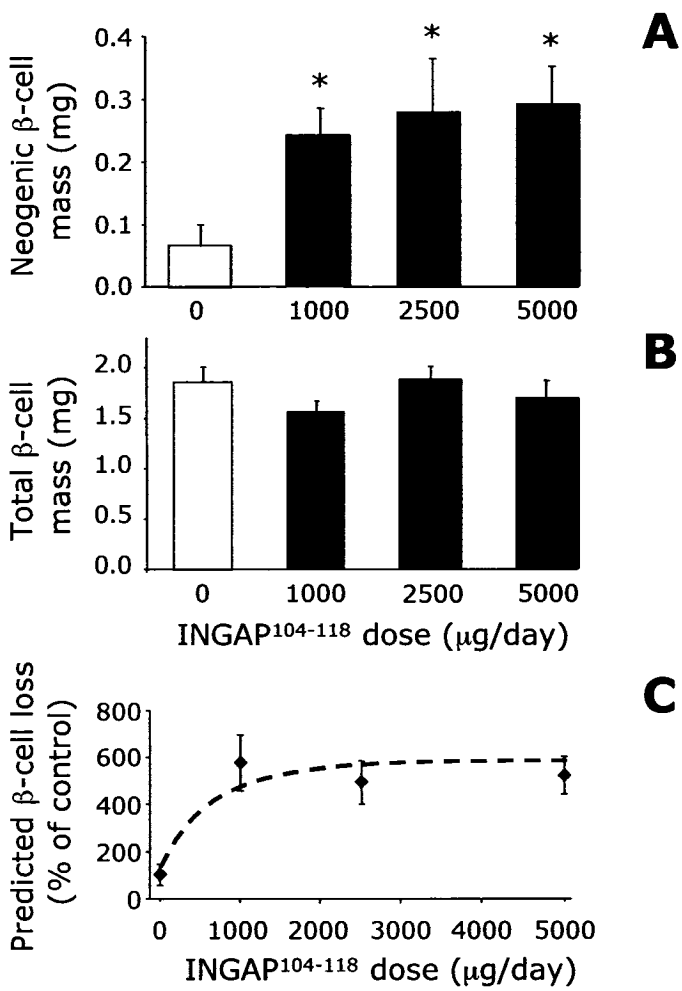

A
Neogenic β-cell mass (mg)
0.4
0.3
0.2
0.1
0.0
*
*
*
0
1000
2500
5000
B
Total β-cell mass (mg)
2.0
1.5
1.0
0.5
0.0
0
1000
2500
5000
INGAP104-118 dose (μg/day)
C
Predicted β-cell loss (% of control)
800
600
400
200
0
0
1000
2000
3000
4000
5000
INGAP104-118 dose (μg/day)

Figure 3.6: (A) Average β-cell size in 31- and 90-day saline (□, n=4 per time point) and INGAP$^{104-118}$-treated mice (■, n=5 per time point); **(B)** percentage of total β-cell nuclei positive for PCNA in CD-1 mice treated with saline (n=4 per time point) or INGAP$^{104-118}$ for 31 or 90 days (n=5 per time point); **(C)** percentage of total β-cell nuclei positive for TUNEL in CD-1 mice treated with either saline or INGAP$^{104-118}$ for 31 or 90 days (n=5 for each group and time point). **(D)** Representative photomicrograph of an insulin and PCNA double-stained pancreatic section from an INGAP$^{104-118}$-treated mouse. **(E)** Representative photomicrograph of a TUNEL-stained pancreatic section from an INGAP$^{104-118}$-treated mouse. *$p<0.05$ versus saline control mice. (bar = 50 μm)

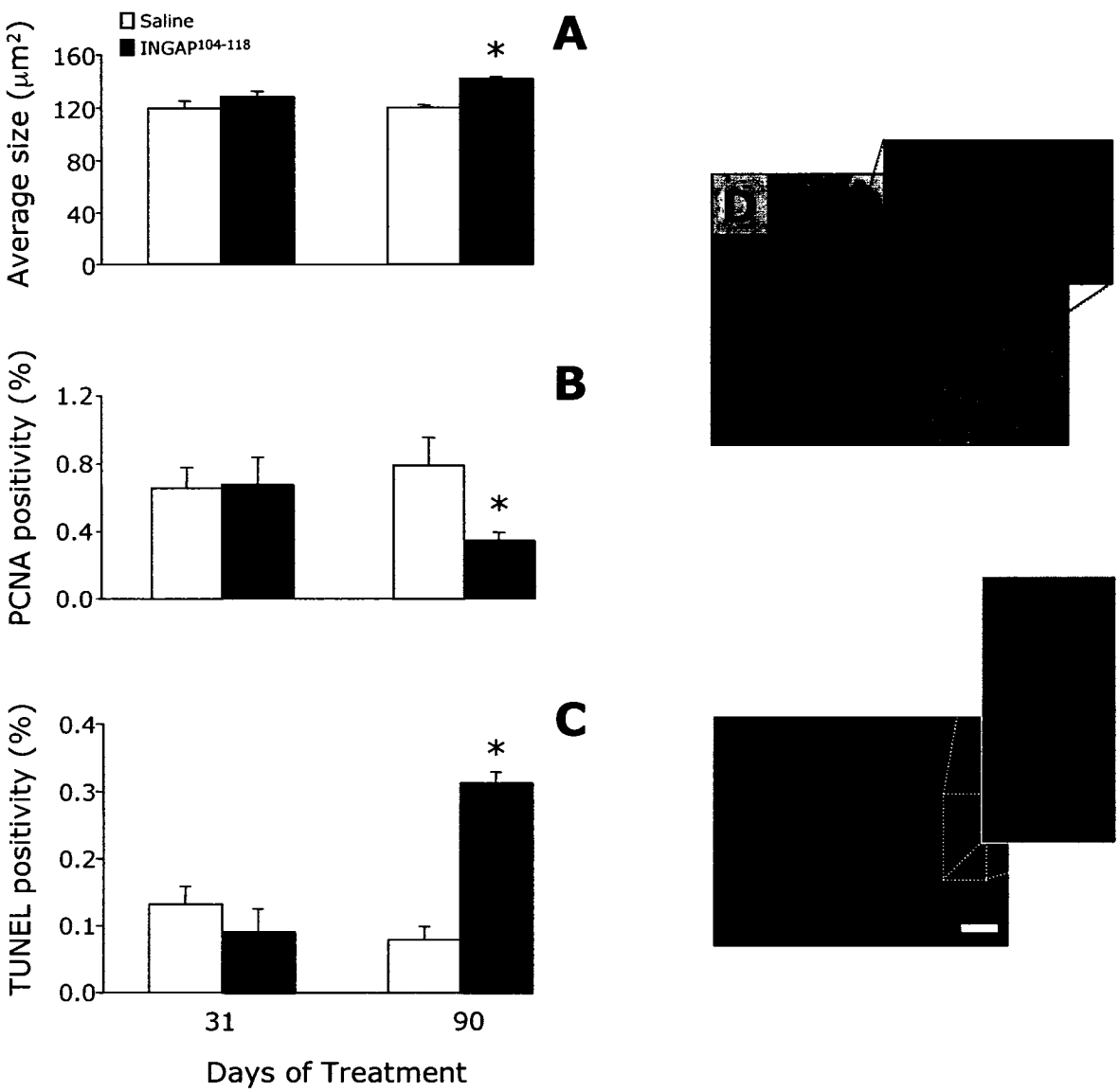
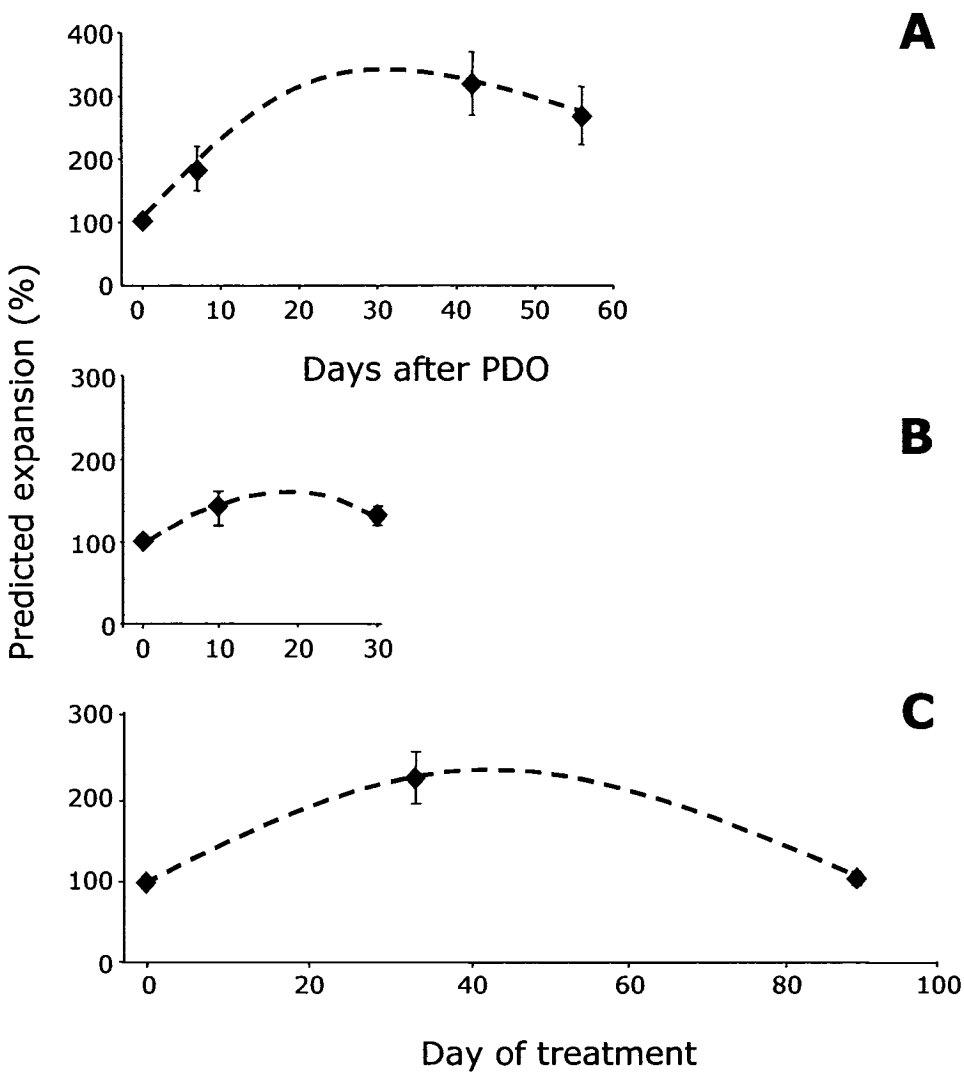

Saline
INGAP104-118
A
Average size (μm²)
160
120
80
40
0
*
B
PCNA positivity (%)
1.2
0.8
0.4
0.0
*
C
TUNEL positivity (%)
0.4
0.3
0.2
0.1
0.0
*
31
90
Days of Treatment

Figure 3.7: (A) Predicted β-cell mass expansion curves based on data from partial duct obstructed hamsters; **(B)** hamsters administered 500 μg/day $INGAP^{104-118}$ for up to 30 days; **(C)** CD-1 mice administered $INGAP^{104-118}$ (2500 μg/day) for up to 90 days. All values represent the percent change from age-matched controls for islet size (μm^2) · islet density ($\#/mm^2$) **(A)** or total β-cell mass **(B)** and **(C)**.


A
B
C
Predicted expansion (%)
Days after PDO
Day of treatment
400
300
200
100
0
0
10
20
30
40
50
60
300
200
100
0
0
10
20
30
300
200
100
0
0
20
40
60
80
100

3.4 DISCUSSION

There is accumulating evidence from different animal models suggesting that pancreatic β-cell mass is dynamic and changes to accommodate the body's needs [79, 89, 226-228, 258, 259]. Here we report that regulatory mechanisms do indeed appear to be operative in the regulation of the insulin-producing β-cell mass, even in the setting of continual stimulation of islet neogenesis. Moreover, the data reported herein suggests that this regulation occurs through decreased β-cell replication and increased β-cell apoptosis.

Although the three studies of induced islet expansion analysed in this report were somewhat different in design, they all shared an initial increase in β-cell mass followed by an involution of this expanded β-cell mass towards age-matched control levels (Figure 3.7). This expansion and subsequent involution is similar to that observed during pregnancy and post-partum periods in rodents [227, 228]. During pregnancy the β-cell mass of the gravid female almost doubles in response to the increased metabolic demands and altered hormonal milieu [227, 228]. However shortly after birth, when the hormonal environment and metabolic demands revert to the non-pregnant state, the expanded β-cell mass involutes, returning to normal levels through β-cell atrophy, increased β-cell apoptosis and decreased β-cell replication [228]. This mirrors quite closely what we have observed in the setting of continual stimulation of islet neogenesis.

The dynamic and highly complex nature of these homeostatic mechanisms is highlighted by the increase in average β-cell size in those animals treated with the Reg3 member $INGAP^{104-118}$ for 90 days. This is perhaps somewhat

counterintuitive, since β-cell hypertrophy would contribute to an expansion of the β-cell mass, not involution, as was seen in 90-day treated mice. However, this finding must be viewed in the context of an approximate 50% reduction in β-cell replication concomitant with a 4-fold increase in apoptotic β-cell death. This observation of β-cell hypertrophy following 90-day INGAP$^{104-118}$ stimulation is in agreement with what has previously been reported in isolated rodent islets [179]. Interestingly, the over-expression or administration of Reg1 has been shown to lead to an increase in β-cell mass through an increase in β-cell replication, although β-cell size was not assessed in these studies [175, 182, 260, 261]. The increase in β-cell replication following Reg1 administration contrasts with our study as we observed that β-cell mass more than doubled following 31 days of treatment in mice, without a concomitant increase in average β-cell size, PCNA labelling of β-cells, or a decrease in TUNEL-positive β-cells. This suggests that the increase in β-cell mass comes from an increase in islet neogenesis. Thus, INGAP$^{104-118}$ may lead to β-cell mass expansion through a different mechanism than the other Reg family members.

Even though enhanced β-cell apoptosis and decreased β-cell proliferation appear to be responsible for the involution of the expanded β-cell mass, the underlying stimulus prompting involution is not readily apparent. The fact that plasma glucose and insulin levels in the saline and INGAP$^{104-118}$-treated hamsters are not significantly different would suggest that other factors are responsible for triggering the process of involution [139].

β-cell involution can result from removal of a β-cell growth/survival factor, the direct stimulation of β-cell death, or both. For example, administration of gastrin is associated with β-cell growth and decreased β-cell apoptosis in the setting of pancreatic regeneration [137]. This suggests that removal of gastrin-stimulated growth could aid in the involution of β-cell mass. Furthermore, gastrin has been shown to regulate the expression of pancreatic Reg genes, suggesting another means of β-cell cell mass regulation [262]. On the other hand, elevated levels of cytokines, specifically, IL-1β, TNF-α and IFN-γ, are directly linked to increased β-cell apoptosis levels [263]. Thus, it is possible that decreased levels of growth factors, as well as increased levels of cytokines could be responsible for initiating the involution of the expanded β-cell mass in our models. We are currently furthering our understanding of the control of endogenous Reg protein and, more specifically, INGAP expression, which may also play a role in these models [264].

Recent information supports the effectiveness of islet neogenesis therapy to reduce HbA_{1c} values [265]. The finding that continual stimulation of islet cell neogenesis does not result in unbridled β-cell mass expansion, suggests that induction of islet neogenesis would be a safe therapy for individuals with diabetes. However, there may still be some issues to resolve. For example, islet neogenesis can occur through one of three pathways: stem cell activation, direct transdifferentiation of mature cell phenotypes or indirect transdifferentiation of mature cell phenotypes. The direct transdifferentiation pathway is simply the differentiation of a mature cell into an insulin-producing cell without the cell going

through a primordial stage, and is suggested by the morphological observation of extra-islet duct- and acinar-associated single β-cells [37, 89, 139]. Indirect transdifferentiation involves the dedifferentiation of previously terminally differentiated cells into a more primitive cell phenotype followed by redifferentiation into a mature β-cell [89, 135-137]. This metaplastic transformation of acinar tissue, leading to the induction of so-called tubular complexes, has been observed in many different models of pancreas regeneration and islet expansion [89, 136, 137, 169, 171, 205]. The significance of these duct-like structures with respect to carcinogenesis remains to be fully explored. Metaplastic transformation of acinar tissue has not been observed in any of the rodent studies of INGAP$^{104-118}$ administration.

Even though the induction of β-cell mass expansion does occur in a regulated manner, there still remains the question as to whether or not pharmaceutical expansion of the insulin-producing cell mass will prove effective in ameliorating both types of this disease. Results from clinical Phase 2a proof of principle trials suggest that new β-cells can be formed in the setting of both type 1 and type 2 diabetes [265]. It still remains to be determined whether, and to what extent, these newly formed β-cells are protected from the autoimmune insulitis that characterizes type 1 diabetes [266].

In conclusion, continual stimulation of islet neogenesis does not lead to unregulated cell expansion. Heightened β-cell apoptosis and diminished β-cell replication serve to regulate induced β-cell mass expansion to meet the body's prevailing needs. This therapeutic approach seems to currently have the greatest

potential in the setting of type 2 diabetes, although with advances in immunosuppression, it could be justifiably applied in the setting of type 1 diabetes.

3.5 ACKNOWLEDGEMENTS

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BRIDGING STATEMENT BETWEEN MANUSCRIPTS

In Chapter 3 it is observed that continual stimulation of islet neogenesis via $INGAP^{104-118}$ administration does not lead to unbridled β-cell proliferation. Induced β-cell mass expansion is regulated through inhibition of β-cell replication as well as through an augmentation of β-cell apoptosis. The observation that homeostatic mechanisms remain active to match the resultant β-cell mass with the body's needs indicates that $INGAP^{104-118}$ may indeed be safe to administer as a human therapy. The next step is to determine if $INGAP^{104-118}$ therapy will be effective in stimulating islet neogenesis in humans, leading to an expanded β-cell mass. Thus, the aim of Chapter 4 was to determine the efficacy of $INGAP^{104-118}$ in stimulating islet neogenesis from non-endocrine human pancreatic tissue.

CHAPTER 4

Documentation of Morphological Plasticity and Neogenic Potential of Adult Human Acinar Tissue

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(Pancreas, in press)

Doctoral Candidate's Contribution - Manuscript 3

The doctoral candidate performed all human tissue collection, experimental and immunohistochemical procedures, data collection and analysis, statistical analyses and manuscript writing. Other co-authors contributed in manuscript preparation and technical expertise in pancreatic digestions.

4.0 ABSTRACT

Objectives: The plasticity of pancreatic tissue is demonstrated in many pancreatic diseases. It has previously been shown that pancreatic islet-to-duct transformation and acinoductal metaplasia have been associated with both pancreatic regeneration and adenocarcinoma in various *in vivo* and *in vitro* settings. Understanding this inherent morphogenetic plasticity of the adult pancreas could lead to new therapeutic approaches to pancreatic disease.

Methods: Cadaveric human pancreases (n=7) were digested and purified acinar tissue, which was ~85% immunoreactive for amylase and ~15% CK19 immunoreactive, was embedded in a type-1 collagen matrix and cultured in a differentiation medium (DM) consisting of DMEM/F12 medium supplemented with cholera toxin (CT, 100 ng/ml), EGF (10 ng/ml) and insulin (24 mU/ml) for 8 days. Following this initial period, the resulting tissues were cultured in DM without CT, supplemented with gastrin (50 nM) and HGF (10 ng/ml), with $INGAP^{104-118}$ (167 nM) or with gastrin + HGF + $INGAP^{104-118}$ for 6 days. Tissue samples were then analysed for amylase, cytokeratin 19, PDX-1, and endocrine hormone immunoreactivity as well as dithizione positivity.

Results: Following 8 days of culture, ~90% of acini transformed into duct-like structures (DLS). This acinoductal transformation was characterized by a complete absence of amylase staining, with virtually all cells CK19 immunoreactive. Addition of $INGAP^{104-118}$ led to an ~18-fold increase in PDX-1 immunoreactivity, though without an observed increase in insulin production as measured by dithizone positivity. However, when acinar-derived DLS were cultured with gastrin + HGF + $INGAP^{104-118}$ the total incidence of dithizone positive

structures increased ~6-fold (10.9±2.9 vs. 1.7±0.4%; p=0.037). Treatment with gastrin + HGF alone led to no significant change in any of the measured parameters.

Conclusions: We have developed a novel *in vitro* model of adult human acinoductal metaplasia that will aid, not only in developing new methods of expanding β-cell mass, but also provide insights into pancreatic carcinogenesis.

4.1 INTRODUCTION

Recently, the potential of adult pancreatic acinar tissue to transform into various cell types has been demonstrated [89, 115, 128, 129, 136, 137]. Direct transformation of adult rodent acini into insulin-containing tissues, as well as indirect acinar-to-islet transformation identified as acinar-to-duct transformation (acinoductal metaplasia) followed by subsequent duct-to-endocrine redifferentiation, can both lead to an expanded β-cell mass. Acinoductal metaplasia, which is observed in numerous *in vivo* settings in association with pancreatic regeneration and β-cell mass expansion, occurs through acinar-to-duct transdifferentiation [115, 129]. These metaplastic structures, commonly referred to as tubular complexes, appear to contain enhanced islet neogenic potential [89, 136, 137, 163, 170]. These findings, together with the fact that acinar tissue makes up approximately 80% of the adult pancreas, suggests that therapies directed at enhancing acinar-derived β-cell mass expansion could be very successful at augmenting the β-cell mass and ameliorating diabetic hyperglycaemia.

Islet neogenesis from pancreatic tubular complexes is thought to occur under the influence of epidermal growth factor (EGF), gastrin, HGF and likely many other growth factors [137, 160, 163, 172, 267]. Since these cytokines have demonstrated efficacy in animal models of islet expansion, it is suggested that they may also have potential to increase human β-cell mass. For example, combination therapy with EGF and gastrin has been observed to increase the insulin content of human pancreatic islet preparations cultured for up to 28 days,

presumably through the differentiation of duct cells present in the semi-purified islet culture [203].

In addition to EGF and gastrin, Reg family members are also upregulated in settings of pancreatic regeneration [158, 162, 175, 180-182, 187, 188, 190]. Islet Neogenesis Associated Protein (INGAP), a hamster Reg3 protein, has been shown to stimulate duct-to-islet neogenesis leading to β-cell mass expansion and reversal of diabetic hyperglycaemia in a mouse model, as well as increase acinar-to-islet differentiation in Syrian hamsters [139].

Strategies to increase β-cell mass are recognized as being increasingly important. The reason for this lies in the ongoing limitations related to widespread application of human islet transplantation as a means to restore normoglycaemia in type 1 diabetic recipients. Donated organs remain a very scare resource and to date, supraphysiologic numbers of islets have been required to achieve long-term euglycaemia. Recently, a number of attempts have been made to increase the efficiency of islet procurement and thus increase the number of people who can benefit from islet transplantation therapy [125, 203, 268-270]. These attempts are based on expanding the number or viability of isolated islets, which only constitute 1-3% of the total pancreatic tissue [125, 203, 268]. If the unused and unwanted pancreatic acinar tissue, could be transformed into islet tissue there would be an immediate increase in β-cell mass available for transplantation. Only a small percentage would need to be transformed in order to greatly expand the transplantable β-cell mass due to the large proportion of acinar tissue in the pancreas. We report here the development of an *in vitro* model of human acinar-

to-duct-to-islet transformation. We demonstrate that the newly formed duct-like tissue exhibits islet neogenic potential and that β-cell mass expansion can be inducted by a combination of gastrin, HGF and INGAP$^{104-118}$. This novel model is not only highly relevant with respect to developing new sources of islet tissue, but it may also provide insight into the role of acinoductal metaplasia in the initiation of other pancreatic diseases.

4.2 MATERIALS AND METHODS

4.2.1 In Vitro Acinar-to-β-cell Differentiation

Pancreases from seven cadaveric humans donors (cause of death was cerebral ischemia with no reported pancreatic disease) were dissected from surrounding tissues and cannulated to permit intraductal infusion with Liberase HI (1.4 mg/ml) (Boehringer Mannheim, Indianapolis, IN, USA) according to established protocols [125]. Purification was achieved using a continuous density ficoll gradient (1.077-1.100 g/ml Biochrom KG, Germany) and a COBE 2991 cell processor (COBE BCT, Denver, CO., USA). Tissue from the highest densities were washed and stained with diphenylthiocarbazone (dithizone, Sigma-Aldrich) to confirm lack of insulin positive cells.

Freshly isolated acinar clusters were embedded in type-1 rat tail collagen and cultured in DM consisting of DMEM/F12 (GIBCO, Burlington, ON, Canada) supplemented with 10% FBS (Montreal Biotech Inc., Montreal, QC, Canada), dexamethasone (1 μM, Sigma-Aldrich, Canada, Oakville, Canada), EGF (10 ng/ml, Sigma-Aldrich), 24 mU/ml insulin (Eli Lilly, Indianapolis, IN, USA) and cholera toxin (100 ng/ml, Sigma-Aldrich) (differentiation media, DM+CT). Cells were cultured in 95% air, 5% CO_2 and 37°C, and the medium was changed on alternate days. Following 8 days of culture, when the acinar tissue had differentiated into duct-like epithelial structures, differentiation media without cholera toxin (DM) was administered to the cells alone or supplemented with gastrin (50 nM, Sigma-Aldrich) and hepatocyte growth factor (10 ng/ml, Sigma-Aldrich) (GH), $INGAP^{104-118}$ (167 nM) (I) or GH and $INGAP^{104-118}$ (GHI) for 6 days,

with the medium changed every 2 days. After six days of treatment, cells were incubated with dithizone and insulin positive islet-like structures were quantified, or tissue was removed from collagen using type XI collagenase (0.25 mg/ml, Sigma-Aldrich) and processed for immunohistochemistry.

4.2.2 Microscopy and Morphometric Analysis

Incidence of islet neogenesis was determined to be the total number of free standing dithizone positive structures (neo-islets) plus the total number of acinar-derived duct-like structures (DLS) that had dithizone positive structures budding from them divided by the total number of structures counted. Following collagen digestion, samples were collected and embedded in paraffin wax.

Pancreatic sections, 4 to 5 μm thick, were de-waxed in xylene and endogenous peroxidase activity was blocked using a 3% solution of hydrogen peroxide and methanol (for sections to be stained with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich)). Sections were then washed in PBS and incubated with blocking buffer (Histostain Plus Kit, Zymed Laboratories, San Francisco, USA) for 15 minutes at room temperature. Slides were stained with one or more primary antibodies, including insulin (1:750; Dako Diagnostics Canada, Mississauga, Canada); a cocktail of glucagon, somatostatin and pancreatic polypeptide (1:750; Dako), C-peptide (1:50; Biogenex, San Ramon, CA, USA), CK19 (1:100; Dako), amylase (1:100; Sigma-Aldrich), or PDX-1 (1:750; a gift from C. Wright, Vanderbilt University). Slides stained for CK19 and PDX-1 were incubated with 0.1M citrate buffer for 9 minutes at 90°C for antigen retrieval before primary antibody application. Slides were incubated with primary

antibodies overnight at 4°C before being washed in PBS and incubated with the secondary antibodies for 20 minutes (Histostain Plus Kit, Zymed) at room temperature. Following washing in PBS, sections were incubated with horseradish peroxidase (Histostain Plus Kit, Zymed) for 20 minutes, and developed using DAB (Sigma-Aldrich). Double stained sections were then incubated with a second set of primary antibodies overnight at 4°C and the above process repeated. Alkaline phosphatase conjugate (Histostain Plus Kit, Zymed) was substituted for horseradish peroxidase and sections were developed with New Fuchsin (Dako). All light immunohistochemical slides were counterstained with Harris' haematoxylin (Sigma-Aldrich) and cover slipped using Permount (Fisher Scientific, Ottawa, Canada).

4.2.3 Statistical Analyses

Comparison between groups was done using student t-test or an ANOVA with post hoc comparison where appropriate. Data are presented as mean ± standard error of the measurement. Significance was accepted at the 5% level.

4.3 RESULTS

4.3.1 In Vitro Stimulation of Acinoductal Metaplasia

Following isolation and embedding into collagen, the human pancreatic acinar tissue begins to lose its native structure and form cystic configurations (Figure 4.1A). Greater than 95% of acinar clusters transformed into duct-like structures (DLS) that were devoid of amylase immunoreactivity and expressed the ductal epithelial marker CK19 (Figure 4.1B). The acinar-to-DLS transformation, characterized by a loss of the central cells resulting in a hollowing of the acinar structure, appeared to initiate at one or multiple foci and then spread throughout the entire structure with duration in culture. By 8 days of culture this acinar-to-duct transformation was complete. Acinar clusters between 50 to 250 μm^2 almost always formed a single DLS whereas acinar clusters larger than 250 μm^2 tended to form multiple DLS (Figure 4.1C). Acini smaller than 50 μm^2 were more likely not to transform into DLS by the end of the 8 day culture period.

4.3.2 Stimulation of Acinar-Derived DLS Transformation to Endocrine Phenotype

Following 8 days of culture, the differentiation medium was replaced with medium that was devoid of cholera toxin and supplemented with various growth factor combinations. Following six days of culture with $INGAP^{104-118}$ there was no morphological change in the acinar-derived DLS, and although there was a moderate increase in dithizone positivity there was no statistically significant difference (average number of structures/flask, p=0.11). When samples were

retrieved and processed for immunohistochemical analysis, a 18-fold increase in PDX-1 immunoreactivity was observed versus controls (Figure 4.2).

When acinar derived cystic structures were treated with a combination of gastrin, HGF and INGAP$^{104-118}$ dithizone positive structures began to bud from the acinar-derived DLS (Figure 4.3). There was a significant increase in the percentage of dithizone positive structures budding from the acinar-derived DLS as well as full neo-islet formation versus controls ($p=0.037$; Figure 4.4). Budding of dithizone positive structures occurred in 1.3±0.6, 1.4±0.2, 2.2±0.9 and 5.1±0.2% of acinar-derived DLS in control, GH, I, and GHI-treated flasks, respectively. The percentage of neo-islet formation versus total structures was 0.4±0.1, 0.9±0.2, 0.4±0.1 and 5.8±3.0% for control, GH, I and GHI-treated acinar-derived DLS, respectively.

In order to rule out the possibility of the new islet-like-structures being dithizone positive due to the sequestering of the insulin in the medium, immunohistochemical analyses for C-peptide and other islet hormones were performed. Immunohistochemical analyses of the growth factor (GHI) supplemented acinar-derived DLS indicated that these budding structures and neo-islets were immunopositive for C-peptide (Figures 4.5A and B), and in addition, were producing other endocrine hormones similar to a normal mature human islet (Figure 4.5C).

Figure 4.1: Transformation of adult human pancreatic acinar clusters into duct-like structures (DLS). **(A)** Inverted micrographs of the differentiation of human acinar tissue into DLS following 8 days of culture. **(B)** Immunohistochemical staining for amylase (red) and the duct epithelial cell marker cytokeratin-19 (brown) during this transformation process. **(C)** Photomicrograph of a large acinar cluster that differentiated into multiple DLS. (bar = 30 μm)

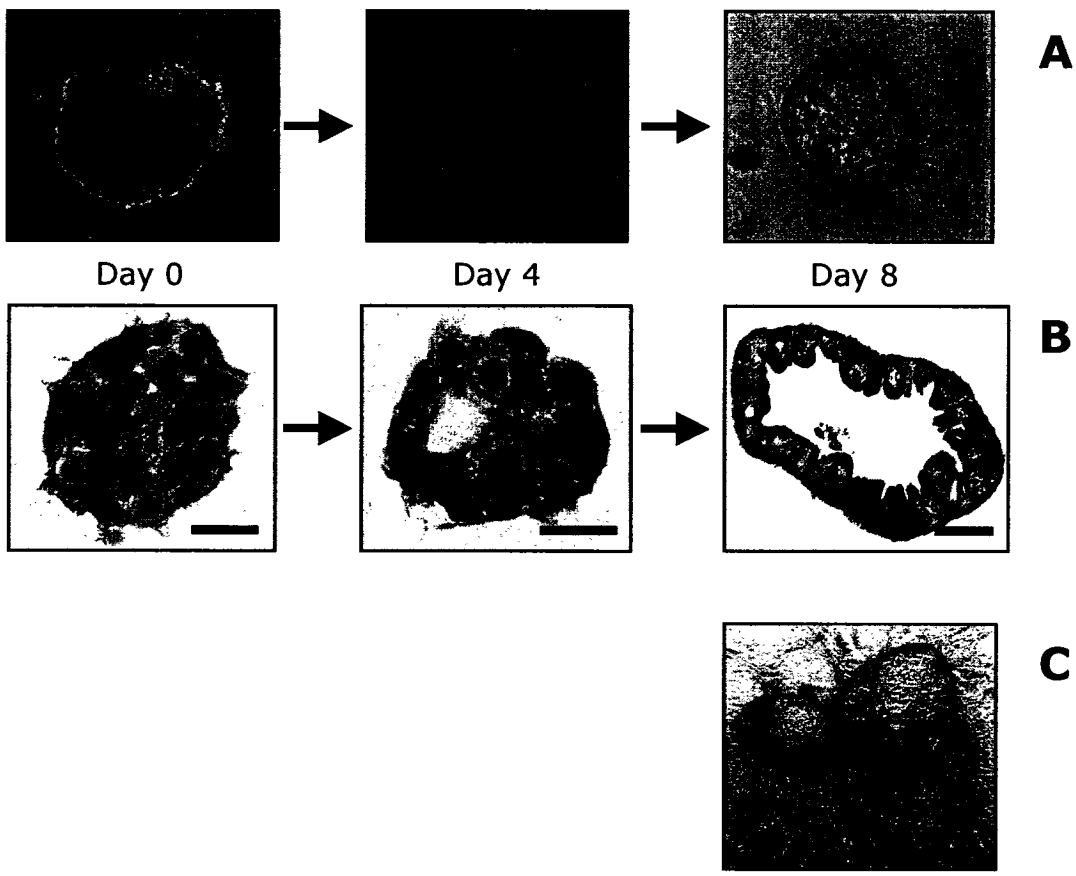

A
Day 0
Day 4
Day 8
B
C

Figure 4.2: PDX-1 immunoreactivity of acinar-derived duct-like structure (DLS) treated with $INGAP^{104-118}$ (167 nM) for 6 days. **(A)** Photomicrograph of PDX-1 stained acinar-derived DLS that was treated with $INGAP^{104-118}$ for 6 days. **(B)** Quantification of PDX-1 positivity of $INGAP^{104-118}$-treated acinar-derived DLS from 3 separate donor preparations. (bar = 20 μm)


A

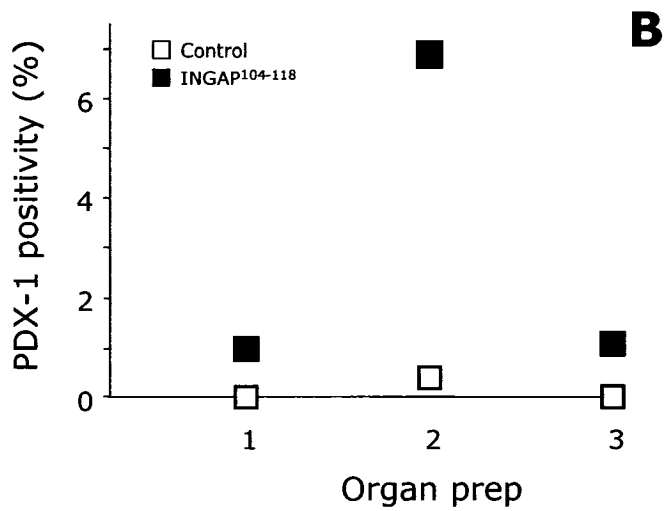

B
Control
INGAP104-118
PDX-1 positivity (%)
0
2
4
6
1
2
3
Organ prep

Figure 4.3: Differentiation of acinar-derived duct-like structure (DLS) into islet-like structures. **(A)** Photomicrograph of dithizone stained acinar-derived DLS after 8 days of culture. **(B)** Photomicrograph of a dithizone stained (red) acinar-derived DLS following four-day treatment with gastrin + HGF + $INGAP^{104-118}$. **(C)** Photomicrograph of dithizone stained islet-like structure derived from a culture of acinar-derived DLS treated with gastrin + HGF + $INGAP^{104-118}$ for 6 days. (bar = 50 μm)

Day 8 Day 12 Day 14

Figure 4.4: Quantification of percentage of dithizone positive structures budding from acinar-derived DLS as well as full islet-like structure formation in cultures of acinar-derived DLS treated with control media, gastrin + HGF, $INGAP^{104-118}$, or gastrin + HGF + $INGAP^{104-118}$ (n=4 separate organ preparations). * $p<0.05$ vs. all other groups.

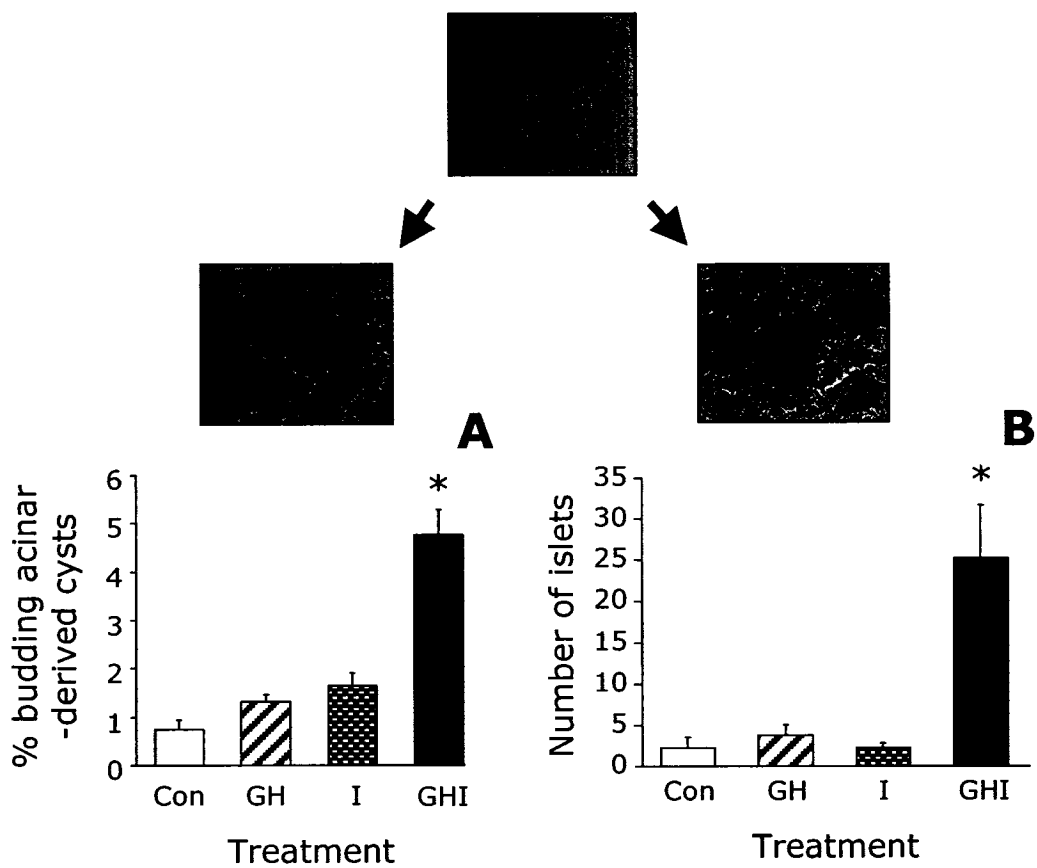
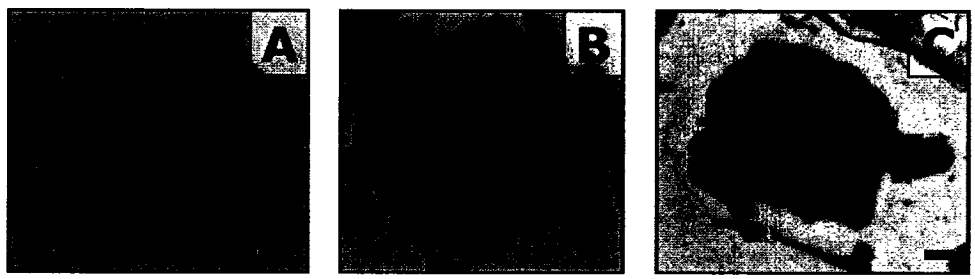

A
B
% budding acinar -derived cysts
Number of islets
0
1
2
3
4
5
6
0
5
10
15
20
25
30
35
*
*
Con
GH
I
GHI
Con
GH
I
GHI
Treatment
Treatment

Figure 4.5: Immunohistochemical analysis of gastrin + HGF + INGAP$^{104-118}$-treated acinar-derived duct-like structure (DLS) cultures. **(A)** Photomicrograph of a C-peptide stained DLS that was treated with gastrin + HGF + INGAP$^{104-118}$ (GHI) for 6 days. **(B)** Photomicrograph of C-peptide staining of an islet-like structure from a culture treated with GHI for 6 days. **(C)** Photomicrograph of a cocktail stained (anti-glucagon + anti-somatostatin + anti-pancreatic polypeptide) islet-like structure from a culture of GHI-treated acinar-derived DLS. (bar = 10 μm)


A
B
C

4.4 DISCUSSION

Islet transplantation therapy has the potential to restore euglycaemia in individuals with type 1 diabetes, however due to the paucity of donor organs this therapeutic approach currently has a very limited impact. This situation could be reversed if an alternative source of islet tissue was identified and developed. In this study we provide evidence that adult human islet tissue can be expanded through the induction of acinar-to-islet transdifferentiation through a duct epithelial-like intermediate. Specifically, a combination of gastrin, HGF and the Reg family member INGAP$^{104-118}$ produces a 6-fold increase in total dithizone positivity (10.9±2.9 vs. 1.7±0.4%) and a dramatic ~15-fold increase in neo-islets compared to untreated control tissue cultures.

Acinar-to-duct-like structure (DLS) interconversion and subsequent DLS-to-islet-like structure transformation has been previously observed in multiple models of pancreatic regeneration [86, 89, 136, 137, 160, 170]. Wang et al. initially indicated that there was a corresponding increase in gastrin and TGFα expression following induction of DLS formation in adult Wistar rats that had been pancreatic duct-ligated [163]. Rooman et al. furthered this work with the critical observation that gastrin treatment of these duct-ligated rats led to enhanced duct-to-islet neogenesis from tubular complexes [137]. This duct-to-islet neogenesis was also associated with an upregulation of PDX-1 in the ductal tissue, similar to that observed in our study. Moreover, we have previously shown that adult human islets also possess the morphogenetic plasticity to transform into DLS and then redifferentiate back into islet-like structures following administration of INGAP$^{104-118}$ [80, 125, 148].

The transformation of adult human acini into DLS is most likely to occur through the extensive remodelling of the extracellular matrix. The pancreas digestion and tissue isolation procedure induces profound changes in the cell-cell and cell-extracellular matrix interactions, which are responsible for the regulation of cell phenotype [120-124]. The re-establishment of cell-matrix interactions using a non-native collagen matrix, together with cholera toxin-induced stimulation of cAMP, are most likely key factors in this phenotypic transformation [148, 271]. Furthermore, poor diffusion of nutrients and oxygen to the central regions of these acinar clusters probably further enhances the central loss of cells contributing to an enhanced formation of the cystic duct-like structures.

Acinar-to-duct metaplasia is also characteristically seen in association with both chronic pancreatitis and pancreatic cancer [20, 128]. An *in vitro* human tissue model that enables the mechanistic study of acinoductal metaplasia, such as the model we describe here, may contribute to a better understanding of the process and origins of pancreatic adenocarcinoma [19, 20].

This model has also allowed us to determine the potential of human acinar tissue to generate new endocrine tissue. Following the addition of INGAP$^{104-118}$ to the acinar-derived DLS there was a dramatic increase in PDX-1 expression (Figure 4.2). However, the administration of the hamster Reg3 peptide INGAP$^{104-118}$ alone was not sufficient to produce a significant increase in insulin-producing cells, in contrast to results observed in islet-derived DLS cultures [125]. However, when INGAP$^{104-118}$ was combined with gastrin and HGF there was a dramatic increase in insulin positive tissue budding from these acinar-derived DLS leading to full islet formation. These *in vitro* results suggest that gastrin and EGF alone,

do not stimulate islet neogenesis from human acinar tissue. This is probably the case *in vivo* as well, as β-cell mass expansion only occurs when these factors are administrated in the setting of enhanced local expression of other growth factors [137, 160, 171, 201, 203].

Currently, islet transplantation therapy is hindered due to a lack of available tissue. Elucidation of a successful means of expanding islet-like tissue will enable a greater number of diabetic individuals to benefit from islet transplantation therapy. The exocrine tissue used in this study is routinely discarded following islet isolation procedures and as such, development of an acinar-to-islet differentiation model could drastically increase the amount of available endocrine tissue with no initial investment of critical pancreatic tissue. Furthermore, as exocrine tissue constitutes the majority of the organ there is ample tissue to dramatically increase the transplantable cell mass. Further studies will be required to determine if these acinar-derived islet-like structures will restore normoglycaemia in diabetic individuals to the same extent as primary islets.

In summary, the model of adult human acinar transdifferentiation described here is an important step in diabetes research that could lead to a new source of endocrine pancreatic tissue for transplantation. Furthermore, elucidation of the mechanisms involved could provide novel insights into the process of pancreatic carcinogenesis.

4.5 ACKNOWLEDGEMENTS

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BRIDGING STATEMENT BETWEEN MANUSCRIPTS

Chapters 2 through 4 have indicated that INGAP$^{104-118}$ therapy can lead to PDX-1 upregulation, islet neogenesis and resultant β-cell mass expansion, which remains under homeostatic regulation preventing unbridled β-cell proliferation. Chapter 4 has indicated the potential of INGAP$^{104-118}$ therapy to increase β-cell mass in humans, through acinar-to-islet transdifferentiation. These results lead to the re-evaluation of the current therapies for diabetes mellitus as well as the determination of effective future strategies for a safe and efficacious treatment of this disease. Chapter 5 looks at the current understanding of type 1 diabetes, the shortcomings of existing therapies for this disease, as well as future therapies leading to the amelioration of this disease.

CHAPTER 5

Current State Of, And Future Avenues For,

The Treatment Of Type 1 Diabetes

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Doctoral Candidate's Contribution - Manuscript 4

The doctoral candidate was responsible for preparing this review manuscript. Reid Aikin performed the experiment in Figure 5.2, and Nikolay Tchervenivanov performed the experiment depicted in Figure 5.3. All other co-authors contributed in manuscript preparation.

5.0 ABSTRACT

Current therapies for type 1 diabetes, including fastidious blood glucose monitoring and multiple daily insulin injections, are not sufficient to prevent complications of the disease. Though pancreas and possibly islet transplantation can prevent the progression of complications, the scarcity of donor organs limits widespread application of these approaches. Understanding the mechanisms of β-cell mass expansion as well as the means to exploit these pathways has enabled researchers to develop new strategies to expand and maintain islet cell mass. Potential new therapeutic avenues include *ex vivo* islet expansion and improved viability of islets prior to implantation, as well as the endogenous expansion of β-cell mass within the diabetic patient. Islet neogenesis, through stem cell activation and/or transdifferentiation of mature fully differentiated cells, has been proposed as a means of β-cell mass expansion. Finally, any successful new therapy for type 1 diabetes via β-cell mass expansion will require prevention of β-cell death and maintenance of long-term endocrine function.

5.1 INTRODUCTION

Type 1 diabetes mellitus afflicts millions of individuals worldwide and its prevalence and incidence continue to rise annually. This disease results from autoimmune mediated destruction of the insulin-producing β-cells of the islets of Langerhans [8]. Despite the widespread use of meticulous blood glucose monitoring and new insulin formulations, most individuals with diabetes will still develop the devastating secondary complications of the disease. Clinical studies suggest that strict blood glucose control by intensified insulin treatment may attenuate or delay, but not prevent the eventual development of complications [62, 256]. As such, exogenous insulin administration cannot match the glucose homeostasis provided by endogenous islets.

Near perfect control of blood glucose levels can be restored by pancreas or islet transplantation, without the risk of serious hypoglycaemic episodes that are associated with intensive insulin therapy [62]. Pancreas transplantation, however, is far more successful in maintaining long-term reversal of hyperglycaemia than islet transplantation [71, 272], which is still an emerging technology. Regardless, both approaches are of limited use due to the shortage of available organs and the need for chronic immunosuppression, relegating transplantation to a last resort therapy for individuals with diabetes. Islet transplantation is further limited by poor isolation efficiency and the unresolved matter of islet death post-isolation [273]. Therefore, alternative strategies will need to be explored in order to develop a widely applicable therapy for the restoration of functional islet mass.

A more innovative approach to islet replacement would be to harness the regenerative capacity of the pancreas. For example, islet regeneration could be

induced *ex vivo* using donor tissue as a means to expand the number of islets available for transplantation. Alternatively, *in vivo* induction of pancreatic regeneration leading to endogenous β-cell mass expansion would also eliminate the necessity for donor tissue and thereby enable the widespread application of a regeneration therapy to restore normoglycaemia. However, due to the autoimmune nature of type 1 diabetes, as well as the immune rejection associated with allogenic islet transplantation, such therapies would have to be coupled with an immunosuppressive regimen that would protect new islets.

5.2 PATHOGENESIS

Type 1 diabetes occurs due to autoimmune destruction of the pancreatic β-cells leading to an insufficient functional β-cell mass. Evidence for the immune basis of the disease can be observed in the elevated levels of circulating auto-antibodies to islet cell cytoplasm (ICA), insulin (IAA) and glutamic acid decarboxylase (GADA) [8].

5.3 β-CELL MASS DYNAMICS

As with virtually every tissue in the body, β-cell mass is dynamic [76, 79]. The magnitude of β-cell mass is determined by the interplay between β-cell mass expansion and reduction mechanisms. Expansion can occur through increases in β-cell hypertrophy, hyperplasia and neogenesis (formation of new β-cells from progenitor cells, Figure 5.1). Conversely, reduction can occur through β-cell atrophy, death or loss of phenotypic stability. Accordingly, β-cell mass expansion

could be achieved through the manipulation of any of these mechanisms, alone or in combination.

5.3.1 Mechanisms of β-cell Mass Expansion

The induction of endogenous β-cell mass expansion could revolutionize diabetes therapy. Nonetheless, the development and implementation of this approach remains stalled due to uncertainty as to whether the principal mechanism of expansion is β-cell hyperplasia or neogenesis. The importance of this distinction rests on the notion that β-cell mass expansion on the basis of hyperplasia requires the persistence of β-cells, a situation that may not exist in type 1 diabetes.

While recent studies indicate a significant contribution of islet neogenesis to β-cell mass expansion [139] another recent study by Dor et al. suggests otherwise [82]. In this study mice were engineered to express an inducible label behind an insulin promoter. Animals were 'pulsed' to label the insulin-producing cells at a set time and 'chased' thereafter to determine the contribution of these labelled cells to β-cell mass. Based on the experimental outcome the authors concluded that replication of pre-existing β-cells was the sole mechanism of β-cell mass expansion. This observation all but precludes the development of therapies based on the induction of endogenous β-cell mass expansion. However, this study characterizes the dynamics of mouse β-cell mass expansion, which differ from human β-cell mass expansion dynamics [12, 236]. For example, Butler et al. have shown that in rodent models of insulin resistance and type 2 diabetes,

endogenous β-cell mass expansion occurs primarily via proliferation [236]; whereas in patients with type 2 diabetes, β-cell neogenesis is markedly upregulated and β-cell replication is unchanged [12]. It is noteworthy that histologic evidence for ongoing islet neogenesis in non-diabetic individuals as well as individuals with early-onset type 1 diabetes has also been reported [37]. These observations suggest that the restoration of β-cell mass in type 1 diabetes may be achievable through the induction of endogenous β-cell mass expansion via islet neogenesis.

5.3.2 Mechanisms of β-cell Neogenesis

β-cell neogenesis may occur through two pathways; stem/progenitor cell activation and/or transdifferentiation of adult pancreatic cells. Stem/progenitors cell-derived β-cell mass expansion refers to the differentiation of a quiescent cell expressing primordial markers to yield insulin-producing β-cells. The existence and identification of such a pancreatic stem cell is still controversial [82, 118, 274]. By contrast, the ability to transdifferentiate from one mature cell phenotype to another cell phenotype reflects a degree of morphogenetic plasticity inherent to adult pancreatic tissue [125]. In this paradigm new β-cells are derived from the transdifferentiation of a mature differentiated pancreatic cell (i.e. duct or acinar) either directly, or through a process of dedifferentiation to a more primitive intermediate cell, followed by redifferentiation into an insulin-producing cell.

5.4 THERAPY

5.4.1 Pharmacological Induction of β-cell Mass Expansion

Current clinical research focuses on the stimulation of β-cell mass expansion using pharmaceutical ligands. Unfortunately, human clinical data are not yet available, though compelling animal and *in vitro* human data does exist.

5.4.1.1 Combined Gastrin and EGF

Combination therapy of gastrin and epidermal growth factor (EGF) has been reported to expand β-cell mass in mice, potentially through increased β-cell neogenesis [171]. Likewise, Suarez-Pinzon et al. have reported that the combination of gastrin and EGF leads to β-cell expansion in long-term cultures of semi-purified human islets, possibly through islet neogenesis [203]. Moreover, it has also been reported that this therapy leads to increased insulin and glucagon content in human islets transplanted into immunodeficient NOD mice [203]. Thus, these studies suggest that the combination of gastrin and EGF may have utility in expanding β-cell mass both *in vitro* as well as *in vivo*.

5.4.1.2 GLP-1

Glucagon-like peptide-1 (GLP-1), an incretin produced by enteroendocrine L-cells of the intestine, can reduce blood glucose levels through the inhibition of appetite, gastrointestinal motility and glucagon release, as well as by the enhancement of glucose-stimulated insulin release [47]. It has also been suggested that GLP-1 can induce β-cell neogenesis from human progenitor cells

[274]. Unfortunately, there are few studies analysing the ability of GLP-1 to expand β-cell mass in the setting of type 1 diabetes. Nonetheless, the use of GLP-1 *in vitro*, and perhaps *in vivo*, may represent another means of manipulating β-cell mass.

5.4.1.3 INGAP$^{104-118}$

Islet Neogenesis Associated Protein (INGAP) is a Reg family member identified in a hamster model of islet expansion. *In vivo* administration of a synthetically produced portion of INGAP (INGAP$^{104-118}$) leads to the dose-dependent expansion of β-cell mass in normoglycaemic rodents and dogs, as well as a reversal of hyperglycaemia in a mouse model of type 1 diabetes [139]. More recently, INGAP$^{104-118}$ has also been used to induce the *in vitro* differentiation of human islet-derived duct-like structures (DLS) back into glucose-responsive islets [125]. Unlike islets, these DLS are highly proliferative, and therefore represent a population of expandable putative progenitors that could be used to increase β-cell mass for the purposes of islet replacement by transplantation. Similarly, acinar-derived DLS, when treated with a combination of gastrin, hepatocyte growth factor (HGF) and INGAP$^{104-118}$, can also be induced to differentiate into islet-like structures. These findings suggest that adult human pancreatic acini also harbour the potential to expand β-cell mass.

Pharmacotherapies based on pancreatic tissue plasticity hold great promise as a means of increasing β-cell mass in patients with diabetes. This is especially true for acinar-based expansion, given that acinar tissue constitutes

approximately 80% of pancreatic tissue and is discarded during routine islet isolation.

5.4.2 Protection of Expanded β-cell Mass From Ongoing Autoimmunity

While restoration of β-cell mass is an obvious requirement for successful reversal of hyperglycaemia, maintenance of the new β-cell mass is equally important. A neogenesis-based therapy for type 1 diabetes will almost certainly require concomitant immunosuppressive therapy to prevent immune-mediated destruction of newly generated β-cells (unless neogenic β-cells are less immunogenic than the original β-cells). New pharmacological regimens are in the early clinical trial phase and are designed to prevent β-cell destruction in new-onset type 1 diabetics and in individuals with latent autoimmune diabetes of the adult (LADA). Immunomodulatory therapeutic regimens focused on GAD [275] and heat shock protein 60 (Diapep277) [255] both appear to have at least marginal efficacy in the inhibition of insulitis and in decreasing insulin requirements. The successful development of these novel immunosuppressive therapies will allow for the protection of exogenously or endogenously expanded β-cell mass from the prevailing anti-β-cell leukocyte population.

5.4.3 Inhibition of Non-Immune Mediated β-cell Death and Maintenance of Islet Phenotype

It has been demonstrated that the standard method of islet isolation leads to the induction of islet apoptosis (Figure 5.2) [273]. During isolation, islets

experience growth factor withdrawal, loss of matrix support, osmotic stress, hypoxia and mechanical stress. As a result, pro-apoptotic signalling pathways are activated resulting in the activation of the intrinsic (mitochondrial) apoptotic pathway [276, 277]. Specific inhibition of early apoptotic signalling events can block the commitment to cell death by preventing activation of the intrinsic pathway, culminating in reduced islet cell apoptosis [276]. This approach could therefore represent a means of increasing islet isolation efficiency and enhancing islet viability.

In keeping with the notion that islet apoptosis may be implicated in loss of graft function, Verchere et al. have recently demonstrated that transplanted islets produce islet amyloid polypeptide (IAPP) at significant levels and that treatment of isolated islets with a hexapeptide inhibitor of amyloid plaque formation leads to a significant reduction in the number of amyloid plaques and apoptotic β-cells, representing another means of increasing isolation efficiency (personal communication, CB Verchere).

Loss of β-cell mass may also occur through the loss of islet phenotype. Isolated islets have the capacity to dedifferentiate back to a duct-like state under certain environmental conditions [125]. Treatment of isolated islets with transforming growth factor-β (TGF-β) prevents islet-to-duct dedifferentiation and preserves islet morphology (unpublished observation). Thus, this would represent another approach to maintaining the function of transplanted islets, thereby improving islet transplant efficacy.

Another factor affecting the outcome of islet transplantation is the engraftment site. Currently, isolated islets are infused via the portal vein to engraft in the liver. This necessarily exposes the islets to elevated concentrations of glucose, lipids and immunosuppressive drugs that are all likely to be β-cell toxic. Better graft sites (Figure 5.3), including those that support islet neogenesis and growth [278], will help to resolve these issues and contribute to making cell-based therapy preferable to whole organ transplantation.

5.5 CONCLUSION

Novel cell-based therapies for diabetes are on the horizon. An increased understanding of the factors contributing to β-cell mass dynamics has enabled researchers to examine new approaches to induce β-cell mass expansion and promote survival. At this stage of development, it appears that any definitive therapy for type 1 diabetes will need to simultaneously address several issues including the induction of new islet formation, promotion of cell survival and long-term maintenance of function, and the prevention of recurrent autoimmunity.

Figure 5.1: Photomicrograph of an insulin stained pancreatic section from an INGAP$^{104-118}$-treated mouse. A duct-associated islet and single extra-islet β-cells indicate ongoing duct-to-islet neogenesis. (bar = 50 μm)

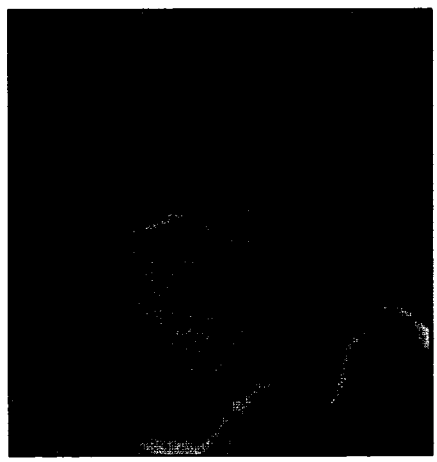

Figure 5.2: Fluorescence photomicrograph of a freshly isolated human islet of Langerhans double-stained with fluorescein diacetate (green: live cells) and propidium iodide (red: dead cells) indicates the islet cell loss that occurs following routine isolation. (bar = 20 μm)

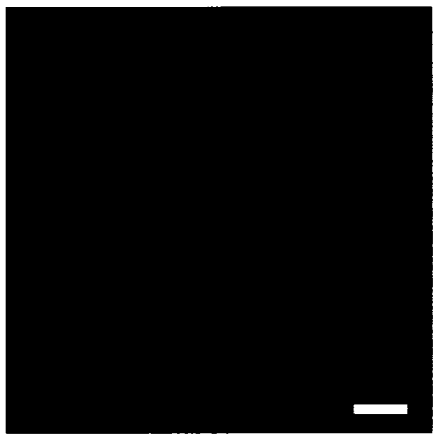

Figure 5.3: Photomicrograph of hamster islets and duct-like structures that were co-transplanted into the submucosal space of the small intestine. Tritiated thymidine incorporation (dark nuclei) by cells in islets and duct-like structures indicate that this site supports progenitor and islet cell proliferation, while the transdifferentiation of duct-like structures into insulin-producing cells (red: insulin) indicate support for progenitor differentiation. (bar = 50 μm)

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CHAPTER 6

Discussion of Results and Concluding Statement

6.0 ABILITY OF INGAP$^{104-118}$ TO STIMULATE ISLET NEOGENESIS

The primary objective of this thesis was to determine the regenerative potential of INGAP$^{104-118}$ in multiple species (Chapter 2). As diabetes results from an inadequate insulin-producing β-cell mass to meet the body's requirements, the formation of new islets from endogenous progenitors would be a significant advancement in diabetes therapy. Before INGAP$^{104-118}$ could be used as a diabetes therapy its efficacy beyond the original hamster setting needed to be determined.

INGAP$^{104-118}$ administration to normoglycaemic hamsters led to a greater than 30% increase in total β-cell mass in comparison to age-matched controls (Figure 2.3A). As total β-cell mass can increase through various pathways (Chapter 1), it was important to perform a stringent analysis of β-cell mass dynamics in order to determine the exact mechanism(s) of β-cell mass expansion in this setting. When a thorough analysis of β-cell mass dynamics was performed, it was found that INGAP$^{104-118}$ therapy stimulated islet neogenesis via extra-islet β-cells associated with both acini and ducts (Figure 2.4). These findings support previous reports of an initial reduction in average islet size, which is a surrogate marker of islet neogenesis [85].

This study then sought to determine whether INGAP$^{104-118}$ had the potential to stimulate β-cell mass expansion in mice. INGAP$^{104-118}$ was administered to normoglycaemic mice for a period of 31 days, after which point, the β-cell mass was observed to increase by more than 120% (Figure 2.5). In addition to this dose-dependent expansion of endogenous β-cell mass in normal mice, INGAP^{104-}

^{118} therapy was observed to expand the β-cell mass in a murine model of type 1 diabetes. This expansion appeared to occur similarly to the β-cell mass expansion observed during embryology, showing a dramatic increase in PDX-1 expression (Figure 2.8). These results suggest that INGAP$^{104-118}$ can lead to endogenous β-cell mass expansion in various rodent settings and that this expansion is sufficient to reverse chronic hyperglycaemia.

It is interesting to note that in these diabetic mice, an autoimmune-mediated destruction of the pancreatic β-cells destroyed the original β-cells but lymphocytic infiltration (insulitis) was not observed to encroach upon the newly formed islets. There are three possible explanations for this occurrence. Firstly, the newly formed β-cells may not express the same antigenic profile as the original β-cells, and thus they are spared from insulitis. Secondly, INGAP$^{104-118}$ may have immunomodulatory properties that inhibit streptozotocin-induced insulitis. Thirdly, the induced insulitis may not appropriately model the autoimmunity observed in type 1 diabetes, and following restoration of β-cell mass via INGAP$^{104-118}$ therapy the autoimmune leukocyte population is less active. Consequently, future studies are required to determine the exact mechanism(s) of neo-islet protection in this model of type-1 diabetes.

When euglycaemic beagle dogs were administered INGAP$^{104-118}$, there was a trend to increase β-cell mass, suggesting that INGAP$^{104-118}$ has the potential to expand β-cell mass in higher mammals (Figure 2.10). This point is further supported by the observation of profound islet neogenesis occurring in cynomolgus monkeys that were injected with INGAP$^{104-118}$ for 90 days (Figure

2.12). Not only was INGAP$^{104-118}$ therapy sufficient to stimulate β-cell mass expansions in various animals, but it was also able to reverse overt hyperglycaemia in a rodent model of type 1 diabetes (Figure 2.6).

6.1 MECHANISMS OF β-CELL MASS EXPANSION

As indicated in Chapter 1, β-cell mass expansion can occur through three cellular pathways including, growth of pre-existing β-cells, differentiation of endogenous stem cells and transdifferentiation of mature cell phenotypes. The studies included in this thesis suggest that INGAP$^{104-118}$ leads to β-cell mass expansion through both direct and indirect transdifferentiation of mature cell phenotypes (Chapters 2-4) as well as through the moderate growth of pre-existing β-cells (Chapter 3). Conclusive evidence that INGAP$^{104-118}$ stimulates activation of endogenous stem cells has not been found. However, it is possible that endogenous stem cells exist and reside amongst mature duct and acinar tissue and are only activated when appropriate changes occur in the extracellular environment that promote their differentiation toward an endocrine phenotype [20, 125, 279].

Changes in the extracellular matrix have been implicated in initiating cellular differentiation [81, 125, 147-149]. It is possible that INGAP$^{104-118}$ binding to appropriate sites on progenitor cells initiates signalling cascades and changes in the extracellular matrix, which in turn enables endocrine differentiation [190]. Changes in the extracellular matrix, along with elevated intracellular cAMP, are involved in the phenotypic conversion of isolated islets and acini into duct-like

structures [148, 149, 280, 281]. The changes in extracellular matrix, along with the addition of INGAP$^{104-118}$, gastrin and HGF lead to endocrine transformation, either through transdifferentiation of dedifferentiated duct-like cells, or through the activation of previously quiescent stem cells adjacent to these structures. Unfortunately, using current cellular labelling techniques it may be difficult to clearly delineate from precisely which tissue types these new endocrine cells are arising in human cultures.

Recently, Dor et al. used a novel experimental technique for tracing the lineage of progenitor cells within the murine pancreas [82]. The authors used a Cre/lox system of inducible progenitor cell labelling to quantify the mechanism(s) of β-cell growth and regeneration in adult mice. Briefly, a transgenic mouse was created in which an inducible label would be expressed in insulin-producing cells and all of the labelled cells' progeny. Following a pulse of the inducer, animals were sacrificed at various time points and the percentage of labelled islets and β-cells were determined. The authors found no change in the percentage of islets and β-cells labelled prior to the chase period in comparison to following, in both normal mice as well as in 60% partial pancreatectomized mice. Dor et al. interpreted these results to mean that β-cell expansion only occurs through duplication of pre-existing β-cells in adults. However, Minami et al. have shown that pancreatic exocrine tissue transforms into insulin-producing tissue using a similar Cre/lox technique. Furthermore, other researchers have indicated possible technical insufficiencies involved in Dor et al.'s study, which may account for their observed results and thus, their controversial conclusion [115, 117, 279, 282].

As stated in Chapter 1, islet neogenesis in adults appears to recapitulate the β-cell mass expansion observed during pancreatic ontogeny. Diabetic mice treated with INGAP$^{104-118}$ showed an increase in duct-cell PDX-1 positivity, consistent with that which is observed during pancreatic organogenesis (Figure 2.8) [91, 97, 98, 102]. This upregulation of PDX-1 in potential ductal progenitors is also seen in duct-like structures derived from human acini treated with INGAP$^{104-118}$ (Chapter 4). In the context of these *in vitro* structures, however, INGAP$^{104-118}$ administration alone was insufficient to stimulate a statistically significant expansion of insulin-producing cells. These results suggest that even though PDX-1 is a critical component of mature β-cell function, it may not be an accurate marker of a fully functional and mature endocrine phenotype [159]. This finding is in keeping with the fact that virtually all cells express PDX-1 during pancreatic ontogeny, though only a small minority (~3%) ever become endocrine cells. Therefore, other, more specific markers of islet neogenesis should be used in addition to PDX-1, such as NGN-3 and/or islet-1, to perform a more robust determination of endocrine differentiation potential.

A critically important finding of this thesis is that administration of INGAP$^{104-118}$ leads to islet neogenesis through a multitude of pathways. We have observed the following processes in settings of β-cell mass expansion: a dramatic increase in PDX-1 upregulation and duct-to-islet differentiation in diabetic mice (Figure 2.8), duct-to-islet and acinar-to-β-cell differentiation in normoglycaemic hamsters (Figure 2.4), duct-to-islet differentiation in normoglycaemic mice (Figure 3.4), and indirect acinar-to-duct-to-islet differentiation in monkeys (Figure 2.11).

Furthermore, INGAP$^{104-118}$ may also induce β-cell mass expansion through β-cell hypertrophy (Figure 3.6A) [179]. Moreover, a mild reduction in β-cell apoptosis was observed at 31 days of INGAP$^{104-118}$ therapy, and although this result is not significant ($p=0.33$), it suggests that INGAP$^{104-118}$ may expand β-cell mass through this mechanism as well. INGAP$^{104-118}$ has previously been shown to promote anti-apoptotic activity in human islet-derived duct-like cells, and a full-length recombinant INGAP was found to prevent peroxide-mediated cell death in isolated human islets (personal communication, Lawrence Rosenberg) [125]. INGAP$^{104-118}$ therefore appears to enhance β-cell mass through multiple pathways, and thus could possibly be used in diabetic individuals to promote β-cell mass expansion and subsequent glucohomeostasis.

The results described herein strongly suggest the occurrence of islet neogenesis in the various described animal settings. Even so, one must be conscious of the potential shortcomings of the techniques used in this thesis work. Currently there is much debate as to whether or not islet neogenesis plays a role in β-cell mass expansion in adult animals [25, 82, 118, 119]. Immunohistochemical techniques were used to quantify the occurrence of islet neogenesis in this thesis. Unfortunately, due to the nature of immunohistochemistry only static measurements of each tissue sample can be made. Islet neogenesis and β-cell mass expansion are dynamic processes and consequently, immunohistochemical analyses do not provide an infallible means of identification and quantification of this process. As described above, one way to circumvent this limitation is to perform genetic manipulations leading to the

labelling of a certain tissue genotype [82, 115]. These cells can then be followed with time in order to determine what cellular factors they come to express. Unfortunately, these manipulations can introduce biases into the animal models used and have a number of idiosyncrasies and imperfections associated with them leading to difficulties in interpreting the resulting data [115, 117, 279, 282]. Hopefully in the near future this technique will become perfected enabling a stringent cellular tracing protocol.

An alternative interpretation of the results of this thesis could be derived based on the knowledge that Reg proteins are duct cell mitogens [158, 184, 190]. The increased duct-associated β-cell mass in these animal models may have arisen not from duct-to-β-cell neogenesis, but from ductal growth into pre-existing islets. The increase in extra-islet duct-associated clusters would suggest against this interpretation, though one cannot rule this out as a possible mechanism for the increased duct-associated β-cell mass. Furthermore, in the setting of human acinar-to-islet transformation, it is possible that endocrine tissue was present in the initial acinar tissue culture, even though it was not visible using dithizone staining. This endocrine tissue could have remained quiescent through the transformation process to duct-like structures. Following the removal of cholera toxin and addition growth factors to the culture medium, these endocrine cells may have then proliferated to give rise to the islet-like structures. An appropriate cellular lineage experiment in which acinar tissue is labelled and then traced throughout the transformation process would enable determination if these newly forming endocrine clusters were truly derived from acinar cell differentiation.

Even with the potential limitations indicated above, it appears that INGAP$^{104-118}$ does contain the ability to stimulate endocrine cell expansion in many different species. As such, INGAP$^{104-118}$ could potentially be used as a therapeutic measure to expand the insulin-producing β-cell mass in individuals with diabetes.

6.2 POTENTIAL OF INGAP$^{104-118}$ THERAPY TO RESTORE EUGLYCAEMIA IN HUMANS

INGAP$^{104-118}$ administration can expand the insulin-producing β-cell mass in a number of species and stimulate islet neogenesis *in vivo* in monkeys, as well as *in vitro* in human tissue cultures. These results imply that INGAP$^{104-118}$ administration to humans would result in expanded β-cell mass and thus could potentially restore euglycaemia in diabetic individuals.

Recently, phase 2 clinical trials were performed to test the efficacy of INGAP$^{104-118}$ in human subjects with type 1 or type 2 diabetes [265]. Analysis of endogenous insulin release and long-term glycaemic homeostasis were performed in INGAP$^{104-118}$ and placebo treatment groups. Endogenous insulin release was determined via measurements of arginine-stimulated C-peptide secretion [265]. Long-term glycaemic control was assessed by measuring the extent of non-specific glycosylation of the haemaglobin A_{1c} site, which normally ranges between 4 and 6% [283].

INGAP$^{104-118}$ administration resulted in a statistically significant increase in arginine-stimulated C-peptide release ($p=0.006$) in type 1 patients as well as a trend towards lowering of glycosylated haemaglobin A_{1c} levels (6.8 vs. 7.1%;

p=0.10). In type 2 patients, there was no statistically significant difference in arginine-stimulated C-peptide release, but a significant decrease in glycosolated haemaglobin levels (HgA_{1C} 7.0 vs. 7.6%; p=0.04). Unfortunately, $INGAP^{104-118}$ was not well tolerated by the type 1 population, and there was a large number of drop-outs and a corresponding reduction in power of the study. Hopefully, advances in peptide stability, administration technique and general efficacy of this peptide will lead to a reduced peptide dosage and a corresponding decrease in associated side effects.

The results of this thesis, as well as the results of the preliminary clinical trials, suggest that $INGAP^{104-118}$ does indeed have the potential to expand the β-cell mass and lead to greater control of diabetic hyperglycaemia.

6.3 SAFETY OF PHARMACOLOGICAL INDUCTION OF β-CELL MASS EXPANSION

Exogenous stem cell therapy has been associated with the induction of numerous incidences of cancer, and as such, there is restricted optimism when considering this therapy as a potential treatment modality [20, 128, 284]. As indicated in Chapter 1, pancreatic adenocarcinoma is a devastating disease with a less than 10% 1-year survival rate. Activation of endogenous progenitors within the pancreas via pharmacological therapy may also lead to an increased incidence of pancreatic adenocarcinoma or other cancers [20, 128]. Multiple β-cell neogenic agents (including EGF, gastrin, Reg1 and Reg4) have been closely associated with gastrointestinal cancers [128, 176, 178, 205, 206]. Therefore, it is

of utmost importance to determine if exogenous administration of these factors would lead to increased incidence of pancreatic carcinogenesis.

Although INGAP$^{104-118}$ is a Reg3 protein, it has similar effects to Reg1 and Reg4, such as stimulating the proliferation of epithelial cells and potentially initiating acinoductal metaplasia [125, 158]. As indicated, acinoductal metaplastic transformation is observed in multiple examples of pancreatic regeneration as well as in the progression of adenocarcinoma [20, 86, 89, 115, 128, 129, 135-138, 170]. It is therefore important to clarify whether INGAP$^{104-118}$ therapy holds the potential to induce acinoductal metaplasia and possible facilitation of pancreatic adenocarcinoma. Even though the metaplastic transformation of acinar tissue was only observed in monkeys administered an extremely high dose of INGAP$^{104-118}$ (400 mg/kg•bw versus the maximum dose of ~8 mg/kg•bw in the human trials) a thorough analysis of carcinogenic potential of any neogenic agent must be performed prior to broad spectrum introduction of such a therapy for the treatment of diabetes mellitus. Overexpression of a Reg protein immunoreactive to an anti-INGAP antibody was found to be associated with reactive hypoglycaemia in an individual following pancreas transplantation, further justifying the need for research into INGAP$^{104-118}$ pharmacodynamics [285].

Our research has taken initial steps towards elucidating whether INGAP$^{104-118}$ induces unbridled pancreatic cell growth (Chapter 3). Findings showed that even at extreme doses, there was no unregulated expansion of β-cells in either mice or monkeys after 90 days of INGAP$^{104-118}$ therapy. Homeostatic regulation of induced β-cell mass expansion occurred through the reduction in proliferation of

mature β-cells and the concomitant 4-fold increase in β-cell apoptosis, indicating that the animals' regulatory mechanisms were working appropriately to keep their β-cell mass at required levels (Figure 3.6).

6.4 PANCREATIC PLASTICITY: THE ACINAR-DUCT-ISLET AXIS

The pancreas has been described as being two separate entities, the exocrine pancreas and the endocrine pancreas [51, 52]. This antiquated description is an inaccurate representation of how the pancreas functions in instance of disease and regeneration. The studies of this thesis, as well as numerous other investigations have indicated that each cell type within the adult pancreas contains the ability to transform into the other pancreatic cell phenotypes [52, 125, 128, 135, 138, 139, 144]. Acini, ducts and islets can all transform into primordial duct-like structures, which can then transform into any of the three mature cell phenotypes. Furthermore, acinar, duct and endocrine cells can transform directly into β-cells. The change in cell phenotype and gene expression of the three main cell phenotypes in the adult pancreas illustrates that the organ has multiple pathways that can lead to appropriate tissue expansion. A better understanding of the mechanism behind cellular plasticity will facilitate new strategies for the effective treatment of disease states.

6.5 FUTURE DIRECTIONS OF PHARMACOLOGICAL INDUCTION OF β-CELL MASS EXPANSION

The studies of this thesis sought to determine if pharmacological initiation of islet neogenesis could be an effective means for the stimulation of β-cell mass expansion and reversal of diabetic hyperglycaemia. INGAP$^{104-118}$ has been shown to stimulate islet neogenesis in a manner similar to the highly regulated process that occurs during pancreatic ontogeny. Significant advancements have been made toward developing an effective pharmacological agent for the successful stimulation of β-cell mass expansion, which may form the basis of future therapies for the treatment of diabetes. It is likely that the most effective therapy for type 1 diabetes will involve a multimodal approach, including neogenic agents (such as INGAP$^{104-118}$), agents to moderate glycaemic profile (such as Amylin, or GLP-1), and selective anti-leukocyte therapies. Such a combination of therapies may finally lead to the restoration of euglycaemia in diabetic individuals and prevent the devastating complications associated with this disease.

6.7 CONCLUSIONS

From the initial hypothesis and objectives of this thesis, to the experiments subsequently performed and the interpretation of their results, the following conclusions can be made:

1. The mechanism of action of INGAP$^{104-118}$ is indeed through stimulation of islet neogenesis, and not through replication of pre-existing β-cells. This effect of INGAP$^{104-118}$ treatment is not species specific, as it can stimulate islet neogenesis in a broad spectrum of mammals, including human. Furthermore, INGAP$^{104-118}$ is effective at stimulating islet neogenesis and subsequent β-cell mass expansion in a dose dependent manner.
2. Similar to that observed during pancreatic organogenesis and new islet formation, INGAP$^{104-118}$ stimulates duct-to-islet transdifferentiation associated with increased PDX-1 expression. As well, INGAP$^{104-118}$ can enhance β-cell mass through both direct and indirect acinar-to-β-cell transdifferentiation, suggesting multiple avenues of potential β-cell mass augmentation.
3. Induction of β-cell mass expansion, through continual administration of INGAP$^{104-118}$ occurs in a highly regulated manner in which the body's homeostatic mechanisms act to prevent unbridled cellular growth, ensuring the resultant β-cell mass remains at the level required.

7.0 ORIGINAL CONTRIBUTIONS TO KNOWLEDGE

"This requirement is mandatory for all McGill University doctoral theses. Elements of the thesis that are considered to constitute original scholarship and an advancement of knowledge in the domains in which the research was conducted must be clearly indicated."

1. *In vivo*, INGAP$^{104-118}$ is effective at stimulating quantifiable islet neogenesis in mice, hamsters and monkeys.

2. INGAP$^{104-118}$ is effective at stimulating islet neogenesis and increasing β-cell mass in a dose-dependent manner in mice.

3. INGAP$^{104-118}$ acts, via a PDX-1 mediated neogenic pathway to enhance β-cell mass.

4. INGAP$^{104-118}$, in combination with gastrin and HGF, can stimulate islet neogenesis and β-cell mass expansion in cultures of human acinar tissue.

5. Induced β-cell mass expansion, via INGAP$^{104-118}$ administration, remains under homeostatic regulation. Following continual exposure to this neogenic agent there is decreased β-cell replication and increased β-cell apoptosis, maintaining the resultant β-cell mass at appropriate levels.

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APPENDICES

Ethics Approval for Animal and Human Tissue Experiments