University of Alberta

BIOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF NEONATAL PORCINE ISLETS

by

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ABSTRACT

Replacement of insulin-secreting β cells by islet transplantation has the potential to restore physiological blood glucose homeostasis. Currently, human cadaveric organ donors are the only source of islets, however, alternative sources such as xenogeneic islets are being investigated. We have established a method for the large scale isolation of islets from neonatal pigs which are glucose responsive and can reverse hyperglycemia in small and large animal models of diabetes.

Islets from neonatal pigs are comprised of a heterogeneous mixture of hormone-producing cells and undifferentiated precursors, which can differentiate, survive and function after transplantation. The objectives of this thesis are two-fold: 1) Examine the differentiation potential of these precursor cells and 2) Assess the survival and functional capabilities of β cells after exposure to high glucose or diabetogenic cytotoxins. The aim of these studies is to indirectly demonstrate that neonatal porcine islets are advantageous for clinical use over adult islets according to the objectives outlined in this thesis.

Precursor cell differentiation was examined after adenoviral mediated over-expression of the transcription factor neurogenin 3. In infected cells, the fraction of glucagon-positive cells was significantly increased. Partial β cell differentiation was achieved by incubation of islets in high glucose culture media (28.0 mmol/l).

Survival and function after chronic exposure to high glucose or brief exposure to the diabetogenic cytotoxin, streptozotocin was also examined. A 7 day culture period in 28.0 mmol/l glucose, failed to irreversibly impair glucose responsiveness, or cause a significant increase in β cell death. Susceptibility to streptozotocin was demonstrated in vitro by significantly reduced islet secretory activity and in vivo by elevated transplant

recipient blood glucose levels. In contrast, graft β cells which differentiated from precursors exposed to hyperglycemia after transplantation into diabetic mice developed streptozotocin resistance.

In summary, islets from neonatal porcine pancreases contain many precursor cells that can be induced to differentiate into endocrine cells with the ability to survive and function after exposure to high glucose and diabetogenic cytotoxins. These advantageous characteristics of neonatal porcine islets may result in better long-term function and protection from cytokine or free radical induced damage after xenotransplantation.

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ABC	avidin-biotin complex
ANOVA	analysis of variance
CK	cytokeratins
DAB	3,3-diaminobenzidinetetrahydrochloride
ES	embryonic stem (cells)
FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Galα(1,3)Gal	galactose-α(1,3)-galactose
GFP	green fluorescent protein
HBSS	Hank's balanced salt solution
IAPP	islet amyloid polypeptide
ICC	islet-like cell cluster
kD	kilo Daltons
MODY	maturity-onset diabetes of the young
NGN3	neurogenin 3
OGTT	oral glucose tolerance test
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen

PCR	polymerase chain reaction
PDX-1	pancreatic and duodenal homeobox gene-1
PERV	porcine endogenous retrovirus
RIA	radioimmunoassay
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of mean
SI	stimulation index
STZ	Streptozotocin
TUNEL	Tdt-mediated dUTP nick-end labelling
XIAP	x-linked inhibitor of apoptosis

CHAPTER ONE

A GENERAL AND HISTORIC DESCRIPTION OF THE ENDOCRINE PANCREAS

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A version of this chapter has been submitted for publication. Harb et al. 2006. Pancreas.

I-1 GENERAL INTRODUCTION

I-1*a* Pancreas

The adult mammalian pancreas is a heterogeneous organ composed of both exocrine and endocrine tissues. The exocrine tissues, which make up over 95% of the pancreatic mass, produce digestive enzymes such as trypsin and amylase. Exocrine tissue is organized into acini, which synthesize the enzymes, and ducts that secrete a bicarbonate rich fluid to deliver the digestive enzymes to the intestine. Islets of Langerhans are spherical clusters of endocrine cells (1-2% of the pancreatic mass) scattered throughout the exocrine tissue (Fig. 1-1), which produce and secrete peptide hormones that regulate glucose metabolism.

The pancreas was discovered by Herophilus (335-280 B.C), a Greek anatomist and surgeon who studied on the island of Cos (1). He is thought to have described the pancreas around the year 300 B.C, but it was not until 400 years later that Ruphos of Ephesus (100 A.D.) named the organ 'pancreas' which in Greek means "all flesh" ('pan'=all and 'kreas'=flesh). Following the discovery of the pancreas, the organ was mistakenly regarded as a protective cushion for the mesenteric vessels by Galen and as a cushion for the stomach 1400 years later by Vesalius and Massa (1). The pancreas was also once proposed to clean and dilute the contents of the upper small intestine before transmitting them to the liver and spleen.

The first resection of the pancreas was performed by Johann Conrad Brunner in 1673 (1). Brunner performed partial pancreatectomies on dogs, admitting that small parts of the organ had to be left behind since the head of the pancreas was difficult to remove from the intestine due to the rich blood supply in that region. Some of the dogs

developed diabetes transiently, which was noticed by increased postoperative drinking and urination. During the nineteenth century, the pancreas was considered a producer of pancreatic juice capable of aiding in the digestion of proteins, fats and starch (1). This realization set the stage for the classic experiments of Claude Bernard (1813-1878), who is considered the father of pancreatic physiology (2). Using dogs with pancreatic fistulaes, he studied the digestive capacity of pancreatic juice, proving that it could degrade proteins, fats and starch into smaller molecules. Although Bernard was correct in asserting a digestive role to the pancreas, he himself sustained, among others at the time, a non-pancreatic origin of diabetes. It was not until eleven years after the death of Bernard, that Joseph Freiherr von Mering (1849-1908) and Oskar Minkowski (1858-1931) demonstrated the relationship between the pancreas and diabetes mellitus (3), thus confirming the role of the pancreas in 'pancreatic diabetes' described by Lancereaux (4). In 1889 Minkowski and Mering debated as to whether an animal could survive without a pancreas. Mering had wanted to study the role the pancreas played in the digestion of fats and so Minkowski suggested total extirpation of the pancreas. That same day, they performed a total pancreatectomy in a dog, thus producing diabetes as determined by abnormal hunger, intense thirst, glycosuria, polyuria, and loss of weight despite good food intake. This was the first clear cut demonstration that a complete pancreatectomy results in diabetes, which was published in *Diabetes mellitus nach pankreasextirpation* [Diabetes mellitus after extirpation of the pancreas] in 1890 (3).

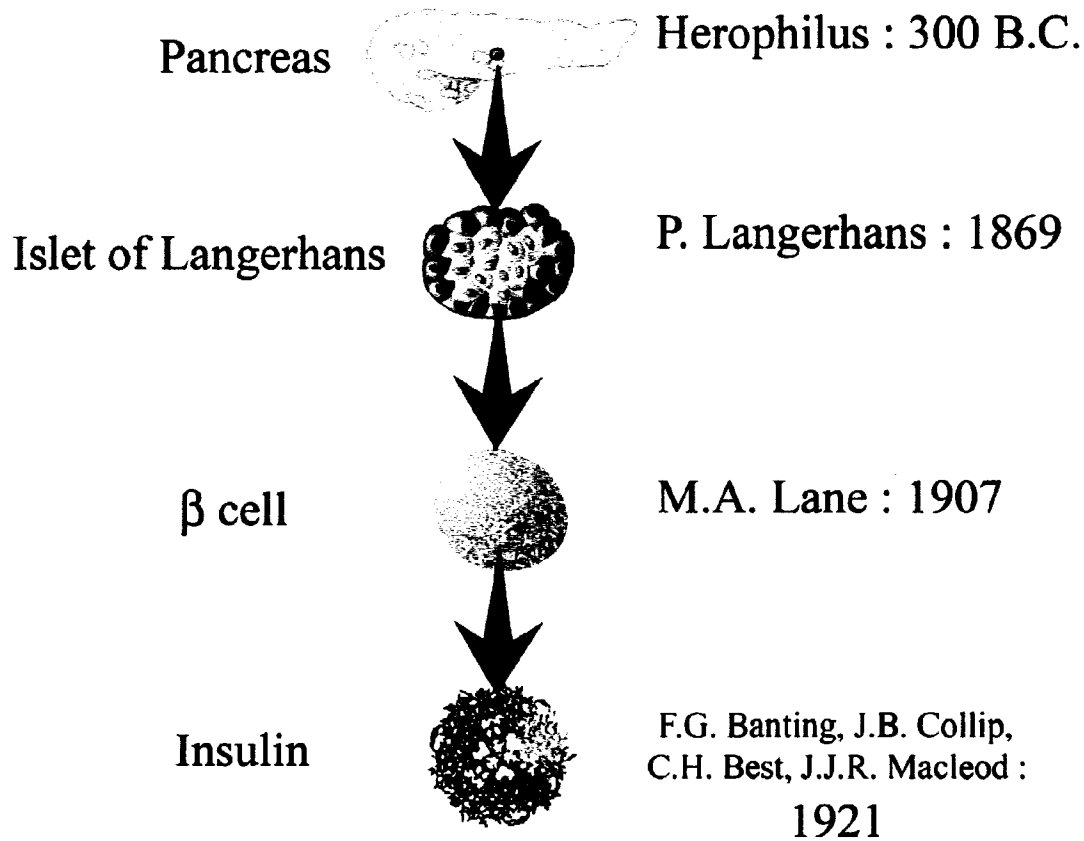

Figure 1-1: The adult human endocrine pancreas consists of several million islets of Langerhans interspersed throughout the gland. Islets are composed of thousands of cells, of which between 30 and 80% are β cells. A single β cell contains between 20-50 pg of insulin. The names of the discoverers and the year each discovery occurred in are shown on the right.

I-1*b* Islets of Langerhans

Islets contain 4 principal endocrine cell types which produce insulin (β cells), glucagon (α cells), somatostatin (δ cells) and pancreatic polypeptide (PP cells). In humans, the proportion of insulin producing β cells within islets varies considerably (28-75%) as does the α cell population (10-65%). An islet contains a few thousand cells and can vary in size from 50 to 500 μm in diameter. Although accounting for less than 2% of

the adult pancreas mass, they receive between 5-10% of the entire pancreas blood supply (5).

The pancreatic isles were first described in 1869 by Paul Langerhans, however their function as a hormone producing endocrine unit would not be described for another quarter of a century. Paul Langerhans (1847-1888) was born on 25 July 1847 in Berlin to a well known and successful physician Paul August Herrmann Langerhans (Paul Sr., 1820-1909) and Anna Luise Caroline Langerhans (1824-1853) (6). While only 12 years old he had revealed his intention to study medicine. As a medical student, Langerhans began his studies at the University of Jena (1865-1866), and then later transferred to the University of Berlin, where he investigated the tactile corpuscles in diseased skin and the central nervous system in 1867. Langerhans then returned to the histological study of the abdominal digestive glands which he defended publicly as his thesis on 18 February 1869. In his 32 page thesis entitled *Beiträge zur mikroskopischen Anatomie der Bauchspeicheldrüse* [Contributions to the Microscopic Anatomy of the Pancreas], Langerhans would describe the microscopic appearance of islets in a rabbit pancreas while admitting he did not know the function of these special cells (7). Langerhans begins the second paragraph of his thesis by apologizing for its scanty contents. "With regret, I must open my communication with the declaration that I cannot in any way put forth the conclusive results of a completed investigation. I can describe, at most, a few isolated observations which suggest a much more complicated structure of the organ investigated than hitherto accepted. The purpose of these lines can therefore at best only be to help draw greater attention to the pancreas than has hitherto been given to it by anatomists."

Langerhans used the pancreas of a rabbit because it possesses a more favorable structure for investigation in that it is a large expansive organ unlike the very compact pancreas of man, guinea pig, dog, cat, snake or frog. Using specimens of pancreas taken from freshly killed rabbits, he studied the duct system which he compared to the branches of a defoliated tree. He then macerated the pancreas in Müller's fluid in order to differentiate the miscellaneous cellular parts of the pancreas. Altogether he identified nine cellular phenotypes within the pancreas: epithelial cells of the peritoneum, connective tissue cells, blood vessel cells, red and white blood cells, nerve cells, acinar cells, epithelial duct cells, spindle cells (centro-acinar), and small cells (endocrine). These small cells which he designated as *Häuflein* were described "...of almost perfect homogenous content, and of polygonal form, which with round nuclei without nucleoli, mostly lying together in twos or in small groups." Although he characterizes their microscopic appearance, he admits to not knowing the exact nature of these cells and only speculates a relationship between them and the nervous system of the gland.

In 1893, a French histologist E. Laguesse (1861-1927) associated these small clumps of cells discovered by Langerhans with those he had been studying in the human pancreas and thus named them *Les ilôts de Langerhans* in honor of Langerhans (8). Laguesse was also the first to postulate that these cells may produce an internal secretion. Later, during the early 1900's more support for a possible connection between the islets of Langerhans and diabetes would emerge (1). Even after the designation by Laguesse, others at the time had noticed the islets dispersed among the pancreatic parenchyma and had named them separately: intertubular cell clumps (Kühne and Lea), secondary cell groups (Harris and Gow), *points folliculaires* (Renaut) and Islands of Langerhans

(American anatomists). Paul Langerhans did not live to see his most significant contribution to science and medicine be truly realized. His life was tragically cut short 5 days before his 41st birthday on 20 July 1888 due to progressive renal failure from chronic tuberculosis. He is buried in the British cemetery in Funchal, on the island of Madeira. His epitaph bears a quotation from the fourth book of Homer's Odyssey, translated: 'Nor did he wish to live any longer nor to see the light of the beaming sun'.

I-1*c* β Cells

The insulin producing pancreatic β cell is a remarkably complex and highly differentiated cell. Often viewed as the body's fuel sensor, the β cell controls insulin secretion by a complex interplay of metabolic and electrogenic events. Glucose-stimulated insulin secretion (GSIS) is made possible by the expression of a number of key transporters and enzymes which allow for the release of insulin within minutes of ingesting a meal. Biphasic insulin secretion involves at least two main signaling pathways, the K_{ATP} channel-dependent and K_{ATP} channel-independent pathways (9). The K_{ATP} channel-dependent pathway begins with glucose uptake by a facilitated-diffusion glucose transporter (GLUT) into the β cell. Immunohistochemical studies have demonstrated that GLUT isoform expression varies in β cells isolated from different species and with developmental age within a given species. For example, with regard to age, compared to GLUT2 expression levels in adult β cells (10), fetal β cells express significantly less GLUT2 (11). This reduced GLUT2 expression has been suggested to impede metabolic flux through glycolysis which results in the immature secretory response observed in fetal β cells (12, 13). Cross species variation in glucose transporter

isoform expression has also been observed in β cells. β cell preparations from human pancreases were compared to rodents and found to contain significantly lower GLUT2 levels (100-fold) (14). The question of whether a high Km glucose transporter such as GLUT2 is necessary for proper β cell function has been the subject of debate (15-17). The importance of GLUT2 is highlighted in cellular engineering studies performed on insulin-secreting cell lines, whereby introduction of the GLUT2 gene confers GSIS (18-20). Moreover, in experiments on GLUT2-knockout mice, β cells had lost the first phase of GSIS, but had a preserved second phase, although reduced in magnitude. In addition, islet cellular composition was altered and a diabetic-like phenotype characterized by hyperglycemia and high circulating free fatty acids was found (21, 22). Re-expression of GLUT2 in these β cells was found to restore normal GSIS (22). However, data from studies on β cells which express only either GLUT1 or GLUT2 suggest the low Km GLUT1 transporter is as efficient as GLUT2 in maintaining normal GSIS (23-25).

Glucose metabolism within the β cell begins with the rate limiting step catalyzed by glucokinase which phosphorylates glucose. Increased glucose metabolism increases the cellular adenosine triphosphate/adenosine diphosphate (ATP/ADP) ratio, which stimulates insulin release via closure of K_{ATP} channels. Glucose induced closure of K_{ATP} channels results in membrane depolarization and opening of L-type voltage-dependent calcium channels. The ensuing increase in intracellular calcium triggers exocytosis of insulin granules responsible for the first phase of insulin release. The second phase of insulin release is due to the K_{ATP} channel-independent pathway (9). The mechanisms described here are undoubtedly more complex since several intracellular messengers are known to modulate the process.

Pancreatic β cells were first described by M.A. Lane while studying the pancreas of a guinea pig in 1905 at the Hull Laboratory of Anatomy, University of Chicago (26). Lane identified the existence of 2 islet cell types which were chemically and morphologically different from each other. At this point in time, Laguesse's idea which suggested that the islets produced an internal secretion and its connection to carbohydrate metabolism had been established and was known as the 'sugar function' of islets. Still, others believed islets were no more than exhausted acini, which after recovery would return to their acinar state. Lane believed the islet cells were not simply exhausted pancreas cells and instead possessed their own 'positive function'.

Using 3 different fixation techniques, alcohol-chrome-sublimate, 70% alcohol, and aqueous-chrome-sublimate, Lane could identify different cell types based on the solubility of their granules. Lane identified 2 types of cells which he called A and β cells. "To avoid cumbersome periphrase and repetition I will hereafter designate the cells in which the granules are fixed in alcohol as A cells, and these in which the granules are fixed with the chrome-sublimate fluid as β cells." Lane points out that none of the granular substances found in either of the 2 types of islet cells are of the same chemical character as the prozymogen or zymogen granules in pancreas cells. Lane then went on to characterize the morphology of each of the A and β cells as well as point out how they differ from each other. The β cell was described as being the smaller cell in the islet but at the same time more abundant, existing as long cords of cells. "The nucleus of the β cell is invariably centrally placed, is smaller than the nucleus of the A cell, circular, markedly less vesicular than the nucleus of the A cell, and is also distinguished from the nucleus of the A cell by the comparatively large quantity of chromatin it contains." In his

concluding remarks, Lane correctly postulates that "...the islets of Langerhans are structures which in all probability have the function of producing a twofold substance which [when] poured into the blood stream, has an important effect upon metabolism." Immunofluorescent localization of insulin in β cells by Lacy and Davis in 1959 later verified that the β cell was in fact a definitive insulin producing endocrine cell (27).

I-1*d* Insulin

Following the discovery of insulin over 80 years ago, much is now known about its structure and biochemistry. Insulin biosynthesis begins as preproinsulin which is processed to proinsulin, folded and subsequently assembled into hexameric insulin for transport (28). The complete amino acid sequence of insulin was first determined by Sanger and co-workers and found to contain 51 amino acids and weigh 5808 daltons (29). The insulin monomer contains 2 peptide chains, the A and B chains which have 21 and 30 amino acids respectively. These 2 chains are cross-linked by 2 disulphide bridges. A third peptide known as C-peptide or connecting peptide serves as a flexible linker between the A and B chains, thereby facilitating the proper formation of the interchain disulfide bridges (30). Detailed knowledge of the amino acid sequence and three-dimensional structure of insulin has resulted in its recombinant synthesis which is now used to treat the vast majority of people with diabetes worldwide.

The contribution of Canadian researchers to the already heavily investigated role of a pancreatic extract on diabetes proved to be the most beneficial for diabetic patients worldwide. The work of 4 researchers from the Universities of Toronto and Alberta proved instrumental in the successful purification and clinical application of their extract.

The beginnings of such instrumental work can be traced to one man, Fredrick Grant Banting (1891-1941), a surgeon by training who initially had no interest in studying or treating diabetes. In 1921, Banting had read an article by M. Baron which described how ligation of the pancreatic duct results in degeneration of the exocrine pancreas while leaving the islets of Langerhans intact (31). This suggested to Banting that the selective destruction of the exocrine pancreas would facilitate the extraction of the internal secretion produced by the islets of Langerhans. Banting took this idea to J.J.R. Macleod (1876-1935), who was Professor of Physiology at the University of Toronto and who assigned Banting work space and an assistant, Charles Best (1899-1978). Later, J.B. Collip (1892-1965), a biochemist from the University of Alberta would join the team when the insulin extraction process proved more difficult than originally thought. The team faced the challenging task of building on work conducted over the past 40 years beginning with Mering and Minkowski, and making it clinically applicable to patients with diabetes. Their experiments, if successful, would provide the final piece of evidence needed to conclusively link the pancreas, islets of Langerhans and β cells to diabetes. "According to Macleod there are two possible mechanisms by which the islets might accomplish this control [of carbohydrate metabolism]: 1) the blood might be modified while passing through the islet tissue, i.e., the islands might be detoxicating stations and 2) the islets might produce an internal secretion." (32).

With this in mind, they began experiments in May 1921 by ligating the pancreatic duct of a number of dogs as well as performing a number of pancreatectomies (33). The pancreatectomies were done to familiarize themselves with the increased sugar contents of blood and urine and the overall general clinical behavior of the animals after the

operation. The results of their experiments which had commenced in May were published in February 1922. On 11 January 1922, the first patients were treated for the first time with insulin at the Toronto General Hospital. Although a lowering of blood and urine sugar levels was noticed, the impure nature of the extract hindered its further use in patients. Due to this impurity, Collip was assigned the job of purifying insulin to lessen the toxic side effects. Following refined purification of the extract by Collip, seven patients in total, including the daughter of the United States Secretary of State, were treated that year with Collip's extract and the results were published in the *Canadian Medical Association Journal* in December 1922. The name for Collip's extract, 'insulin' as it would eventually be named after the Latin word *insula*, meaning island, was not initially used by Banting or his co-workers, in fact the word 'iletin' was used instead. Later it was realized that Macleod had always insisted on using the word 'insulin', which had previously been used in Europe by J.D. Meyer in 1909 and by E.A. Schafer in 1916 (1). Meanwhile, in the United States, William D. Sansum working at the Potter Metabolic Clinic in Santa Barbara, California, injected Charles E. Cowan, the first diabetic patient to receive U.S. made insulin, on 31 May 1922 (34). Eventually, collaboration was setup between the University of Toronto and Eli Lilly Company of Indianapolis to allow for the large scale production of insulin since the Connaught Laboratories at the Toronto University could not keep up with the increasing clinical demand for insulin in both Canada and the United States (33).

Although insulin was first crystallized by Abel and co-workers in 1925, its primary structure was not described until 1959 by Fred Sanger (29) earning him a Nobel prize in 1958. Insulin was the first circulating peptide hormone to be measured by

radioimmunoassay in 1959 by R.S. Yalow and S.A. Berson (35) earning Yalow a Nobel prize in 1977. Now with the ability to measure insulin levels, Curry, Bennett and Grodsky, demonstrated insulin secretion is biphasic, suggesting compartmentalization of insulin storage in the β cell (36). The three-dimensional structure of insulin was solved by Dorothy Crowfoot Hodgkin and co-workers in 1969 using X-ray crystallographic methods (37). In 1982, genetically engineered human insulin was introduced and is now used to treat the vast majority of people requiring insulin treatment. Recombinant insulin replaced the porcine-bovine insulins initially used after the discovery of insulin in 1921. Today there are over 14 different insulin formulations available from ultra-short-acting to long-acting, which when used in combination come close to mimicking the body's natural insulin production.

The route of insulin delivery in diabetic patients may one day change as companies are racing to develop an inhaled insulin product which could potentially replace injectable forms of insulin (38). Currently, dry powder and liquid droplet forms of insulin are being developed and tested for any unwanted pulmonary side effects. If such a technology succeeds, the cumbersome use of insulin injections may one day become a thing of the past.

I-2 ENDOCRINE PANCREAS DEVELOPMENT

I-2*a* Embryonic Specification and Differentiation of Islet Cells

Development of the endocrine pancreas can be divided into 3 developmental stages (transitions) (39). The first developmental transition occurs when dorsal and ventral buds protrude from regions of primitive gut endodermal epithelium early in

development [embryonic day 8.5 (E8.5) to E9.5 in mice, E28 in humans]. These buds undergo branching morphogenesis and become separate pancreatic buds of undifferentiated ductal epithelium. The second developmental transition begins when levels of mRNAs encoding endocrine hormones increase by 100-1000 fold at (E13.5) and β cell terminal differentiation is initiated (40). The third transition marks the beginning of islet formation when just prior to birth (E18-19) the endocrine cells cluster together into islets and subsequently undergo maturation/acquire full nutrient responsiveness 2-3 wks after birth.

A detailed description of the cellular and molecular mechanisms underlying each transition is beyond the scope of this chapter and hence the reader is referred to a number of excellent reviews (39, 41-45). What is important to consider during embryonic pancreas development, is that a number of intra- and inter-cellular molecules specify pancreas morphogenesis and islet cell differentiation. Prior to pancreas formation, signals from the surrounding mesenchyme and ectoderm induce endoderm prepatterning into a pancreatic fate. The neighboring notochord releases factors which permit pancreas development by expressing inductive signals such as activin-βB and FGF2 which repress endodermal sonic hedgehog (Shh) activity allowing for expression of pancreatic genes (46). Interestingly, development of the dorsal, but not ventral pancreatic bud is dependent on these signals from the notochord (47). The ventral pancreas which develops in close proximity to the liver develops by default due to a lack of instructive signals from cardiac cells to induce liver formation (41). Specification of endocrine islet cells within the developing pancreas occurs by the Notch signaling pathway which is also responsible for the generation of neurons during neurogenesis (48, 49). Notch signaling controls the fate

of developing pancreatic cells to become either differentiated endocrine cells or remain as undifferentiated progenitor cells (48, 50). When Notch receptor activation is blocked, high levels of a pro-endocrine transcription factor neurogenin 3 (ngn3) is expressed which promotes endocrine cell differentiation. The expression of several transcriptional regulators early in pancreas development later reappear to induce final commitment of islet cell fate. Mutations in the genes encoding these transcription factors results in a subtype of diabetes called maturity-onset diabetes of the young (MODY) characterized by autosomal dominance inheritance, non-ketotic hyperglycemia, an age of onset <25 years and a major defect in β cell function (51). Expert detailed reviews are available which summarize the results of numerous studies using transcription factor specific knock-out/in mice in which key genes have been disrupted or over-expressed (39, 52, 53).

Pancreatic endocrine cells arise from endodermal cells, while sharing many of the developmental transcription factors important for neuronal differentiation. Specification of pancreatic cells is initiated by homeodomain transcription factors such as Hb9 and Pdx-1 which become restricted to β cells later in development (42). Pdx-1 regulates a number of key β cell genes required for maintaining the mature hormone-producing phenotype such as insulin (54), islet amyloid polypeptide (55), GLUT2 (56) and glucokinase (57). It is not uncommon for transcription factors which play a role in islet development to also be required for β cell function later in adult life. After specification of the pancreas, endocrine cells derive from progenitors expressing ngn3, a basic helix loop helix protein (58) which is absent from differentiated endocrine cells (50, 59). Another bHLH transcription factor, NeuroD is expressed in all endocrine cells including β cells where it acts as a key regulator of β cell products such as insulin (60). Members

of the paired-box homeoprotein family, Pax4 and Pax6, are expressed early in pancreas development, then later reappear during β cell differentiation prior to birth (61). Members of the NK2 family of homeoprotein transcription factors, Nkx2.2 and Nkx6.1 are also important in islet cell differentiation. Nkx2.2 is expressed just after Pdx-1 in the primitive pancreatic epithelium and later becomes restricted to $ngn3^+$ progenitors and differentiated islet cells (52). Nkx6.1 is found only in β cell progenitors where it plays a role in β cell terminal differentiation.

Many of the transcription factors involved in pancreatic endocrine cell development, of which only some are briefly mentioned here, serve dual roles in initiating endocrine cell differentiation as well as later maintaining the terminally differentiated phenotype in postnatal life.

I-2*b* Postnatal β Cell Growth

As was once thought about brain cells, it used to be thought that we are born with a fixed number of β cells to sustain us for our life time. However, we now know that the insulin-producing β cell mass is dynamic and controlled by a delicate balance of proliferation, neogenesis, growth and death (62-64). Although recent studies debate the origin of new β cells in postnatal life, contributions from both β cell proliferation as well as neogenesis are most likely responsible. Prenatal β cells arise from endodermal epithelium and hence differentiation dependent changes in the expression patterns of individual cytokeratin (CK) polypeptides can be used to study β cell differentiation before and after birth (65). Cytokeratins belong to one of three groups of cytoskeletal systems known as intermediate filaments. Intermediate filaments are made up of

filamentous proteins (type I-V) that have no known enzymatic activity, but are encoded for by at least 67 genes in humans (66). Cytokeratins are intermediate filament heteropolymers composed of type-I and type-II intermediate filament proteins which comprise a family of approximately 20 different polypeptides (CK1-CK20). The cell-type specificity of CKs makes them useful markers to determine cell identity as well as assess (trans)differentiation. In the adult human pancreas, acinar cells express CK8 and 18, while ductal cells express CK7 and 19 (67, 68). Islets transiently express CK7, 19, (and 20 in rats) during fetal and neonatal stages of development (67-69), but later lose this expression in adulthood (70, 71). These data along with the high reported proportion of β cells found budding from ductal epithelium as well as the presence of CK^+/$insulin^+$ cells suggest ductal cells represent a pool of β cell precursors (69, 72, 73). In addition to occurring normally in postnatal development, islet neogenesis from ductal epithelium can also be stimulated experimentally, for example by partial pancreatectomy (72) and has recently been proposed to be responsible for the regeneration of β cells in patients with long-standing type 1 diabetes (74).

β cell replication is another major contributor to new β cells in postnatal life (75). Replication is highest during the neonatal and adolescent periods of life, then later slows to as little as 0.07% per day in adults (76-78). Ongoing β cell loss occurs to maintain a balanced β cell mass which would otherwise double within one month if there was no concomitant β cell loss (79). Several hormones such as growth hormone, hepatocyte growth factor and prolactin can also stimulate β cell replication in postnatal life in response to increased insulin demand (80, 81). In addition, nutritional alterations, such as

high levels of circulating plasma glucose during fetal development can also affect postnatal β cell growth and function (82-84).

I-3 DIABETES MELLITUS: WHEN THE ENDOCRINE PANCREAS FAILS

"Man may be the captain of his fate, but he is also the victim of his blood sugar"

Wilfrid Oakley [*Trans. Med. Soc. Lond.* 78, 16 (1962)]

I-3*a* Etiology and Pathophysiology

Diabetes mellitus, for which there is as yet no cure, is a complex metabolic disease characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. Diabetes is diagnosed by plasma glucose levels above 7.0 mmol/l after an 8 hour fast or above 11.1 mmol/l at 2 hours after an oral glucose tolerance test (85). Classic symptoms of hyperglycemia are also used for diagnosis and include polyuria, polydipsia, unexplained weight loss, polyphagia and blurred vision. The majority of diagnosed cases of diabetes fall into two main categories characterized by either absolute insulin deficiency (type 1) or a combination of insulin resistance and an insufficient insulin secretory response (type 2).

Over 200 million people worldwide suffer from diabetes, with only half being diagnosed, making it the third most common disease and fourth leading cause of death in North America. In the Unites States alone, there are over 20 million people with diabetes, most of them with type 2, which represents about 7% of the population. Around 2 million Americans suffer from type 1 diabetes, which is the number of combined

Canadians with both types 1 and 2 diabetes. The prevalence of diabetes is increasing at such an alarming rate worldwide that by 2025 there will be over 330 million people in the world with the disease (86, 87).

The alarming rise in the incidence of diabetes has much to do with the rise in obesity. Over 50% of the world's population is overweight or obese and is responsible for 60% of all cases of diabetes (86). Even more alarming is the rate at which this once adulthood disease is being reported in children, in some countries accounting for 80% of all cases of juvenile diabetes (88). The frequency of type 1 diabetes is low relative to type 2, but can vary significantly. Clinical type 1 diabetes represents end-stage insulitis with only 10-20% of β cells still present at the time of diagnosis. The pathogenesis of type 1 diabetes is multifactorial involving genetic susceptibility, autoimmune mechanisms, and environmental factors. By disease onset, the majority of the β cell population has been lost leaving patients with no residual insulin secretion and fully dependent on exogenous insulin therapy for survival. Failure to maintain strict glycemic control with exogenous insulin increases the risk of developing chronic complications of hyperglycemia which includes damage to the eyes, kidneys, nerves, heart and blood vessels. Many patients face an increased risk of hypoglycemia caused by too much insulin at the wrong place and/or time when attempting to manage blood glucose levels in the normal range. Fluctuating levels of blood glucose in patients with type 1 diabetes affects the levels of reactive oxygen species in cells and accelerates micro- and macrovascular disease. Therefore, more physiologic control of glycemia is required to avoid development of complications associated with fluctuating blood glucose levels. By

providing an artificial endocrine pancreas by β cell replacement therapy, proper blood glucose homeostasis can reliably be achieved for the treatment of diabetes.

I-3*b* The Discovery of Diabetes Mellitus

Diabetes was first described around 1500 B.C. in what is now known as the Ebers Papyrus, named after Georg M. Ebers (1837-1898) a scholar of ancient Egypt who translated the papyrus (89). In the papyrus, there is a description of patients who become thin, are hungry continuously, urinate frequently and are extremely thirsty. The phenomena of polyuria had been observed in several other ancient papyri, including the Hearst papyrus (1600 B.C.) and the Brugsch papyrus (1300 B.C.) as well as ancient Hindu, Greco-Roman, and Chinese texts (90).

The Greek term *διαβητης* [diabetes] was coined by the physician Aretaeus Cappadocia (81-138 A.D.), which means 'to run through' or 'to siphon' since large amounts of urine were noticed to pass through the kidneys in this disease (91). The sweet taste of this urine was first described in the seventeenth century by Thomas Willis (1621-1675) (1). Ancient Arab, Indian, Japanese, and Chinese physicians had also noticed large amounts of sweet tasting urine from their diabetic patients, however, Willis was the first to distinguish diabetes mellitus from diabetes insipidus, the latter lacking glycosuria. The presence of sugar in the urine became such a characteristic feature of diabetes that mellitus, from the Latin noun *mell*, meaning honey, was added to distinguish it from diabetes *insipidus* (without taste; insipid) (1). In 1797 John Rollo noticed the smell of acetone on the breath of diabetics as well as diabetic cataracts (92). Almost a century had passed before anyone would attempt to subdivide diabetes as a disease further. In 1887

Lancereaux described two types of diabetes basing his distinction on whether the patient was fat or thin and thus introduced the terms *diabète Maigre* [thin] and *diabète Gras* [fat] (1). In addition, Lancereaux is credited with being the first to discover an atrophic pancreas in patients suffering from 'thin diabetes' and thus also introduced the term 'pancreatic diabetes'. Decades later, patient responsiveness to insulin was used to distinguish between the two types of diabetes, thus further confirming the existence of insulin-sensitive and resistant forms of the disease. Roughly ten years later, comparisons of certain physical characteristics of diabetic patients by the physical anthropologist Dupertuis, lead him to designate patients as either Group I or II (93). Group I patients were further subdivided into IA and IB; IA patients exhibited skeletal linearity and delicacy while Group IB patients had obscured muscle sculpturing due to the overlying panniculus fat. These designations were re-emphasized in the 1950's, that there are two broad groups of diabetics, the young, thin, acute onset and the older, obese, insipidious onset, which were referred to as type I and type II, respectively. In 1976, Cudworth assumed 2 aetiologically distinct forms of the disease as determined by differences in age onset, relative abruptness of onset, tendency to ketoacidosis, and dependence on insulin (94). He recognized that both types of primary diabetes which he called type 1 and type 2 in favor over 'juvenile onset' and 'maturity onset' respectively are caused by both genetic and environmental factors. Although type 1 diabetes has only been considered an autoimmune disease since the 1970s, many of the original essential features outlined by Cudworth (94) have remained the same today and have only been modified in detail by the revolution of molecular biology.

I-4 INVESTIGATIONAL SOURCES OF β CELLS FOR THE TREATMENT OF TYPE 1 DIABETES

"For people who already have the disease [type 1 diabetes], replacement of the damaged β cells can conceivably be achieved by stimulating the patient's own pancreas progenitor cells to accelerate the rate of β cell neogenesis. However, our present understanding of this process, let alone the ability to manipulate it in vivo, precludes such a possibility."

- Dr. S. Efrat, (Taken from Efrat Trends Mol Med 2002)

Physically replacing the deficient β cell mass in patients with type 1 diabetes has long been proposed as an ideal therapy for restoring physiologic blood glucose homeostasis. β cell transplants in diabetic rodents have been shown to survive and reverse hyperglycemia (95, 96). Nonetheless, the only current β cell transplants are in the form of allogeneic islet cell transplants or whole pancreas transplants which include non-β cells and pancreatic non-endocrine cells. Ultimately, endogenous regeneration of a patients own β cells by pharmacological stimulation of the pancreases reparative process may one day circumvent the need to transplant foreign insulin-producing cells. Nonetheless, researchers are currently investigating alternative sources of β cells for transplantation including xenogeneic islets and embryonic or adult stem cells to replace the use of organ derived allogeneic islets which are in limited supply (97).

I-4*a* Allogeneic Islets

Currently, allogeneic islets isolated from cadaver organ donors are being used for islet transplants to treat brittle type 1 diabetic patients with a high incidence of hypoglycemic unawareness (98). The most common indication for clinical islet

transplantation is metabolic instability marked by recurrent hypoglycemia. However, for islet transplantation to become the gold standard treatment for diabetes, an unlimited supply of islets which are immunologically accepted by the recipient must first be found. Human allogeneic islet transplantation currently involves implanting ABO-compatible, HLA-mismatched islets from 2-4 cadaveric donor pancreases into the portal vein of immunosuppressed patients (Fig. 1-2). The goals of allogeneic islet transplantation involve: insulin independence, long-term islet graft function, normalization of HbA1c levels and elimination of severe hypoglycemic episodes. At the University of Alberta, a team of researchers and surgeons led by Dr. Ray Rajotte was able to achieve enhanced insulin secretion and insulin independence in isolated cases of islet transplantation in the late 1980's/early 1990's. Patients (C-peptide negative) received large grafts (10000 IEQ/kg BW) consisting of multidonor fresh and cryopreserved islets and were immunosuppressed with polyclonal antilymphocyte serum, cyclosporine, steroids, and azathioprine (99). This success was achieved due to several technological advancements in islet isolation and pharmacological improvements in immunosuppression which had occurred over a period of twenty years, culminating in enhanced clinical outcomes (100). Between 1974 and 1999, over 353 islet allografts had been performed in type 1 diabetic patients in many European and North American centers as reported by the International Islet Transplant Registry (ITR) (101). Of these however, only 8% of recipients were insulin-independent at one year posttransplant. In 2000, the group at the University of Alberta reported a 100% insulin independence rate at 1 year posttransplant in seven islet recipients treated with a steroid-free, sirolimus-based immunosuppression protocol (98). Following this milestone development, several centers across North America began

performing islet transplants according to the Edmonton protocol and have now transplanted over 470 patients in only 5 years [there are now more than 45 centers worldwide attempting clinical islet transplantation]. In April 2004 the Collaborative Islet Transplant Registry (CITR) released the results of 12 islet transplant centers which had performed 86 islet transplants (102). At 12 months posttransplant, 60% were reported to be insulin-independent, whereas insulin independence rates of >80% have been achieved at the most experienced islet transplant centers (103). Even with islets from 2-4 donor pancreases and a glucocorticoid-free, calcineurin inhibitor based immunosuppression regimen, the functional capacity of transplanted islets is estimated to correspond to only 20-40% of that in a non-diabetic person (104, 105). In experimental rodent models of islet transplantation, as much as 50-70% of the transplanted islet mass is thought to be lost in the immediate posttransplant period (106). One of the main reasons for this initial loss of islet mass in the immediate posttransplant period is the activation of a thrombotic/inflammatory reaction between the islet cells and ABO-compatible blood (107). Islet cell expression of inflammatory mediators such as tissue factor (TF) and MCP-1 triggers the rapid binding to and activation of platelets to the islet surface, activation of the coagulation and complement systems and leukocyte infiltration (108). This reaction which is referred to as the instant blood-mediated inflammatory reaction (IBMIR) is triggered within minutes after islet infusion into the portal vein and remains a major obstacle to implementing successful tolerance protocols in islet allotransplantation. Another reason for the selective loss of β cell mass immediately posttransplant, is the changes in the energy status and balance of pro-apoptotic and anti-apoptotic mediators which are influenced by the preservation and isolation procedures prior to transplantation

(109). Transplanted β cells also suffer from metabolic stress, hypoxia and cytokines which ultimately results in cell death. β cell function continues to deteriorate over time posttransplant and is due to several reasons including: chronic allograft rejection, undiagnosed acute rejection, local islet toxicity from immunosuppressive drugs, recurrent autoimmunity and failed islet regeneration.

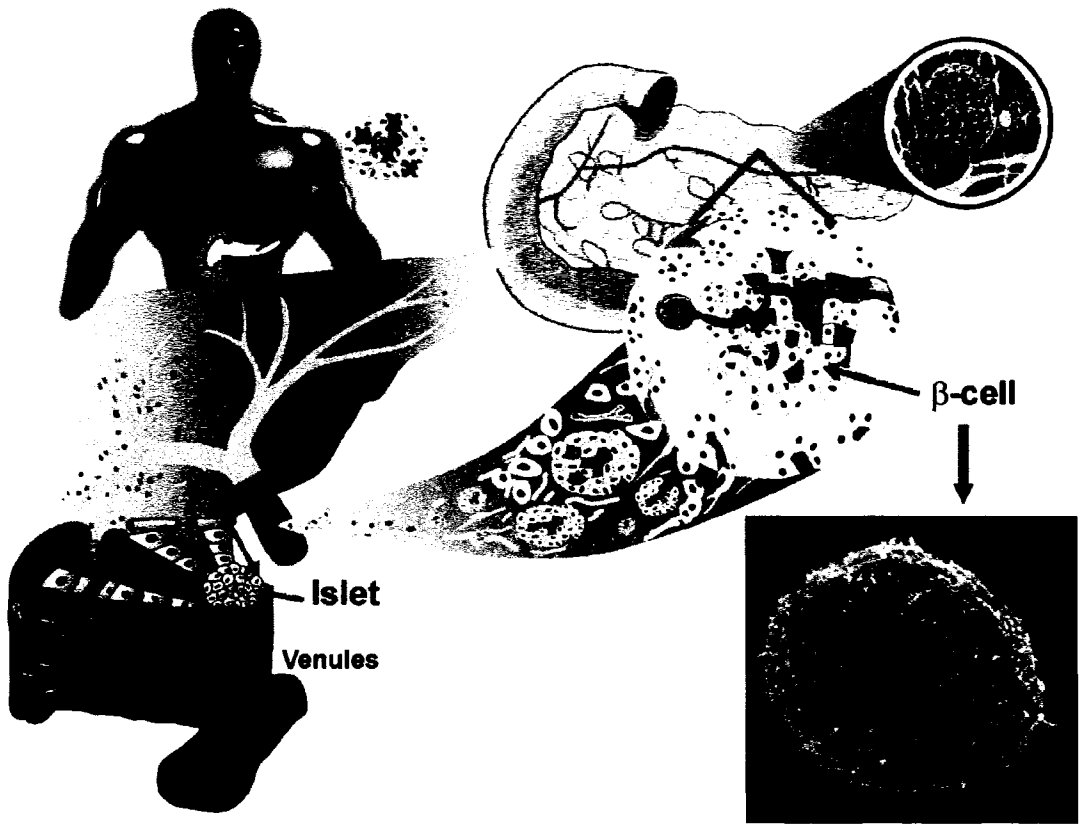

Figure 1-2: Human allogeneic islet transplantation.

Despite only a limited number of insulin independent patients at 5 years posttransplant, the reduced risks of metabolic lability, recurrent hypoglycemia and reduced glycated HbA1c are perceived as continual graft function and transplant success (110). Indeed, 83% of recipients continue to demonstrate persistent islet function at 5

years when measured by C-peptide secretion. However, progress is still needed in critical areas involved in human allogeneic islet transplantation before it can become a standard procedure for a larger number of patients with type 1 diabetes and eventually patients with insulin-dependent type 2 diabetes. For example, an extravascular site which circumvents IBMIR would be desirable such as the omental pouch. Also, by avoiding the use of toxic immunosuppressive drugs such as calcineurin inhibitors which impair islet function, viability and proliferation, posttransplant β cell survival and function may be prolonged (111). One way of avoiding toxic immunosuppressive drugs is to encapsulate islets and protect them from rejection and autoimmune destruction in diabetic patients. However, encapsulation technology which was developed over twenty years ago has progressed little and still fails to protect islets from long-term allogeneic and/or autoimmune destruction (112-114). In addition, a large supply of islets will also need to be found for this type of therapy to become more widely used. One attempt at reducing the number of donor pancreases required for islet recipients is highlighted in the recent success rates of single-donor islet transplants. Insulin independence after single-donor islet transplantation has already become possible on a more consistent basis (115, 116). Interest in single-donor or even the more controversial live islet donor transplants may become applicable to young diabetic patients and contribute to the reduction in the shortfall between supply and demand.

However, much more research is needed before allogeneic islet transplantation becomes a routine therapeutic treatment for clinical diabetes since islet, just as pancreas transplantation is hindered by the shortage of organ donors and the chronic use of

immunosuppression. With the current protocols available, human allogeneic islet transplantation continues to fall short of the definitive diabetes cure.

I-4*b* Xenogeneic Islets

Sources of non-human β cells are being investigated as a potential source of insulin producing cells for replacement therapies in patients with type 1 diabetes. Historically, animal sources of insulin-producing tissues (eg. sheep) were considered long before the use of human tissues (117). Among the many animal sources of insulin producing β cells available, bovine islets (118), fish-Brockman bodies (119, 120) and porcine islets are the most frequently considered.

Pigs have been advocated as an ideal donor source of xenogeneic islets for clinical use for a number of reasons including shared physiology, plentiful supply, short gestation, and more recently, the generation of transgenic animals. In addition, porcine insulin has been used for years to treat type 1 diabetes and pig islets can maintain blood glucose levels in the same physiologic range as humans. Moreover, pigs can be raised under gnotobiotic (germ free) conditions to prevent the passage of lethal microorganisms (121). Islets from pigs have been frequently shown to reverse hyperglycemia in experimental animal models of diabetes (122-126). The use of adult pig islets for widespread basic or clinical use has been met with limited success however, because adult pig islets are difficult to isolate (127), fragment easily in culture (128) and possess very limited growth potential (106, 129). Due to these disadvantages, fetal and neonatal pigs are currently being more commonly researched as potential donors of islets for clinical transplantation. Insulin secretion studies performed on islets isolated from pigs

aged 5-24 weeks suggest in vitro insulin secretion from younger pigs may be greater than that exhibited by islets of older animals (130). Indeed the insulin secretory response of adult pig islets to many insulin secretagogues is lower compared to human islets (131). However, advantages of using islets isolated from older versus younger animals (132) include faster normalization of blood glucose levels after transplantation (133). Interestingly, the percentage volume density of β cells within the total pancreas does not change as the pancreas matures (132). There are more β cell groups with diameters less than 50 μm in the pig pancreas at 5 weeks compared to larger cell groups found in the older pig pancreas. β cells do not cluster together into small islets until 10-13 days after birth (134). Islets from younger animals are often referred to as islet-like cell clusters (135) since less than half the proportion of cells within the clusters are differentiated endocrine cells with the majority being epithelial precursor cells. This property of immature islets makes them ideal for studying islet endocrine cell development and maturation as a model of in vivo islet development (136, 137). Moreover these islets are more robust in culture and have great proliferation and maturation potential. Islets isolated from fetal pig pancreases are comprised of only 6-8% β cells and require up to 1-5 months to reverse hyperglycemia in chemically-induced diabetic mice. Despite the functional immaturity of fetal porcine β cells, in vitro incubation with butyrate, nicotinamide, or glucagon-like peptide 1 (138-140) as well as implantation into diabetic mice (141, 142) can increase the insulin content, morphology, and insulin secretory activity of β cells.

Postnatal maturation of pancreatic islets makes neonatal pigs a more attractive source of functional β cells than fetal pig pancreases. By as early as the 1980's, attention

began to focus on islets from neonatal pigs (143). Studies demonstrated that unlike fetal pig β cells, β cells from neonatal pig pancreases could respond to in vitro glucose stimulation (143-146). Oral or intravenous glucose infusion in neonatal pigs results in significant increments of plasma insulin levels and restoration of fasting blood glucose levels after 60 min. This demonstrates that neonatal pig β cells respond to hyperglycemia, unlike fetal pig β cells (147). In 1996, Korbutt et al. developed a simple tissue culture method for the reproducible large scale isolation of islet cell aggregates from neonatal pig pancreases (148). In this study, aggregates were characterized in great detail for cell composition, insulin secretory activity and the ability to reverse hyperglycemia in chemically-induced diabetic mice. Following this initial report, others have reported on the isolation and characterization of neonatal pig islets as an ideal source of islets for transplantation (149-151). These reports have shown islets from neonatal pigs are comprised of approximately 20% β cells (Fig. 1-3) which are capable of increasing insulin release 5-fold when stimulated with glucose. Although the β cells are glucose responsive, when neonatal porcine islets are perifused with 16.7 mmol/l glucose, they exhibit a poor first phase insulin secretory response which is indistinguishable from the second phase (150). These data suggest insulin-positive cells within neonatal islets are not as functionally mature as adult β cells and hence are still capable of undergoing significant functional maturation. In large animal transplantation studies using islets from neonatal pigs, diabetes was reversed in adult pigs as well as non-human primates in both a timely fashion (~2 weeks) and without the transmission of porcine viruses to the recipient host (124, 126).

The majority of cells which make up the neonatal porcine islet are epithelial precursors identified by CK7 expression. Yoon et al. examined the use of other cytokeratins such as CK19 and CK20, as markers of ductal cells in the neonatal pig pancreas and found CK7 was most effective for staining all and only ductal cells (149). Following transplantation into diabetic mice, the β cell mass increases significantly due to at least 3 mechanisms: 1. (trans)differentiation of CK7-positive precursors, 2. replication of pre-existing β cells and 3. an increase in β cell size (129). The expansion of the β cell mass in transplanted grafts is associated with a 20- to 30-fold increase in cellular insulin content which becomes comprised of over 90% β cells (149). Maturation of neonatal islets can be achieved in vitro by providing serum and a three-dimensional matrix such alginate microcapsules (152-154). However, the extent of maturation achieved in vitro is never as significant as that achieved in vivo following transplantation. Nevertheless, an alginate matrix is beneficial for maintaining the function and morphological integrity of porcine islets in long-term in vitro culture (152, 155).

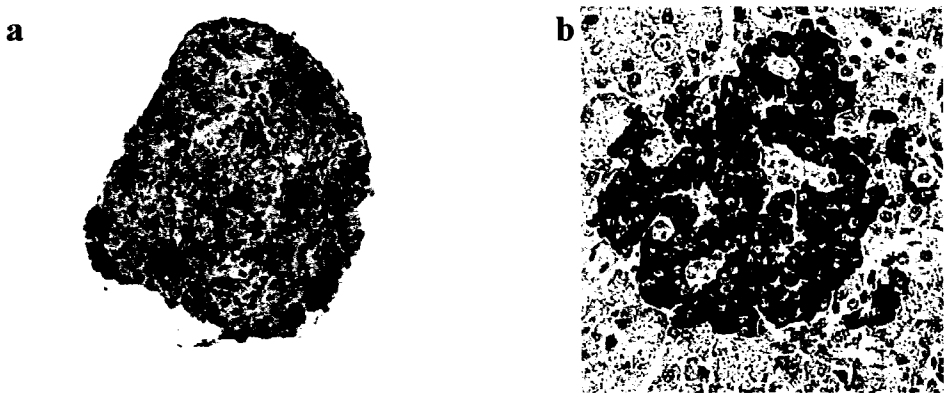

Figure 1-3: Neonatal porcine islet in culture (**a**) and an adult porcine pancreatic islet (**b**) stained for insulin (brown) (x200 magnification).

The elimination of glucocorticoids as an immunosuppressive drug in clinical islet transplantation will benefit the use of neonatal pig islets, since glucocorticoids have recently been found to suppress the expansion and transdifferentiation of neonatal pig islet precursors into β cells (156). In addition to their enormous growth potential, neonatal pig islets may also be more advantageous for clinical use than adult pig islets since Bloch et al. found that T cells from type 1 diabetic patients were less reactive to islets from neonatal versus adult pigs (157). Despite their suspected lower immunogenicity, neonatal pig islets cells express the xenoreactive antigen Galα(158;159)Galβ(158;160)GlcNAc-R (Gal epitope) and are susceptible to human antibody/complement-mediated cytolysis (161). In addition, pig neonatal islet cells are capable of stimulating human $CD4^+$ T cells (162) and are recognized by the host MHC class II-restricted indirect pathway of antigen recognition (163). However, by inhibiting T cell costimulatory signals with monoclonal antibodies specific for costimulatory molecules, T cell unresponsiveness can result in xenograft survival and tolerance (164). In addition to T cell mediated rejection, the presence of porcine endogenous retrovirus (PERV) presents another major concern when considering pig-to-human islet transplantation. In immune deficient mice receiving pig islets, PERV expression was detected in peripheral host tissues, as were pig cells (165). However, when pig islet cells are prevented from mixing with host cells as in the case of transplanting encapsulated pig islets, no evidence of PERV is detected in host tissues (166). Despite the safety concerns associated with PERV transmission and the immunological rejection of pig islet transplants, clinical trials of pig-to-human islet transplants have commenced. In the early 1990's 10 diabetic renal transplant patients in Sweden received fetal porcine ICCs (167).

Fetal porcine ICCs were injected intraportally into 8 immunosuppressed type 1 diabetic patients with end-stage diabetic nephropathy. Porcine C-peptide was excreted in the urine of four recipients for 200-400 days posttransplant (167). Despite urinary C-peptide detection, insulin requirements were not reduced due to the pig islet grafts, but patients did tolerate the procedure well and experienced no adverse effects. More recently, a group in Mexico reported on the controversial results of a clinical trial in which neonatal pig islets and Sertoli cells were transplanted subcutaneously into 12 non-immunosuppressed patients (168). The authors claim half of the patients had a 50% or greater reduction in their insulin requirements with two patients becoming transiently insulin independent as a result of the grafted porcine islets, despite the fact that no porcine C-peptide was detected in any of the recipients. An encouraging result from this and the previous reported case of pig-to-human islet transplantation was that no evidence of PERV infection was found in any of the recipients of pig islets (168-170).

I-4*c* Embryonic Stem Cells

The application of pluripotent embryonic stem (ES) cells in regenerative medicine for the treatment of diabetes is theoretically very promising and currently under intense investigation (Fig. 1-4). ES cell lines are obtained from the inner cell mass of blastocysts and can be maintained as undifferentiated cells with the capacity to differentiate into several specific cell lineages (171). Unfortunately, the current protocols available for producing insulin-expressing cells from ES cells are in a very preliminary stage and in need of further refinements before fully functioning glucose responsive β cells are available for clinical transplantation.

The generation of insulin-producing cells from ES cells has been reported to occur by either spontaneous or directed differentiation (172). Spontaneous differentiation in the absence of lineage-specific differentiation factors in vitro has been shown to result in the expression of insulin and other pancreatic hormones (173). However, during development, several tissues such as fetal liver, yolk sac, brain and other neuronal tissues generate insulin-containing cells which never ultimately become true β cells. Attempts to specifically induce β cell differentiation by in vitro culture protocols suggest the field is still in its infancy. This has recently been emphasized by reports which demonstrate that the insulin present in these cells is due to uptake from the culture medium and hence are not true β cells (174, 175). Many studies using directed differentiation methods, design their protocols on the known developmental pathways of pancreatic endocrine cells. Differentiation protocols to enrich for insulin-positive cells can be classified into three categories: cell trapping or gating selection protocols which confer antibiotic resistance or express fluorescent proteins under the control of the Nkx6.1 or insulin promoters (176, 177); coaxial methods which employ different growth factors to the culture medium (178-180); and forced over-expression of key transcription factors implicated in β cell function such as Pax4 or Pdx-1 (181, 182).

Fewer studies have been performed using human ES cells, however few have reported human ES cells can spontaneously differentiate into insulin-producing cells which secrete insulin and express other β cell markers (183, 184). Recent reports by Segev and Khoo demonstrate that exposure to basic fibroblast growth factor enhances β cell specific gene expression and increases the numbers of insulin-expressing cells (185, 186). Rather than attempt to directly produce endocrine pancreatic cells, Baetge and

colleagues derived definitive endoderm from human ES cells, which is a significant first step towards making endoderm derived tissues such as pancreas and liver (187).

Despite the very limited, yet encouraging preliminary results to date, the need to further optimize differentiation protocols and prevent teratoma formation following transplantation will hinder the clinical use of ES cells for many years (188).

I-4*d* Adult Pancreatic Stem Cells

Adult tissues are considered a more ethical and less controversial source of stem cells for cell-based replacement therapies (Fig. 1-4). Stem cells have been reported to exist in the adult pancreas, residing within both the exocrine and endocrine compartments (189, 190). However, a lack of definitive markers and reliable in vitro isolation protocols has hampered the characterization and verification of such cells. Many putative markers which are transiently expressed in developing embryonic islets, have been suggested to be indicators of islet stem cells; these include cytokeratins (70), β-galactosidase (191), tyrosine hydroxylase (192), GLUT2 (193), and several transcription factors (39) which are important in determining the cell lineages of islet cells. Several reports have isolated cells from the ductal epithelium with the ability to differentiate into insulin-producing cells both in vitro and in vivo (190, 194, 195).

Multipotent precursor cells located in both the ductal epithelium and islets themselves have recently been isolated from adult murine pancreases and differentiated into all endocrine islet cell types (190, 196). A recent study by Street et al. found islet grafts transplanted with a high proportion of ductal-epithelial cells ($CK19^+$) resulted in better long-term metabolic success in patients as assessed by intravenous glucose

tolerance tests performed 2 years posttransplant (197). Still others believe acinar cells can generate new β cells by transdifferentiation due to their ability to dedifferentiate into a primitive precursor cell followed by redifferentiation into islet cells (198). Studies by

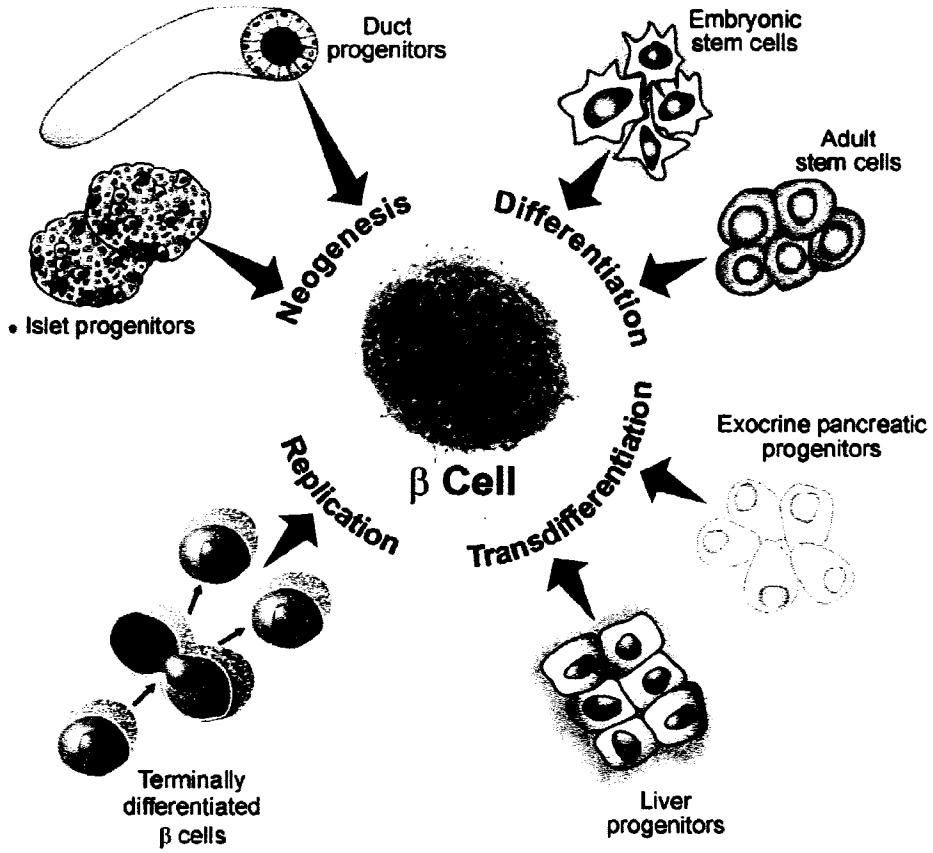

Figure 1-4: Potential stem cell sources of new β cells for replacement therapy to treat patients with type 1 diabetes.

Schmied et al. suggest islet endocrine cells can act as pancreatic precursor cells due to their ability to transdifferentiate into acinar, ductal and undifferentiated intermediary cells (199, 200). Recent evidence suggests glucose responsive insulin-secreting cells can also derive from adult pancreatic acinar cells (201). Studies have also found islet cells can lose hormone expression and dedifferentiate into mesenchymal cells (199, 202, 203).

Epithelial-to-mesenchymal-to-epithelial transition (EMET) is a process whereby β cells dedifferentiate into proliferating mesenchymal precursor cells that can be induced to redifferentiate into insulin-expressing cells (203). A study by Seeberger et al. also demonstrated the existence of multipotent mesenchymal stem cells in the exocrine portion of adult human pancreas which can differentiate into mesodermal (bone, adipose, and cartilage) and endodermal (hepatocytes and pancreatic endocrine cells) tissues (189). However, a recent study by Dor and colleagues has demonstrated that new β cells can only arise from preexisting β cells both in the normal adult pancreas and after partial pancreatectomy, questioning the very existence of an adult pancreatic endocrine stem cell (75).

I-5 THESIS OBJECTIVES AND GENERAL OUTLINE

The studies in this thesis focus on the differentiation, survival and function of neonatal islet pancreatic precursors. While the source of these precursors, the neonatal pig pancreas, is currently at the center of intense investigation for its potential use to treat type 1 diabetes, it also serves as a useful model to study islet cell differentiation and function. The islets from these pancreases are unique and even considered advantageous for clinical use over adult islets for many reasons, including: they contain numerous cytokeratin-positive precursor cells in addition to endocrine cells, are easier to isolate and maintain in culture and are less immunogenic than adult pig islets. The studies reported in this thesis, provide further evidence in support of the clinical use of these islets over adult sources of islets.

In Chapter 2, precursor cells from neonatal islets are induced to differentiate into endocrine cells by over-expressing the developmental transcription factor neurogenin 3 (ngn3) which results in α cell differentiation, assessed by glucacon mRNA and protein expression. The forced over-expression of embryonic transcription factors involved in pancreas development has been shown to result in islet cell differentiation in a number of stem/precursor cell models. Previous studies have demonstrated predominant α cell development from prenatal pancreatic cells forced to over-express ngn3. The protocol for ngn3 over-expression used in this study was adopted from a study which attempted to differentiate adult human pancreatic ductal cells left over from an islet isolation into β cells. This group reported only limited differentiation of ductal cells into an insulin-producing phenotype in vitro. The ultimate goal would be to (trans)differentiate all non-endocrine pancreatic cells into insulin-producing β cells that would be eligible for transplantation. However, due to the limited (trans)differentiation potential of committed adult pancreatic cells, early postnatal pancreatic cells such as those from a neonatal pancreas, may possess more (trans)differentiation potential. In this study, we show that the majority of neonatal islet precursor cells can easily be manipulated in vitro to (trans)differentiate into endocrine cells. Additional studies are required to determine what further manipulations are required to induce complete and functional β cell differentiation.

Chapter 3 provides data to suggest that not only is exposure to high glucose sufficient to induce partial β cell differentiation from precursors, but that the function and survival of neonatal β cells is not compromised after prolonged exposure. These results have important implications for the clinical use of these islets to treat diabetes, since adult

human islets are particularly susceptible to high glucose exposure both in vitro and in vivo.

In addition to having demonstrated the feasibility of inducing an endocrine cell phenotype from the differentiation of neonatal porcine islet precursors as well as neonatal β cell resistance to high glucose, Chapter 4 demonstrates that the high glucose-dependent differentiation of precursors into β cells can result in a diabetogenic cytotoxin resistant phenotype. The ability of a β cell to resist cell death caused by cytotoxic agents, such as streptozotocin, may have important implications for the ability of these cells to withstand cytokine, autoimmune or hypoxia induced injury. The susceptibility of adult pig β cells to streptozotocin has previously been demonstrated, with no evidence of β cell regeneration from either precursors or β cell proliferation. In contrast, when neonatal islet precursors are transplanted into diabetic mice, where they develop into β cells after 6-8 weeks, these newly differentiated β cells develop resistance to the diabetogenic effects of streptozotocin, a phenomenon not observed with transplanted adult islets.

In summary, this thesis provides evidence to further support the use of neonatal porcine islets in the clinic because of precursor cell differentiation and reduced overall susceptibility to noxious stimuli. The general vulnerability of adult islets in the immediate posttransplant environment contributes to substantial β cell loss after transplantation and reduced long-term islet function. The ability of neonatal porcine β cells to survive and maintain glucose responsiveness following prolonged exposure to high glucose as well as the ability of precursor cells to differentiate into streptozotocin resistant β cells suggests transplanted neonatal porcine islets may better survive and function in the immediate posttransplant period as well as long-term.

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CHAPTER TWO

ECTOPIC EXPRESSION OF NGN3 IN NEONATAL PIG PANCREATIC PRECURSOR CELLS INDUCES (TRANS)DIFFERENTIATION TO FUNCTIONAL α CELLS

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II-1 INTRODUCTION

Type 1 diabetes is an autoimmune disease in which insulin-producing pancreatic β cells are destroyed. Therefore, lifesaving exogenous insulin must be administered to promote glucose utilisation by the body and maintain normal blood glucose levels. Insulin injections, however, fail to mimic the body's natural release of insulin in response to glucose. The replacement of insulin-producing β cells by islet transplantation may provide patients with a more physiological therapeutic alternative to insulin injections (1–3). Yet for islet transplantation to become the current treatment of choice for diabetes, an unlimited supply of islets must first be found. Currently, extensive research is being devoted to the study of xenogeneic islets as a potential source of tissue for transplantation into patients with type 1 diabetes. In particular, neonatal pig pancreatic islet preparations may be preferred over adult pig pancreatic islets, since our group and others have shown that neonatal islets are easier to isolate and maintain in culture, contain many cytokeratin-7 (CK7)-positive endocrine precursor cells, and are less immunogenic than adult pig islets (4-7). While pancreatic cells from neonatal pigs are composed of only ~25% β cells, the differentiation of the CK7-positive precursor population into β cells is necessary to reverse hyperglycaemia in diabetic mice, which normally takes 6 to 9 weeks (4, 5). Thus, inducing the differentiation of β cells from precursors within the neonatal pancreas in vitro prior to transplantation would perhaps lead to enhanced in vivo function after transplantation. This has been demonstrated to be the case when neonatal pancreatic cells are provided an alginate extracellular matrix or cultured with nicotinamide (4, 8).

Another approach to induce β cell neogenesis is by ectopic expression of embryonic transcription factors such as PDX-1 or NeuroD1, since they are involved in

islet cell development (9-11). In particular, neurogenin 3 (ngn3), a member of the basic helix–loop–helix transcription factor family, is expressed in pancreatic epithelial precursor cells prior to endocrine differentiation, which, if absent, results in an absence of the endocrine component of the pancreas (12-15). Following ectopic expression of ngn3 in adult human pancreatic ductal cells, a neuroendocrine phenotype was induced with an increased fraction of insulin-positive cells 10 days after transduction (16). Similarly, ngn3 transfection of retinoic acid-treated mouse ES cells was shown to induce insulin transcription and other endocrine genes (17). In contrast, in mouse embryos with premature expression of ngn3 in most pancreatic precursor cells no β but rather α cells developed (13, 15), suggesting that additional signals are required to deviate the differentiation of precursor cells from α cells into β cells. In addition, Grapin-Botton et al. demonstrated predominant α cell development and some δ cell differentiation from chick endoderm following ectopic ngn3 expression (18). A recent report by Dominguez-Bendala et al. demonstrated that direct administration of cultured embryonic pancreatic explants with ngn3 protein rather than by genetic manipulation with the ngn3 gene can also promote α cell differentiation at the expense of other pancreatic cell types (19). The aim of this study was to induce endocrine cell differentiation in pancreatic cells from neonatal pigs by ectopic adenovirus-mediated expression of ngn3.

II-2 MATERIALS AND METHODS

II-2*a* Preparation and Culture of Neonatal Pig Pancreatic Cells

Cells were obtained from 1- to 2-day-old Duroc pig pancreases (Swine Research and Technology Center, University of Alberta, Edmonton, AB, Canada). Neonatal

pancreatic cell aggregates were isolated and cultured as previously described by Korbutt et al. (4). Briefly, neonatal pigs were anesthetized with halothane and subjected to laparotomy and exsanguination. The pancreases were removed, cut into small pieces and digested with 1.0 mg/ml collagenase XI (Sigma, St Louis, MO, USA). After filtration through a nylon screen (500 μm), spherical-shaped islet cell aggregates were formed during 7 to 9 days of suspension culture in Hams F10 media (Gibco, Burlington, Ontario, Canada) containing 10 mmol/l glucose, 50 μmol/l isobutylmethylxanthine (IBMX; ICN Biomedicals, Montreal, Ontario, Canada), 0.5% bovine serum albumin (BSA; Sigma), 2 mmol/l L-glutamine, 10 mmol/l nicotinamide (BDH Biochemical, Poole, Dorset, UK), 100 U/ml penicillin, and 100 μg/ml streptomycin (Cambrex Bio Science, East Rutherford, New Jersey, USA) at 37°C in a humidified atmosphere of 5% CO_2-95% air with media changes every two days.

II-2*b* Adenoviral Transduction

A replication-deficient adenovirus containing the genes for murine ngn3 as well as enhanced green fluorescent protein (*GFP*) as a reporter, both driven by separate cytomegalovirus promoters, was used. Cells were infected using a modification of a previously described protocol (16). Prior to infection, pancreatic cells were collected in a cation- and phenol-red-free Hank's balanced salt solution (Sigma, St Louis, MO, USA) supplemented with 1 mmol/l EGTA and 0.5% BSA (4), with frequent pipetting for 10 min before infection in order to break down the extracellular matrix and enhance access of viral particles within the aggregates. Preliminary experiments showed this treatment to significantly increase infection efficiency over untreated aggregates (data not shown).

Cells were then seeded in 60-mm non-tissue-culture-treated plates (Fisher Scientific, Edmonton, AB, Canada) at 10×10^6 cells/5 ml Hams F10 medium supplemented with 10% neonatal pig serum. These cultures were infected at a multiplicity of infection of 50 with either a replication-deficient adenovirus expressing ngn3 and *GFP* (Ad-Ngn3) or a control replication-deficient adenovirus expressing only *GFP* (Ad-GFP) for 4 h at 37°C, and were subsequently washed to remove viral particles and then cultured in Hams F10 plus 10% neonatal pig serum for either 3 or 8 days. A non-infected control group received no virus and was cultured in the same medium as the infected group for a similar time period.

II-2c Post-infection In Vitro Assessment

Prior to infection, intact cellular aggregates were washed with PBS, fixed in 4% paraformaldehyde for 30 min and stored in PBS. Samples were subsequently embedded in a 2% low melting point agarose solution and allowed to harden at 4°C before processing, paraffin embedding and sectioning (5 μm).

At 3 and 8 days after infection, infected and control preparations were assessed for cellular insulin and DNA content as well as for cellular composition by immunocytochemistry. Insulin and DNA content were analysed as previously described (4) and expressed as per cent recovery compared with controls. Cell composition was determined by dissociating aggregates into single cell suspensions by gentle agitation in a 37°C water bath in cation- and phenol-red-free Hank's balanced salt solution, supplemented as above and also with trypsin (25 μg/ml; Boehringer Mannheim, Indianapolis, IN, USA) and DNAse (4 μg/ml; Boehringer Mannheim). Cells were then

placed on positively charged Histobond microscope slides (Paul Marienfeld GmbH & Co. KG, Lauda-Koenigshofen, Germany) and fixed in a water-based Bouin fixative for 12 min before storage at 4°C in 70% ethanol (20).

To assess which cells expressed ngn3 after infection, fluorescence-activated cell sorting was used to separate GFP-positive (i.e. ngn3-positive) and -negative cell populations. Pancreatic single cell suspensions as described above were obtained on either days 3 or 8 after infection and analysed by flow cytometry (EPICS Elite ESP flow cytometer; Coulter, Hialeah, FL, USA). Pre-sorted and GFP-positive and -negative cells were collected and assessed for cell composition by immunocytochemistry. Immunostaining was visualised using the avidin-biotin complex (ABC) method with peroxidase and diaminobenzidine (DAB) as the chromagen. Endogenous peroxidase was quenched with a 10% H_2O_2 methanol solution. Microwave antigen retrieval for ngn3, CK7 and synaptophysin staining was performed in 10 mmol/l citrate buffer (pH 6.0). Blocking was performed with 20% normal goat serum (Fischer) for 15 min. Primary antibody concentrations were as follows: 1:1,000 guinea-pig anti-porcine insulin (Dako Diagnostics Canada, Mississauga, ON, Canada), 1:5,000 guinea-pig anti-glucagon (Linco Research, St Charles, MO, USA), 1:50 rabbit anti-human synaptophysin (Dako), 1:200 mouse anti-human CK7 (Dako) and 1:1,000 rabbit anti-mouse ngn3 (Michael German, UC San Francisco, CA, USA). Primary antibody incubations were for 30 min followed by two washes in PBS before addition of secondary antibody. All biotinylated secondary antibodies were obtained from Vector Laboratories (Burlingame, CA, USA) and used at a concentration of 1:200 for 20 min. For insulin and glucagon, anti-guinea-pig IgG was used, while for synaptophysin and ngn3, anti-rabbit IgG and for CK7 anti-mouse IgG

secondary antibodies were used, respectively. ABC complex (Vector) incubation time was for 40 min and visualisation was with DAB (BioGenex, San Ramon, CA, USA). Single cell counts were performed on a minimum of 500 cells per sample and percentages calculated as a number of positive cells per 500 cells.

II-2*d* RT-PCR Analysis

Infected and control samples taken on days 3 and 8 were suspended in Trizol reagent and stored at −80°C for molecular analysis. All samples were subsequently thawed and RNA extracted according to the manufacturer's protocol (Gibco, Burlington, ON, Canada). cDNA was transcribed from 1 µg mRNA with 10 U (200 U/µl) Superscript reverse transcriptase in 1×buffer containing 0.01 mol/l dithiothreitol, 0.5 mmol/l dNTPs and 0.02 µg/µl oligo dT15. For each sample, 2 µl of cDNA were used per 25 µl reaction along with 1×PCR buffer, 2 mmol/l $MgCl_2$, 0.2 mmol/l dNTPs, 1 U Taq polymerase (5 U/ml), and 0.5 mmol/l of each primer pair. For all PCR reactions, 35 cycles were performed, with 30 s denaturation at 94°C, 30 s annealing at 58°C and 30 s extension at 72°C. Final polymerisation was at 72°C for 10 min followed by a 4°C hold. Products were separated on a 2% ethidium bromide-stained agarose gel and images captured with Alpha Digidoc software (Perkin-Elmer, Boston, MA, USA). Primer sequences were as follows: 5'-GCT TCT TCT ACA CGC CCA AG-3' (forward) and 5'-CCA GCT GGT AGA GGG AAC AG-3' (reverse) (insulin 102-bp fragment); 5'-CCC AAG ATT TTG TGC AGT GGT T-3' (forward) and 5'-GCG GCC AAG TTC TTC AAC AAT-3' (reverse) (glucagon 221 bp fragment); 5'-CCG GAT GAC GCC AAA CTT ACA-3' (forward) and 5-ACA CCA GTG CTC CCG GGA G-3' (reverse) (murine ngn3 288-bp

fragment); and 5'-TGT ATT CCC CTC CAT CGT G-3' (forward) and 5'-GGA TCT TCA TGA GGT AGT CTG TC-3' (reverse) (β-actin 500-bp fragment). Negative controls consisted of water (Sigma) instead of experimental cDNA.

II-2*e* Transplantation and Follow-up

Immune-deficient C57BL/6-rag1$^{tm1/mom}$ (B6 *rag1*−/−, H2^{b}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and used as recipients. Mice were rendered diabetic by a single i.p. injection of 175 mg/kg streptozotocin (Sigma) dissolved in cold acetate buffer (pH 4.5) 2 days before transplantation. Diabetes was defined as a minimum of two consecutive blood glucose measurements ≥20 mmol/l. Blood from the tail vein of each recipient was collected once a week to monitor glucose levels using a glucose meter (One Touch Ultra; Lifescan, Milpitas, CA, USA). On the day of transplantation, mice were anaesthetised using halothane and grafts of 2,000 aggregates were transplanted in a single operation beneath the left renal capsule using a micromanipulator syringe as previously described (4). In addition to non-infected control and Ad-ngn3-infected groups, an Ad-GFP-infected control group was included to assess the effect of GFP on endocrine function after transplantion. Transplanted animals were monitored once a week for blood glucose levels. All mice were housed and fed under specific pathogen-free conditions and were cared for according to the guidelines of the Canadian Council on Animal Care.

II-2*f* Analysis of Graft Hormone Content and Morphology

Graft insulin or glucagon content was assessed before and after transplantation. For hormone extraction, grafts were homogenised and then sonicated at 4°C in 2 mmol/l acetic acid (0.25% BSA). After 2 h at 4°C, tissue homogenates were resonicated, centrifuged (8,000×*g*, 25 min), then supernatants were collected and the pellets further extracted by sonication in acetic acid, then stored for 1 h at 4°C. The second supernatant was collected after centrifugation, combined with the first supernatant, the total volume was measured, and samples were assayed for insulin or glucagon content. For morphological assessment, harvested grafts were immersed in Z-fix solution (Anatech, Battle Creek, MI, USA) and embedded in paraffin. Sections, 5 μm thick, were immunostained for the presence of insulin-, glucagon- and CK7-positive cells, as described above and counterstained with Harris' haematoxylin for 2 min.

Apoptosis was detected by Tdt-mediated dUTP nick-end labelling (TUNEL) staining (Promega, Madison, WI, USA) according to the manufacturer's instructions. Sections were counterstained with an anti-insulin antibody and phycoerythrin-conjugated goat anti-guinea-pig IgG secondary antibody (1:200; Jackson Immunoresearch, West Grove, PA, USA). A positive control was prepared as described by the manufacturer by treating cells with DNase I. Cell proliferation was assessed using a monoclonal mouse anti-PCNA (proliferating cell nuclear antigen) (1:50, Dako) and Cy3 anti-mouse IgG secondary antibody (1:200; Jackson Immunoresearch).

Incubation times for primary and secondary antibodies were 1 h at room temperature in a humidified chamber.

II-2*g* Statistical Analysis

Data are expressed as means±SEM of *n* observations. Statistical significance of differences was determined using one-way ANOVA with SPSS statistical software, version 12.0 for Windows (Chicago, IL, USA). $P<0.05$ was considered to be statistically significant.

II-3 RESULTS

II-3*a* In Vitro Assessment of Neonatal Pancreatic Cells Infected with Ad-Ngn3

Following collagenase digestion of neonatal pancreases and 7 to 9 days of tissue culture, cellular aggregates formed in suspension (Fig. 2-1). Immunocytochemical staining of aggregates for insulin (Fig. 2-1a), glucagon (Fig. 2-1b), CK7 (Fig. 2-1c) and synaptophysin revealed that 23.5±3.3, 17.6±4.3, 39.7±8.6 and 55.3±4.3% of cells were immunopositive, respectively. After infection with the Ad-Ngn3 virus, ectopic expression of ngn3, as visualised by GFP fluorescence and ngn3 mRNA expression, remained detectable at 3 and 8 days after infection in all preparations examined (Fig. 2-2). No GFP fluorescence or ngn3 mRNA were detected in non-infected controls. In addition to ngn3, mRNAs encoding insulin and glucagon were detected on days 3 and 8 after infection (Fig. 2-2a).

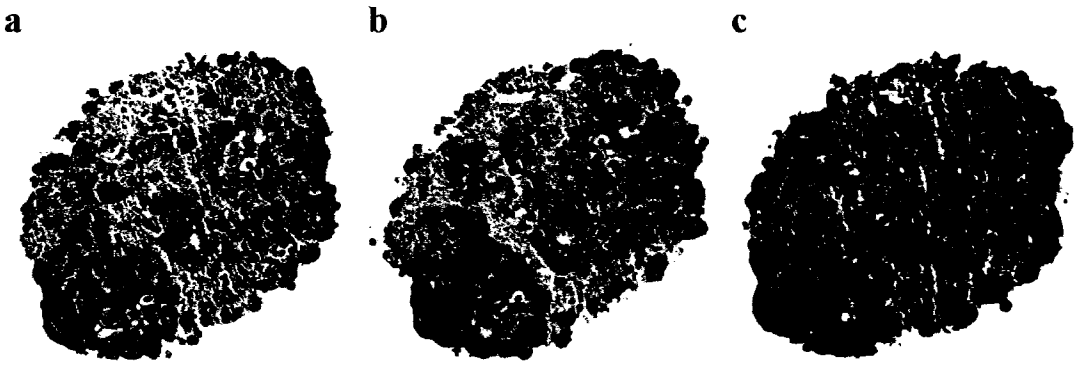

Figure 2-1: Immunohistochemical staining of representative cellular aggregates for insulin (**a**), glucagon (**b**) and CK7 (**c**) prior to infection (x400 magnification). The cellular composition of aggregates was determined by dissociating aggregates into single cells and calculating the proportion of insulin-, glucagon-, and CK7-positive cells.

The proportion of cells which produced ngn3 was 40.3±1.5 and 35.6±2.9% at days 3 and 8 after infection, respectively (Table 2-1). The percentage of α, β or CK7-positive cells in the total population of pre-sorted Ad-Ngn3-infected cells did not differ significantly from non-infected controls on either days 3 or 8 (Table 2-1).

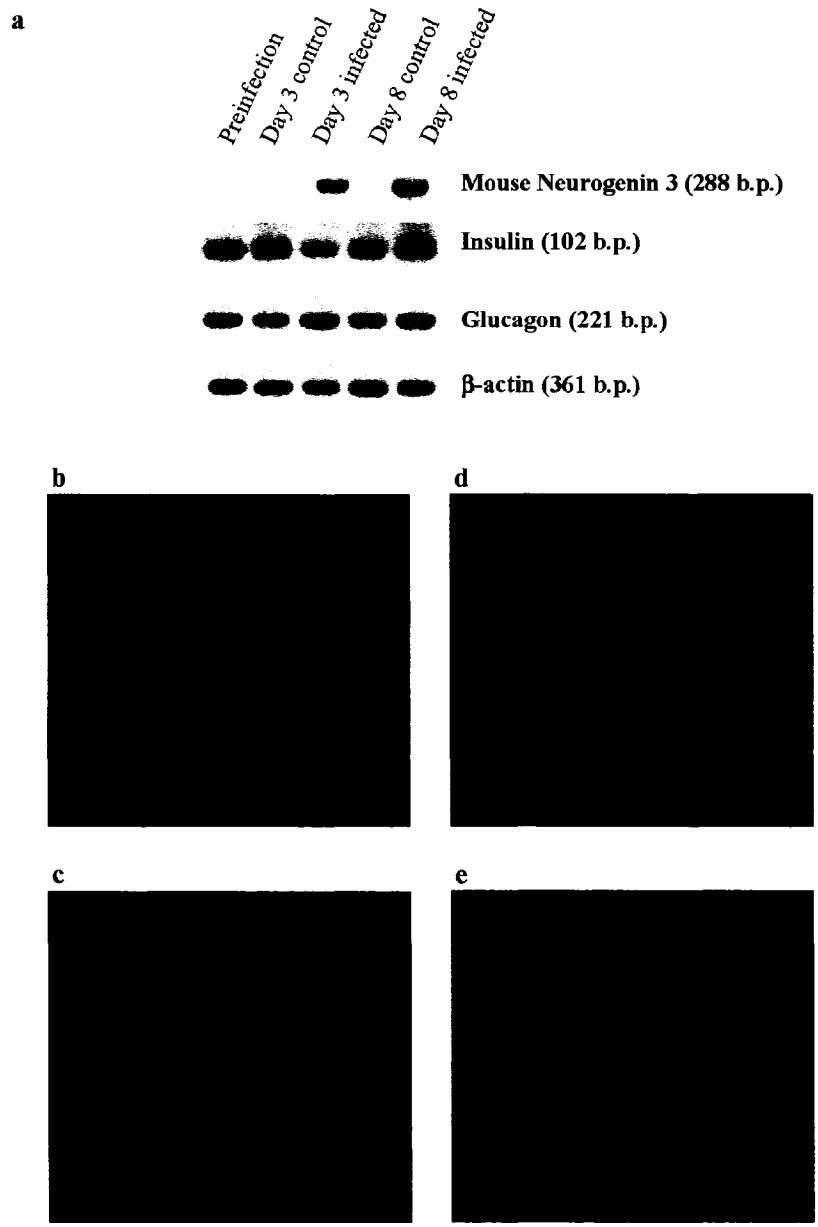

Figure 2-2: a Representative RT-PCR analysis of non-infected control and Ad-Ngn3 infected cells after 3 or 8 days in vitro culture. Samples were assessed for transcription of murine neurogenin 3, insulin and glucagon genes along with actin as an internal control (n=3). Ngn3 mRNA was not detected in non-infected control cells. Expression of the reporter gene, green fluorescent protein (*GFP*) in pancreatic preparations 3 (**b**) and 8 (**c**) days after infection suggests successful transduction of neonatal pig pancreatic cell cultures with Ad-Ngn3 virus. Non-infected controls expressed no *GFP* on days 3 (**d**) or 8 (**e**) (x200 magnification).

Similarly, in Ad-Ngn3-infected preparations, the percentage of synaptophysin-positive cells did not differ compared with non-infected controls on day 3 (53.5±10.9 vs 59.6±9.9%, respectively) or day 8 (55.1±7.2 vs 58.8±3.1%, respectively) after infection (Table 2-1). The cellular DNA content was assessed on days 3 and 8 after infection and served as an accurate assessment of the number of viable cells (21). Following infection with Ad-Ngn3 virus, the cellular DNA content of pancreatic cells remained similar to non-infected controls (100.0%) (Fig. 2-3). In contrast, the cellular insulin content on day 3 after infection was reduced by 42.7±6.3%, $P<0.05$ (Fig. 2-3); however, by day 8, the recoverable cellular insulin content was decreased by only 25.3±4.6%, $P<0.05$ (Fig. 2-3).

Table 2-1. Cellular composition of Ad-Ngn3 infected neonatal pancreatic cells on days 3 and 8 post-infection

	Day 3				Day 8			
	Control	Ad-NEUROG3			*Control*	Ad-NEUROG3		
		Presort	*GFP*$^+$	*GFP*$^-$		*Presort*	*GFP*$^+$	*GFP*$^-$
Neurogenin 3	0±0	40.3±1.8	96.9±0.6	2.8±0.7	0±0	35.6±2.9	96.2±1.3	2.4±1.1
Insulin	17.3±1.6	15.4±1.5	16.9±3.5	14.4±1.0	26.7±2.3	28.2±5.2	22.8±2.3	28.9±3.5
Glucagon	15.1±2.2	18.3±3.4	19.7±1.3	22.6±3.7	27.0±1.1	31.0±1.0	73.8±6.9*	21.4±3.2
CK7	43.2±3.6	41.2±4.5	45.6±3.2	46.7±2.3	34.5±5.6	36.3±2.1	5.2±4.5*	33.8±5.7
Synaptophysin	59.6±9.9	53.5±10.9	nd	nd	58.8±3.1	55.1±7.2	nd	nd

Results are mean±SEM (n=4). *$P<0.05$ compared to GFP$^-$ cells on day 8
nd: not determined

To assess the phenotype of the Ad-Ngn3-infected cells, single cell suspensions of GFP-positive and -negative cells were sorted and stained for ngn3, insulin, glucagon and CK7. The GFP-positive cell population contained ~96% ngn3-expressing cells, whereas <3% of the cells in the GFP-negative population expressed ngn3 (Table 2-1). On day 3, the percentage of insulin-, glucagon- or CK7-positive cells in the GFP-positive population did not differ significantly from that found in either the pre-sorted condition,

GFP-negative population or non-infected controls (Table 2-1). However, by day 8, the majority of GFP-positive cells were glucagon-positive α cells (73.8±6.9%), contrasting with the pre-sorted condition, the GFP-negative population or non-infected controls, which contained 31.0±1.0, 21.4±3.2 and 27.0±1.1% α cells, respectively (Table 2-1). There was no difference in the percentage of insulin-positive cells in both GFP-positive and -negative populations, whereas there was a significant decrease in the percentage of CK7-positive cells in the GFP-positive population (5.2±4.5 vs 33.8±5.7%, $P<0.05$; Table 2-1).

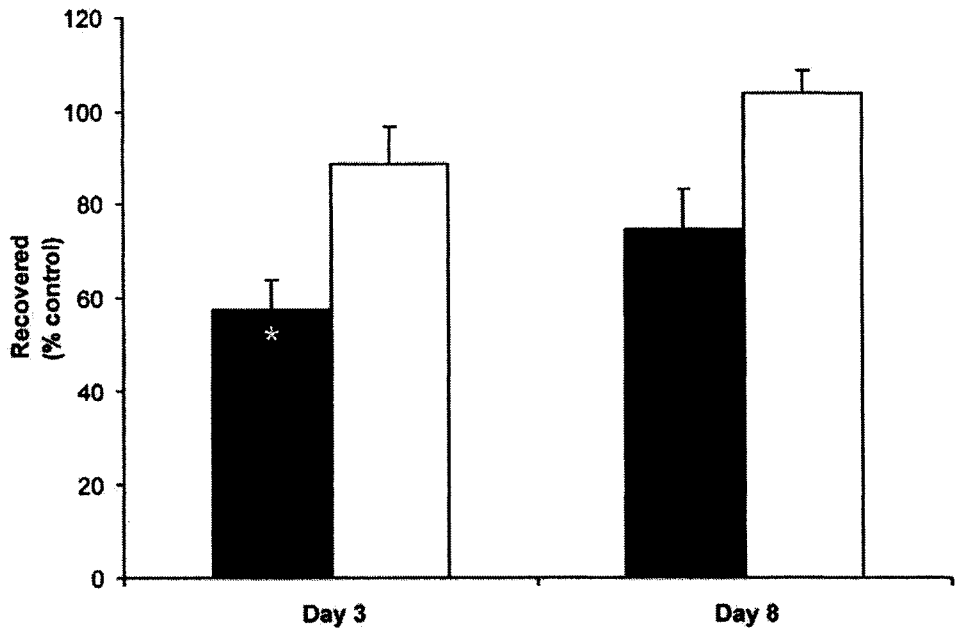

Figure 2-3: Cellular insulin (black bars) and DNA (white bars) contents were assessed in non-infected control and Ad-Ngn3 infected neonatal pig pancreatic cells after 3 and 8 days of culture. Ad-Ngn3 infection significantly reduced the cellular insulin content by day 3 of culture. Data are expressed as means±SEM, (n=4); *$P<0.05$ vs. control.

II-3*b* In Vivo Assessment of Transplanted Neonatal Pancreatic Cells Infected with Ad-Ngn3

Twenty-four hours after infection, Ad-Ngn3, Ad-GFP and non-infected control groups were transplanted into immune-deficient streptozotocin-diabetic mice. Graft characterisation prior to transplantation revealed that all groups of pancreatic preparations contained a similar cellular insulin content and β cell number (Table 2-2). Mice receiving control (n=5) or Ad-GFP-infected grafts (n=4) achieved normoglycaemia by day 70 after transplantion (blood glucose value ≤10 mmol/l) whereas recipients of Ad-Ngn3-infected cells (n=6) did not achieve euglycaemia (blood glucose levels ≥20 mmol/l) throughout the 109-day follow-up period (Fig. 2-4a). In recipients of both non-infected control and Ad-GFP-infected cells, removal of the graft-bearing kidney after normoglycaemia resulted in a rapid return to hyperglycaemia, indicating that the grafts were responsible for the normoglycaemic state (Fig. 2-4a).

Table 2-2. Composition of grafts prior to transplantation

	Control	Ad-NEUROG3	Ad-GFP
Beta cells (x 10^6)	0.6±0.5	0.6±0.3	0.6±0.6
Insulin content (μg)	4.7±0.5	4.2±0.6	4.1±0.9

Results are mean±SEM (n=4).

Grafts retrieved from non-infected control and Ad-GFP groups of animals contained similar levels of cellular insulin and glucagon (Fig. 2-4b). The graft insulin content of non-infected controls increased 23.7±3.2-fold (from 4.7±0.5 to 110.3±13.7 μg) after

transplantion and Ad-GFP-infected grafts increased 31.1±1.6-fold (from 4.1±0.9 to 140.6±13.5 μg). Ad-Ngn3-infected grafts, on the other hand, increased only 2.2±0.5-fold (from 4.2±0.6 to 9.1±1.3 μg), $P<0.05$ (Fig. 2-4b). When the glucagon content of the grafts was compared, Ad-Ngn3-infected grafts contained significantly more glucagon (57.0±3.4 μg) when compared with non-infected control (25.6±4.9 μg) and Ad-GFP-infected grafts (33.1±2.5 μg), $P<0.05$ (Fig. 2-4b).

Histological examination of the Ad-Ngn3-infected grafts taken from mice on day 109 after transplantion revealed glucagon-positive α-cell-rich areas (Fig. 2-5h) but very few faint insulin-positive cells (Fig. 2-5g). In contrast, non-infected control and Ad-GFP-infected grafts consisted predominantly of well-granulated insulin-positive cells (Fig. 2-5a, d) with few glucagon-positive cells (Fig. 2-5b, e). Similar staining patterns of CK7-positive cells were seen in all groups of transplanted grafts (Fig. 2-5c, f, i). Randomly scattered ngn3-positive cells were detected in Ad-Ngn3-infected grafts up until 2 weeks after transplantion, after which none were detected (data not shown). No marked differences in morphology were observed between the non-infected control and Ad-GFP groups.

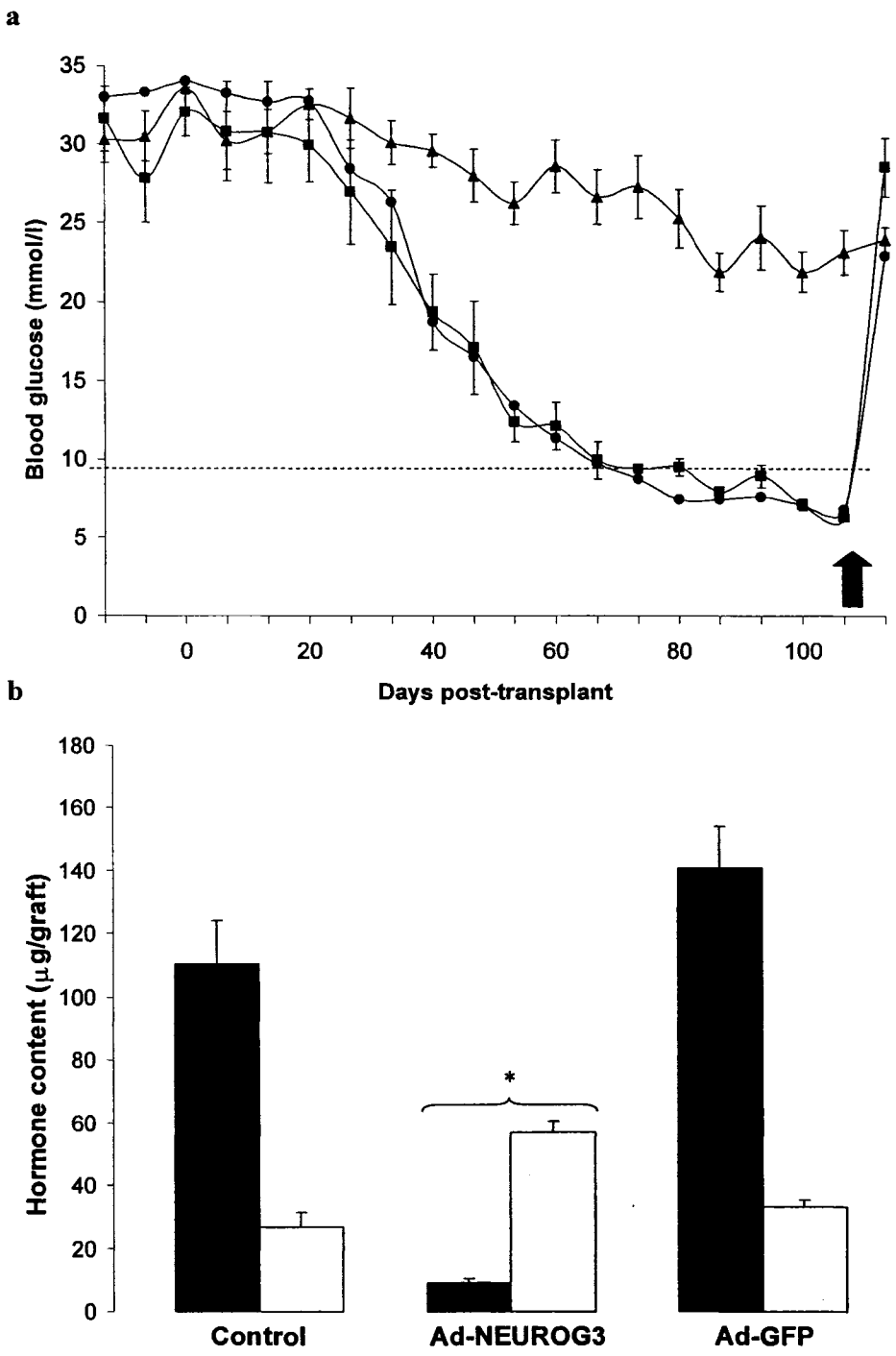

Figure 2-4: **a** Blood glucose values of mice following transplantation of control (●; *n*=5), Ad-GFP (■; *n*=4) or Ad-Ngn3 (▲; *n*=6) infected grafts. Transplantation was performed 24 hrs after adenovirus infection and grafts were harvested (arrow) for histological and hormone content analysis 109 days post-transplant. Recipients of Ad-Ngn3 grafts failed

to achieve normoglycaemia in the time recipients of non-infected and Ad-GFP control grafts were able to. Values are means±SEM. **b** Cellular insulin (black bars) and glucagon (white bars) contents of graft bearing kidneys harvested from animals 109 days post-transplant. The insulin content of Ad-Ngn3 grafts was significantly reduced compared to non-infected and Ad-GFP control grafts. Ad-Ngn3 graft glucagon content was greater than controls. (n=3); *P<0.05 vs. control and Ad-GFP.

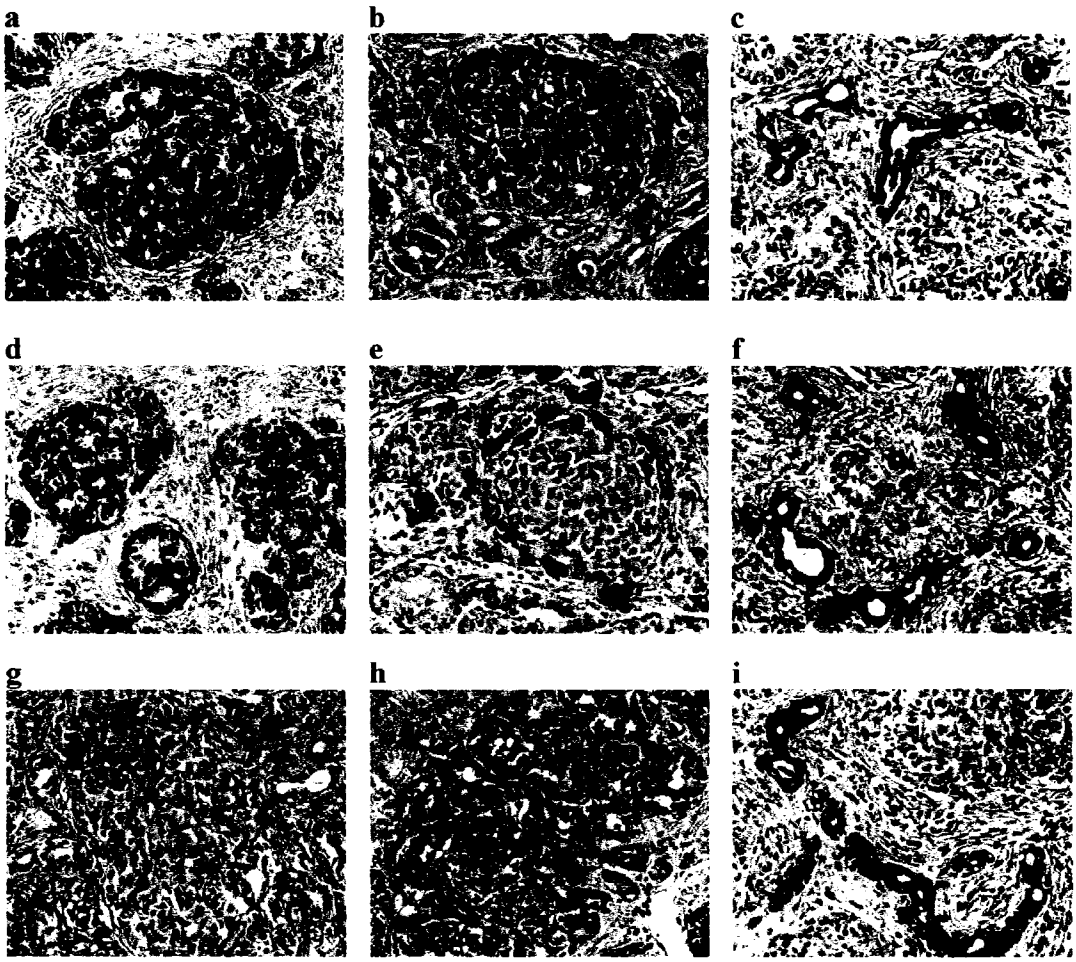

Figure 2-5: Immunohistochemical staining of non-infected control (**a-c**), Ad-GFP (**d-f**) and Ad- Ngn3 (**g-i**) infected grafts for insulin (**a, d, g**), glucagon (**b, e, h**) and CK7 (**c, f, i**). Abundant glucagon-immunopositive cells were seen in Ad-Ngn3 infected grafts which were almost devoid of insulin-positive cells. Grafts were harvested 109 days post-transplant, fixed and embedded in paraffin. Immunopositive cells were identified using ABC/DAB and counterstained with Harris' hematoxylin (x400 magnification).

Assessment of apoptotic cells in Ad-Ngn3-infected grafts 2 weeks following transplantation was performed using the TUNEL assay (Fig. 2-6a, b). In both non-

infected control and Ad-Ngn3-infected grafts, very few apoptotic β cells were detected. In contrast, the number of proliferating β cells was markedly reduced in Ad-Ngn3-infected grafts compared with non-infected control grafts at 2 weeks after transplantion (Fig. 2-6c, d).

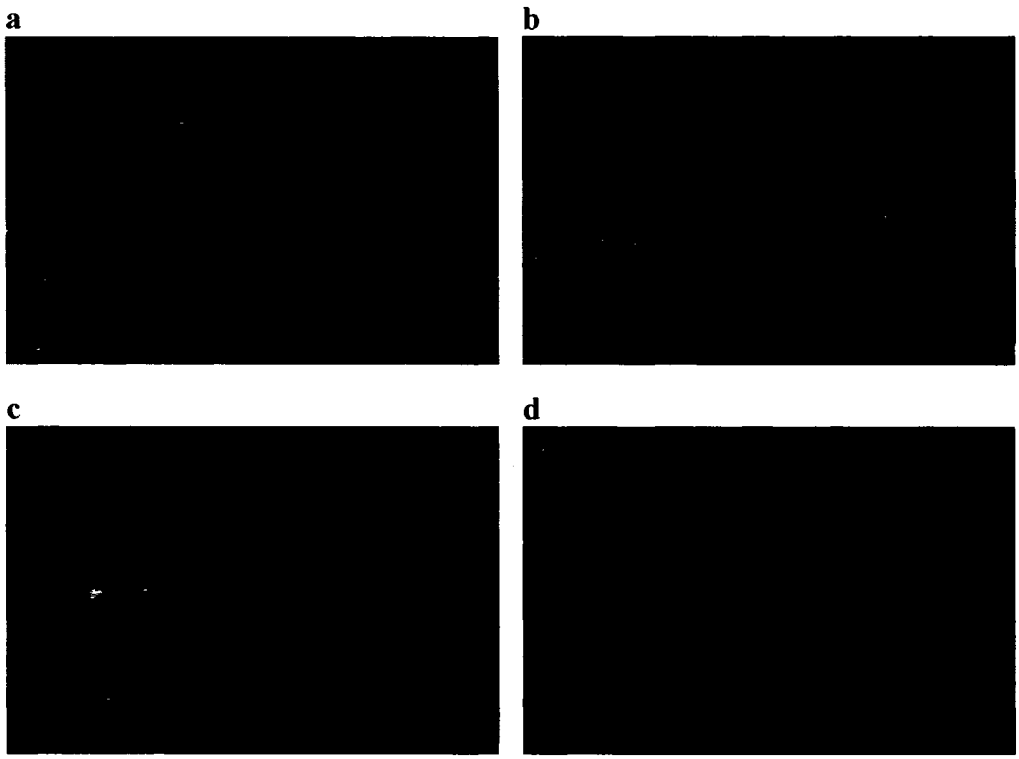

Figure 2-6: In vivo assessment of cell death (**a, b**) and proliferation (**c, d**) in Ad-Ngn3 infected grafts. **a** Representative non-infected control and (**b**) Ad-Ngn3 infected grafts showing very few apoptotic β cells (insulin staining, *red*; TUNEL labeling with FITC, *green*) at 2 weeks after transplantation. **c** Numerous proliferating β cells (insulin staining, *green*; PCNA staining, *orange*) were seen in non-infected control grafts, whereas Ad-Ngn3 infected grafts (**d**) contained very few proliferating cells (x400 magnification).

II-4 DISCUSSION

This study shows that ectopic expression of ngn3 in neonatal pig pancreatic precursor cells induces (trans)differentiation to functional α cells. In vitro assessment of pancreatic cell composition after Ad-Ngn3 infection showed no effect by day 3, but by day 8 there was a marked increase in the proportion of α cells in the ngn3-positive population. In addition, the increase in the α cell fraction was associated with a significant decrease in the proportion of CK7-positive cells, while the fraction of β cells remained unchanged. These data suggest that CK7-positive precursor cells expressing ectopic murine ngn3 differentiate into α cells. The observation that Ad-Ngn3 induces α cell differentiation by day 8 after infection and not day 3 suggests that sufficient time is necessary for the gene-expression events controlling α cell differentiation to occur. Whether the porcine ngn3 gene was ever expressed in pancreatic cells remains undetermined, since the murine ngn3 primers or antibody used in this study did not detect ngn3 mRNA or protein, respectively, in non-infected control cells. Similarly, when fetal porcine pancreatic cells were examined for ngn3 mRNA using the same murine ngn3 primers used in this study, none was detected (H.H. unpublished observation). Adenoviral infection of pancreatic cells had no significant effect on cellular DNA content recovery and thus the infection procedure does not appear to cause significant cell death. This supports the assumption that the decrease in the proportion of CK7-positive cells in the ngn3-positive population in vitro is not due to selective cell death, but rather the differentiation of these precursor cells into α cells.

Ngn3 mRNA and protein are normally not detected in differentiated insulin- and glucagon-producing cells (13, 15). However, in this study, ectopic murine ngn3 protein

was produced in hormone-producing α and β cells. In a study by Mellitzer et al. (22), transgenic mice were developed in which ngn3-expressing cells of the developing pancreas were labelled with enhanced yellow fluorescence protein (EYFP). The experiments showed that some islet progenitors expressing ngn3 (EYFP-positive) also co-stained for insulin or glucagon. The detection of ectopic murine ngn3 and not porcine ngn3 suggests forced expression of ngn3 in neonatal pig pancreatic cells does not necessarily affect hormone production, at least in the short-term. Perhaps if the endogenous porcine ngn3 gene were activated, hormone expression would eventually be extinguished. This has been reported to be the case at least in adult human β cells forced to proliferate in vitro (23). Expression of ngn3 was reported in dedifferentiated in-vitro-expanded human β cells along with an accompanying loss of expression of genes characteristic of β cell differentiation. Similarly, in an adult mouse model of β cell regeneration, intra-islet endocrine progenitor cells transiently expressed ngn3 mRNA, implicating reactivation of endogenous ngn3 during regeneration of β cells (24). However, a recent report by Lee et al. found β cell regeneration after partial pancreatectomy does not involve ngn3 gene reactivation (25). The subsequent loss of insulin production in transplanted Ad-Ngn3-infected grafts may be due to a lack of β cell differentiation because of forced ngn3 production. However, unlike β cells, α cells appear unaffected by the presence of ngn3 and thus were able to maintain glucagon production even in vivo after transplantation. However, until we are able to detect endogenous porcine ngn3 gene expression we cannot be certain.

The induction of glucagon-positive α cell differentiation in neonatal pig pancreatic cells is not surprising since others have reported similar findings resulting

from overexpression of ngn3 in pancreatic precursor cells (16, 17, 26). The developmental age, and hence plasticity, of the infected tissue appears to affect the differentiation potential, as it would appear neonatal precursors behave similarly to precursor cells of embryonic origin in their ability to become α cells following ngn3 overexpression.

Genetic engineering of pancreatic islets may enhance metabolic function or reduce graft immunogenicity after transplantation. In this study, the metabolic function of Ad-Ngn3-infected pancreatic cells was assessed in an in vivo transplantation model. Unlike non-infected control and Ad-GFP-infected cells, Ad-Ngn3-infected cells failed to reverse streptozotocin-induced diabetes when transplanted into immune-deficient mice. Assessment of the proportion of β cells and insulin content prior to transplantation revealed a similar β cell mass and insulin content was transplanted in all groups. The use of an Ad-GFP control ruled out the possibility that GFP expression in pancreatic cells may interfere with endocrine function. Consistent with our data, others have reported Ad-GFP expression in transplanted pancreatic islets does not affect in vivo function (27).

The failure of Ad-Ngn3-infected grafts to reverse hyperglycaemia in animals was due to an inadequate β cell mass in grafts, as determined by significantly reduced insulin content. Prior to infection with Ad-Ngn3, the insulin content was similar to that found in non-infected and Ad-GFP control groups; unfortunately, however, glucagon content was not assessed. Despite only a 2.2-fold increase in insulin content, significantly increased glucagon content was observed in Ad-Ngn3-infected grafts after transplantion compared with controls. The increased α cell mass suggests a biased differentiation of precursor cells in neonatal pig pancreatic cells towards an α cell phenotype, which was made

possible by ectopic ngn3 overexpression. Exposure of infected precursor cells to the hyperglycaemic environment of the recipient after transplantation may also have contributed to the increased α to β cell mass, which is also seen in diabetes (28). To make full use of the potential of pancreatic precursor cells and achieve optimal metabolic function in recipients of differentiated islet precursor cells, other islet cell types will ultimately be required.

What is the mechanism by which ngn3 promotes transdifferentiation? Previous studies have indicated that ngn3 activates islet transcription factors such as Nkx2.2, Pax4 and IA1 in addition to endodermal transcription factors like Foxa2 and HNF1α, while repressing its own promoter (29-32). In this study, interactions between these transcription factors in vitro and in vivo resulted in the rapid transdifferentiation of neonatal pig pancreatic cells into α cells. Nonetheless, insulin content was significantly reduced in vitro by day 3 after infection, but was partially restored by day 8. Culture supplements affecting β cell proliferation and differentiation such as nicotinamide, 3-isobutyl-1-methyl-xanthine and pig serum may have contributed to this recovery in vitro, but because they are not present in the murine post-transplantion environment in vivo, the insulin content (i.e. β cell mass), was not successfully restored to meet the critical threshold level of insulin secretion required to reverse hyperglycaemia in diabetic mice (4). Evidence to suggest β cell proliferation was markedly impaired in Ad-Ngn3-infected grafts was apparent in 2-week-old Ad-Ngn3-infected grafts, which contained significantly reduced PCNA-positive β cells. β cell death does not appear to be a major contributing factor to the reduced β cell mass seen in Ad-Ngn3-infected grafts,

since no significant changes in measurable cellular DNA content in vitro and apoptotic β cells in vivo were found compared with non-infected controls.

Co-expression of ngn3 with other downstream transcription factors may be necessary to induce differentiation of all endocrine islet cell types. By co-expressing multiple transcription factors together or in sequence in neonatal pig pancreatic cells, islet cell differentiation may be repeated, as occurs in situ during development, thus resulting in an increased β cell mass. A better understanding of the mechanisms involved in the differentiation of islet endocrine cells is likely to be essential in directing the formation of such cells from precursor cells for use in cell-therapy treatment of diabetes mellitus.

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CHAPTER THREE

EFFECT OF PROLONGED IN VITRO EXPOSURE TO HIGH GLUCOSE ON NEONATAL PORCINE ISLET FUNCTION, DIFFERENTIATION AND SURVIVAL

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III-1 INTRODUCTION

Pancreatic β cells depend on glucose as a source of fuel, but also require it as a growth factor as well as for the regulation of glucokinase and insulin gene transcription (1-3). Elevated glucose concentrations can have either beneficial effects such as stimulating β cell growth, as well as detrimental effects however, such as causing impaired insulin secretion and even cell death (4, 5). The adult human β cell has been shown to be particularly susceptible to high glucose resulting in reduced glucose-stimulated insulin secretion, disproportionately elevated proinsulin secretion, and reduced islet insulin content (6-9). In addition to impaired β cell function, a recent study demonstrated high glucose can cause increased β cell death by tilting the balance of proapoptotic and antiapoptotic Bcl proteins towards apoptosis (10). In addition, exposure to high glucose can impair glucose signaling in β cells by decreasing GLUT2 and glucokinase gene expression (11). However unlike adult β cells, fetal β cells appear less susceptible to the detrimental effects of chronic exposure to high glucose (12). Immature fetal β cells respond poorly to glucose (13), whereas after birth, β cells mature becoming glucose-responsive (14). Islets from neonatal animals such as pigs provide an interesting model to study the effects of high glucose exposure on β cell function and survival as well as differentiation from precursors. Neonatal porcine islets contain differentiated endocrine cells as well as their precursors which are identified by cytokeratin-7 (CK7) immunoreactivity (15, 16).

These islets can reverse chemically-induced hyperglycemia in diabetic rodents and large animal models (17, 18, 19). This in vivo transplantation model has been shown to be effective for recapitulating the growth and differentiation of islet cells (20). In the

time required for this to occur, neonatal porcine islet grafts contain a suboptimal β cell mass which is insufficient to reverse hyperglycemia until 6-8 weeks posttransplant. During this time, the neonatal porcine islet graft is exposed to prolonged hyperglycemia which may affect islet function, growth and survival. The effects of high glucose exposure have not yet been studied to date, therefore in the present study, neonatal porcine islets were exposed to slightly elevated (10.0 mmol/l) and high (28.0 mmol/l) glucose concentrations and assessed for β cell function, growth and survival in culture.

III-2 MATERIALS AND METHODS

III-2*a* Neonatal Porcine Islet Isolation and Culture

Islets were isolated from Landrace-Yorkshire neonatal pigs (Swine Research and Technology Center, University of Alberta) aged 1-2 days old (1.5-2.0 kg body weight) of either sex as previously described by Korbutt et al. (17). Briefly, the pancreases were removed, cut into small pieces and digested with 1.0 mg/mL collagenase XI (Sigma, St Louis, MO, USA). After filtration through a nylon screen (500 μm), islets were cultured for 7 days in Hams F10 medium (Gibco, Burlington, Ontario, Canada) containing 5.6 mmol/l glucose supplemented with 50 μmol/l isobutylmethylxanthine (IBMX; ICN Biomedicals, Montreal, Ontario, Canada), 0.5% bovine serum albumin (BSA; Sigma), 2 mmol/l L-glutamine, 10 mmol/l nicotinamide (BDH Biochemical, Poole, Dorset, UK), 100 U/mL penicillin, and 100 μg/mL streptomycin (Cambrex Bio Science, East Rutherford, New Jersey, USA) at 37°C in humidified 5% CO_2-95% air with media changes every two days. Following the initial week of culture in 5.6 mmol/l glucose, islets were divided into three groups and cultured in supplemented Hams F10 medium

containing either 5.6, 10.0, or 28.0 mmol/l glucose for an additional 7 days with media changes every two days. Islets from each group were then returned to 5.6 mmol/l glucose for an additional 2 days recovery period to assess if the effects of high glucose are reversible.

III-2*b* Cellular Insulin and DNA Content Analysis

Determination of cellular insulin content was accomplished by measuring duplicate samples by radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA). Samples were sonicated in 2 mmol/l acetic acid containing 0.25% BSA, centrifuged (800 g, 15 min) then supernatants were collected and stored at -20°C until the time of assay. DNA content was analyzed using PicoGreen, an ultra-sensitive fluorescent nucleic acid stain for double-stranded DNA (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Duplicate aliquots were washed in citrate buffer (150 mmol/l NaCl, 15 mmol/l citrate, 3 mmol/l EDTA, pH 7.4) and stored as cell pellets at -20°C. Cell pellets were resuspended in lysis buffer (10 mmol/l Tris, 1 mmol/l EDTA, 0.5% Triton X-100, 4°C, pH 7.5) sonicated and then analyzed (17).

III-2*c* Glucose-Stimulated Insulin Secretion

Islet insulin secretory activity in response to an in vitro stimulatory glucose challenge was assessed using a static incubation assay (17). Islets were washed twice with Hams F10 media containing 0.5% BSA and 2.8 mmol/l glucose and samples were taken for determination of cellular insulin content. Islets were cultured in 24 well plates, and incubated in 1.5 ml of Hams F10 media supplemented with 0.5% BSA and either 2.8

or 20.0 mmol/l glucose for 120 min. At the end of the incubation, supernatants were collected for measurement of insulin release by radioimmunoassay. Insulin secretion was calculated by dividing the insulin released into the supernatant by the cellular insulin content of the islets (percent of content). Stimulation indices were calculated by dividing the percentage of insulin released at 20.0 mmol/l glucose by that released at 2.8 mmol/l glucose. Insulin release per β cell was calculated by dividing the amount of insulin released into the culture medium by the number of β cells. Based on the determination of total islet cellular DNA content and the percentage of insulin-positive cells, the following equation was used to calculate the total number of β cells (17):

$$\frac{\text{Total DNA content}}{\text{7.1 pg DNA/cell}} \times \frac{\text{\% insulin positive cells}}{100} = \text{number of } \beta \text{ cells}$$

III-2*d* Differentiation, Proliferation, and Apoptosis

To determine the percentage of insulin-positive β cells, CK7-positive ductal epithelial cells and proliferating cells, islets were dissociated into single cell suspensions to facilitate quantification of stained cells (17). Islets were dissociated by mechanical disruption using siliconized glass pipettes at 37°C in calcium free HBSS media supplemented with 1 mmol/l EGTA and 0.5% BSA for 7 min before addition of trypsin (25 μg/ml; Boehringer Mannheim, Laval, Canada) and DNAse (4 μg/ml, Boehringer) and further pipetting for 10 min. Cells were washed, resuspended in phosphate buffered saline (PBS), and allowed to adhere onto Histobond microscope slides (Marienfeld, Germany) before fixation in Bouin's fixative for 12 min. Microwave antigen retrieval for CK7 staining was performed for 1.5 min in 50 ml of Target Retrieval Solution, pH 9 (S2368, Dako, Mississauga, Canada). Blocking was performed with 20% normal goat

serum (Fischer) for 30 min. Primary antibodies were purchased from Dako and used at the following concentrations: 1:1000 guinea pig anti-porcine insulin, 1:25 mouse anti-human CK7, and 1:100 mouse anti-rat PCNA for 1 hr at room temperature in a humidified chamber followed by 2 washes in PBS before addition of the secondary antibody. Secondary antibodies were: fluorescein isothiocyanate (FITC)-conjugated goat anti-guinea pig and Cy3-conjugated donkey anti-mouse, both purchased from Jackson Immunoresearch Laboraties Inc. (West Grove, PA, USA) and used at a 1:200 dilution for 1 hr. Double staining for insulin and PCNA was performed in sequential order of primary followed by secondary antibodies. The percentage of PCNA-positive islet cells was calculated by dividing the number of PCNA-positive cells by the total number of cells. The percentage of proliferating β cells was determined by dividing the number of PCNA and insulin double positive cells by the total number of insulin-positive cells. Apoptotoic cells were detected by TUNEL staining (Promega, Madison, WI) according to the manufacturer's instructions on intact islets fixed in 4% paraformaldehyde for 30 min then embedded in a 2% low melting point agarose solution and allowed to harden at 4°C before processing, paraffin embedding, and sectioning (5 μm). Sections of islets were stained for apoptotic cells and counterstained for insulin. Slides were coverslipped with Vectashield (Vector) and viewed using a Zeiss Axioskop II fluorescent microscope with a Coolsnap camera. The percentage of TUNEL-positive cells was determined by dividing the number of TUNEL-positive cells by the total number of cells. The percentage of apoptotic β cells was determined by dividing the number of TUNEL and insulin double positive cells by the total number of insulin-positive cells. Cell counts were performed

on a minimum of 500 cells per sample and percentages were calculated as the number of positive cells per 500.

III-2*e* Statistical Analysis

Results are presented as means±SEM of 3 independent experiments performed with islets prepared from at least 3 pancreases. Statistical comparisons were performed using the one-way ANOVA test with SPSS statistical software, version 12.0 for Windows (Chicago, IL, USA). A *P* value of less than 0.05 was considered to be significant.

III-3 RESULTS

III-3*a* Effect of High Glucose on Neonatal Porcine Islet Recovery and Insulin Secretory Activity

Immediately after collagenase digestion of neonatal porcine pancreases, digested tissue was cultured in Hams F10 media containing 5.6 mmol/l glucose for 1 week. The resultant islets were then switched to media containing either 5.6, 10.0, or 28.0 mmol/l glucose for an additional week to compare the effects of prolonged exposure to high glucose on islet recovery and insulin secretory activity. Islets cultured in high glucose (28.0 mmol/l) contained the least amount of recoverable cellular insulin after one week of culture (42.0±4.7%), although a week of culture in 5.6 and 10.0 mmol/l glucose also significantly reduced cellular insulin content as well (63.2±6.4 and 55.8±3.8% respectively, Table 3-1). Similarly, β cell insulin content decreased dramatically following a week of culture in 28.0 mmol/l glucose compared to 5.6 or 10.0 mmol/l glucose ($P<0.05$; Table 3-1). The decrease in insulin content after incubation at 28.0 mmol/l glucose was accompanied by increased cellular DNA recovery compared to islets

cultured at 5.6 or 10.0 mmol/l glucose ($P<0.05$; Table 3-1). After a week of culture in different glucose concentrations, islets from each group were returned to physiological glucose (5.6 mmol/l) for 2 additional days of culture. During these 2 days, islet cellular insulin content was partially restored as both the total amount of recoverable islet and individual β cell insulin contents increased (Table 3-1).

To evaluate the insulin secretory activity of islets after culture at different glucose concentrations, we compared the percentage of insulin released by islets after stimulation with low (2.8 mmol/l) or high (20.0 mmol/l) glucose. Following the first week of culture in 5.6 mmol/l glucose, islets exhibited a stimulation index of 3.9±0.1 in response to a 20.0 mmol/l glucose challenge (Table 3-2). The insulin released per β cell in response to a high glucose challenge was 5.5±0.7 x 10^{-4} ng, or 4.0±0.2% of the total intracellular insulin stores. After exposing islets to 10.0 mmol/l glucose for one week, the insulin secretory response to 20.0 mmol/l glucose (9.4±0.2% insulin release) was significantly higher compared to islets cultured at 5.6 or 28.0 mmol/l glucose for one week (6.2±0.3% vs. 7.7±0.2%, respectively; $P<0.05$; Table 3-2). However, the stimulation indices of islets were similar after culture at 5.6 and 10.0 mmol/l glucose (3.4±0.4 vs 3.9±0.0, respectively), despite a non-statistically significant higher basal insulin release after culture in 10.0 mmol/l glucose (1.8±0.1% and 2.4±0.1%, respectively; Table 3-2). One week of culture in 28.0 mmol/l glucose caused significantly increased basal insulin secretion (3.0±0.3%), while only slightly reducing stimulated insulin release in response to 20.0 mmol/l glucose (7.7±0.2%, $P<0.05$, Table 3-2), resulting in a significantly reduced ($P<0.05$) stimulation index (2.6±0.2) compared to islets cultured at 5.6 and 10.0 mmol/l glucose.

Table 3-1. Recovery of cellular insulin and DNA content as well as β cell insulin content after culture at different glucose concentrations

	Medium glucose (mM)	% Recovery		Insulin content (pg per β-cell)
		Insulin	DNA	
Week 1	5.6	100.0[a]	100.0[b]	14.3±1.6
Week 2	5.6	63.2±6.4	82.8±4.1	9.9±1.5
	10.0	55.8±3.8	73.0±2.1	9.1±2.1
	28.0	42.0±4.7	104.8±3.2[c]	3.7±0.7[d]
2 Day Recovery	5.6 - 5.6	59.5±5.4	77.7±1.6	10.2±2.2
	10.0 - 5.6	62.2±4.4	78.0±0.7	10.3±1.9
	28.0 - 5.6	68.4±9.5	80.1±1.9	7.2±2.0

Cellular insulin (5.1±0.7 μg) and DNA (12.5±0.2 μg) content values are expressed as recoveries of week 1 values arbitrarily set at 100.0%. Insulin content was calculated by dividing the total insulin content of islets by the number of β cells (n=3). [a]P<0.05 vs week 2 and 2 days recovery values; [b]P<0.05 vs week 2 – 5.6 and 10.0 mmol/l and 2 days recovery values; [c]P<0.05 vs week 1, 2 and 2 days recovery values; [d]P<0.05 vs week 1, 2 and 2 days recovery.

Despite there being no change in β cell insulin content after culture in either 5.6 or 10.0 mmol/l glucose (9.9±1.5 and 9.1±2.1 pg, respectively), islets cultured in 10.0 mmol/l glucose were more responsive to a glucose challenge and hence released more insulin compared with those cultured at 5.6 mmol/l (9.2±1.7 vs 4.3±0.2 x 10^{-4} ng, respectively, P<0.05, Table 3-2). Islets which were cultured in 28.0 mmol/l glucose, contained the least cellular insulin content (3.7±0.7 pg/β cell), and thus released even less insulin (2.5±0.6 x 10^{-4} ng) upon glucose stimulation.

TABLE 3-2. Glucose stimulated insulin release after culture at different glucose concentrations

	Medium	Insulin secretory activity (% content)		Insulin release /β-cell (x 10^{-4} ng)		Stimulation Index
	glucose (mM)	2.8 mM	20.0 mM	2.8 mM	20.0 mM	
Week 1	5.6	1.0±0.2[a]	4.0±0.2	1.7±0.1	5.5±0.7	3.9±0.1[e]
Week 2	5.6	1.8±0.1	6.2±0.3	1.7±0.3	4.3±0.2	3.4±0.4[f]
	10.0	2.4±0.1	9.4±0.2[b]	2.4±0.1	9.2±1.7[d]	3.9±0.0[g]
	28.0	3.0±0.3	7.7±0.2[c]	1.0±0.1	2.5±0.6	2.6±0.2
2 Day	5.6 - 5.6	2.6±0.2	4.2±0.1	2.9±0.3	4.9±0.8	1.6±0.1
Recovery	10.0 - 5.6	2.8±0.1	4.5±0.3	2.7±0.4	4.2±0.6	1.6±0.3
	28.0 - 5.6	3.1±0.8	5.0±1.0	1.8±0.3	3.1±0.7	1.7±0.1

A 120 min in vitro static glucose-stimulated insulin release assay was conducted after culture at different glucose concentrations. The stimulation indices were calculated by dividing the amount of insulin released at high glucose (20.0 mmol/l) by that released at low glucose (2.8 mmol/l) (n=3). [a]P<0.05 vs 28.0, 28.0-5.6 mmol/l; [b]P<0.05 vs all except 28.0 mmol/l; [c]P<0.05 vs all except 5.6, 10.0 mmol/l; [d]P<0.05 vs 5.6, 28.0, 10.0-5.6, 28.0-5.6 mmol/l; [e,f,g]P<0.05 vs 28.0, 5.6-5.6, 10.0-5.6, 28.0-5.6 mmol/l.

Stimulation indices of islet from all groups were restored to similar values after the 2 day recovery period (5.6 mmol/l glucose) compared to before, but were also lower due to increased basal insulin release as well as reduced stimulated insulin release (Table 3-2).

III-3*b* Effect of High Glucose on Neonatal Porcine β cell Differentiation, Proliferation and Apoptosis

The porcine β cell mass is dynamic and can increase by both precursor differentiation and β cell replication (16, 21). To examine the effect of high glucose on β cell differentiation, we assessed the proportion of CK7-positive ductal epithelial cells in islets exposed to different glucose concentrations in vitro. Islets contained 30-35% CK7-positive cells after 7 days of culture in either 5.6 or 10.0 mmol/l glucose. This percentage

was reduced to 18.8±1.7% after culture in 28.0 mmol/l glucose, while the percentage of β cells increased to 29.3±0.6% (Fig. 3-1a).

Neonatal porcine islet cell proliferation has been shown to occur both in vitro (22) and in vivo (16) and was assessed here after exposure to different glucose concentrations for 7 days. To determine the percentage of proliferating β cells, dispersed neonatal porcine islet cells were immunostained for both insulin and PCNA. PCNA protein expression is strictly correlated to cell proliferation and to the active phases of the cell cycle (23). The number of PCNA-positive cells (30%) was similar to the number of Ki-67-positive cells (25%) reported in neonatal porcine islets after 7 days culture in 10 mmol/l glucose (22). By day 14 of culture in 5.6 mmol/l glucose, the percentage of both the total population of $PCNA^+$ islet cells and $PCNA^+$ β cells had dropped significantly by 32.3±3.7% and 53.3±7.4% respectively ($P<0.05$; Fig. 3-1b). Culture in 10.0 and 28.0 mmol/l glucose did little to affect the percentage of proliferating islet and β cells significantly compared to 5.6 mmol/l glucose (Fig. 3-1b).

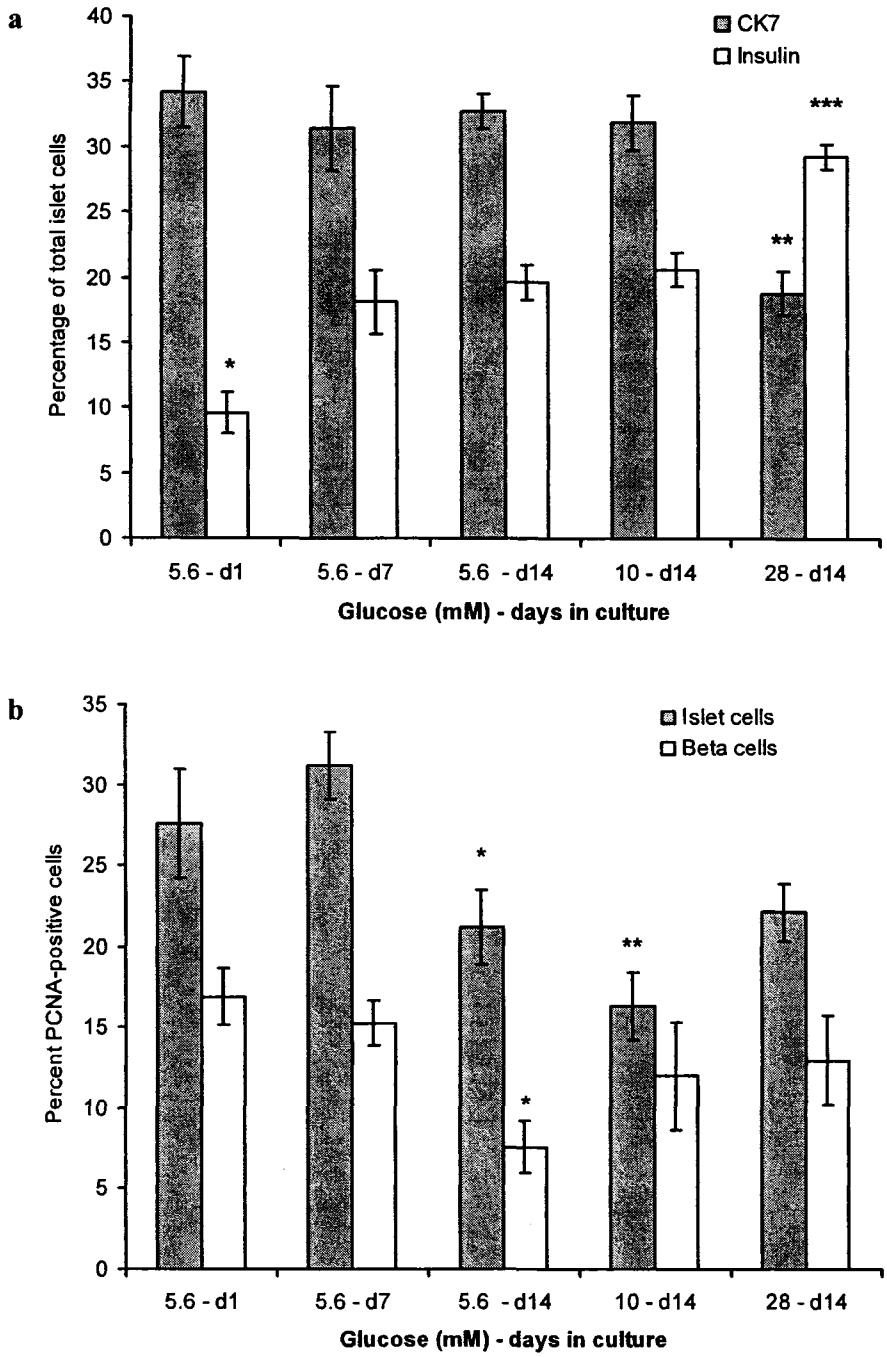

Figure 3-1: Effect of high glucose culture on neonatal porcine β cell differentiation and proliferation. (a) Neonatal porcine islets were cultured in 5.6 mmol/l glucose for 7 days then switched to either 10.0 or 28.0 mmol/l glucose for an additional 7 days. The percentage of β cells in islets cultured for 7 days in 28.0 mmol/l glucose had significantly increased while the percentage of CK7-positive cells decreased (n=3). *P<0.05 vs insulin-positive cells cultured in 5.6-d7-d14, 10-d14, 28-d14. **P<0.05 vs CK7-positive

cells cultured in 5.6-d1,d7,d14, 10-d14. ***$P<0.05$ vs insulin-positive cells cultured in 5.6-d1,d7,d14, 10-d14.
(b) Proliferating islet cells and β cells decreased by the end of two weeks in culture in 5.6 mmol/l glucose. High glucose culture had no effect on the percentage of proliferating cells (n=3). *$P<0.05$ vs proliferating islet (β and non-β cells) and β cells after culture in 5.6 mmol/l glucose for 1 and 7 days. **$P<0.05$ vs proliferating islet cells after culture in 5.6 mmol/l glucose for 1 and 7 days.

Next, neonatal porcine islet cell apoptosis was examined using the TUNEL method to identify DNA fragmentation (Fig. 3-2). Islet cell apoptosis was assessed in intact islets embedded in agarose using the TUNEL method and co-stained for insulin to identify apoptotic β cells. Representative islets examined after 14 days culture in 5.6 mmol/l glucose showed extensive TUNEL labeling (Fig. 3-2a). Only few of the cells in islets undergoing apoptosis are insulin-positive (Fig. 3-2b-c). Both whole islet and β cell apoptosis were not significantly increased after exposure to high glucose concentrations (28.0 mmol/l) for 1 week in culture (Fig. 3-2d).

d

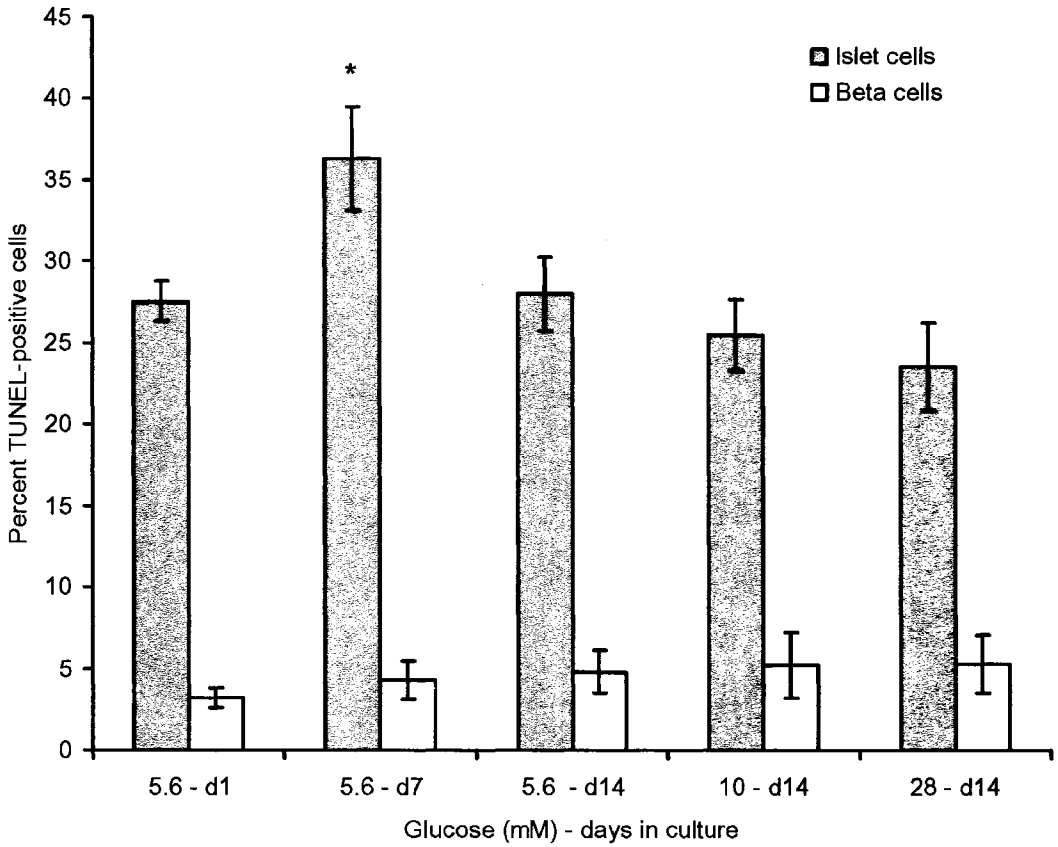

Figure 3-2: Effect of high glucose culture on neonatal porcine β cell apoptosis. **(a-c)** Representative islets cultured for 14 days in 5.6 mmol/l glucose were examined for apoptosis using a fluorescent DNA fragmentation assay (TUNEL stain). Microscopic fields of islets were photographed individually to detect apoptotic cells **(a)** and β cells **(b)** and merged to detect apoptotic β cells **(c)**. x200 magnification.

III-4 DISCUSSION

The data obtained in the present study suggest neonatal porcine islets exposed to high glucose concentrations in vitro leads to a proportional increase in β cells while only moderately affecting β cell function and survival. Exposure to elevated glucose concentrations in culture decreased the insulin content of neonatal porcine islets in a similar way to that described for fetal porcine and adult human islets in tissue culture (9, 24). In addition to the observed β cell degranulation, function was also modified by prolonged in vitro exposure to different glucose concentrations. Islets cultured in 10.0 mmol/l glucose exhibited higher insulin secretion when compared to islets exposed to either 5.6 or 28.0 mmol/l glucose. However in these islets, the insulin secretion in response to 2.8 mmol/l glucose was also increased, resulting in a stimulation index comparable to that for islets cultured in 5.6 mmol/l glucose. Exposure to 28.0 mmol/l glucose also increased basal insulin release and reduced stimulated insulin release at 20.0 mmol/l glucose, thereby resulting in a significantly decreased stimulation index.

Restoration of the glucose concentration to normal (5.6 mmol/l) for 2 days, restored the stimulation index to levels similar to those for islets which had been previously cultured in 5.6 and 10.0 mmol/l glucose suggesting the detrimental effects of high glucose are reversible. One reason for this may be the increased insulin biosynthesis which occurs in β cells that are allowed to recover in 5.6 mmol/l glucose following chronic stimulation for one week in 28.0 mmol/l glucose. Increased insulin biosynthesis after culture at 5.6 mmol/l glucose could then account for the greater insulin content and release per cell seen after the recovery period.

It is also noteworthy that the high glucose exposure induced more severe impairments in adult human β cell function (6) than what has been observed for neonatal porcine β cells in this study. One possible reason to explain this difference may be due to the presence of nicotinamide used in the neonatal islet culture media. Nicotinamide has been shown to protect against the cytotoxic action of streptozotocin on β cells as well as significantly increase glucose-stimulated insulin secretion (17, 25). When cultured with nicotinamide, neonatal porcine islets have been shown to be protected from β cell desensitization following prolonged exposure to high glucose (26). The use of nicotinamide in culture media in this study may explain why islets cultured at 28.0 mmol/l glucose still managed to respond with increased insulin release in response to a 20.0 mmol/l glucose stimulation. The Hams F10 media used in this study contains niacin, a derivative of nicotinamide, and is also supplemented with additional nicotinamide to a final concentration of 10.0 mmol/l. In many of the studies examining the effects of high glucose concentrations on adult human islets, media was used which either contained no (6, 8, 9, 10) or very little (2.0 mmol/l) nicotinamide (7).

High glucose concentrations in culture can influence precursor cell differentiation into β-cells with only a minimal effect on cell replication and apoptosis. In vivo evidence in support of the role of hyperglycemia on precursor cell differentiation was reported by Yoon et al. (27). Their group as well as ours (unpublished observation) have noticed a greater tendency for neonatal porcine islet grafts to develop a more substantial β cell mass when implanted into diabetic (hyperglycemic) animals compared to nondiabetic (normoglycemic) recipients (27). Recent studies have shown that adult stem cells cultured in high glucose medium are able to transdifferentiate into insulin-producing cells

(28, 29, 30). The ability of islets in postnatal life to respond to hyperglycemia by increased precursor cell differentiation has previously been reported to occur in vivo (31).

In addition to precursor differentiation, replication of preexisting β cells has been reported to contribute to the increased β cell mass seen in transplanted neonatal porcine islet grafts (16). The lack of an effect of high glucose on islet cell replication contradicts the results of King et al. who found elevated glucose concentrations (16.5 to 27.5 mmol/l) increased the frequency of replicating neonatal rat β cells in culture (32). However, one study reported that high glucose (28 mmol/l) was ineffective and may even inhibit β cell proliferation in islets from 3 day old neonatal rats (33).

Quite possibly the most significant result of this study is the demonstration that high glucose does not significantly increase the percentage of apoptotic β cells in neonatal porcine islets. Unlike adult human islets which contain 48% apoptotic β cells after exposure to 16.7 mmol/l glucose for 5 days (10), neonatal porcine islets contained less than 5% apoptotic β cells even after exposure to 28.0 mmol/l glucose for 7 days. Despite the low number of apoptotic β cells, the total percentage of islet cells undergoing apoptosis was approximately 30% which has also been reported by others (27). This high percentage of cell death which is present even at the beginning of the culture period (day 1) has been attributed to the isolation procedure since 30% of islet cells have been reported to be undergoing apoptosis immediately following the isolation and culture of both human and porcine pancreases (34, 35). The ability of the neonatal porcine islet cells to resist the toxic effects of high glucose is of paramount importance for their ability to survive posttransplantation. Sustained exposure to hyperglycemia in the posttransplant environment can contribute to increased β cell apoptosis and reduced β cell mass (36).

While many of these studies were performed using adult islets, donor age can significantly affect cell survival. Neonatal islets are known to be more robust and to survive better in tissue culture compared to fragile adult islets. This robustness may be due to the metabolic immaturity of neonatal islets which generate lower ATP and thus a lower secretory response to glucose compared to adult islets (37). Neonatal islets are also known to be better protected than adult islets against oxidative stress due to their more active peroxidase system (38). Indeed we have found significantly higher expression levels of x-linked inhibitor of apoptosis (XIAP), which is known to protect against apoptosis, in neonatal compared to adult porcine islets (unpublished observation, 39). High glucose has also been shown to have a beneficial effect on neonatal islets by enhancing the H_2O_2 scavenger system, a phenomenon not observed in adult islets (38).

Based on in vivo data which found exposure to hyperglycemia can increase the β cell mass of neonatal porcine islet grafts by β cell differentiation from CK7-positive cells, we have now confirmed this observation in vitro (15-17). Of major significance is the fact that β-cell apoptosis does not appear to increase in frequency following exposure to high glucose which is in contrast to the situation with adult human islets. Given the ease by which neonatal porcine islets can be obtained and their enormous growth potential, these insulin-producing cells are a potentially useful source of insulin-producing tissue for clinical transplantation.

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CHAPTER FOUR

NEONATAL ISLET PRECURSORS DIFFERENTIATE INTO STREPTOZOTOCIN RESISTANT β CELLS AFTER TRANSPLANTATION

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IV-1 INTRODUCTION

Streptozotocin (STZ) is a diabetogenic toxin which induces insulin deficiency due to its selective pancreatic β cell cytotoxicity. The deoxyglucose moiety of STZ enables selective uptake via the low affinity glucose transporter GLUT2 (1, 2). Once inside the β cell, genomic DNA is damaged by alkylation of the base pairs causing strand breaks (3). The excision repair process responsible for removing the alkylated lesions involves activation of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) (4, 5). PARP repairs DNA strand breaks by catalyzing the addition of long branched chains of poly (ADP-ribose) (6, 7). Ultimately, cellular energetics and function are compromised however, when a massive depletion of cellular NAD occurs as a result of NAD consumption as substrate for the building of ribose units (8). The β cell then dies by a regulated form of necrosis (9).

Inhibitors of PARP such as nicotinamide can protect against β cell damage and diabetes development (10). Moreover, genetically engineered PARP-deficient mice are resistant to STZ-induced diabetes (11, 12, 13). In addition to PARP-induced cell death, susceptibility to STZ-mediated damage can also be influenced by the expression of GLUT2 (14) and functional maturation (15) of β cells. For example, human β cells, which express very low levels of GLUT2 (16) have been shown to be resistant to STZ (17, 18). Furthermore, immature fetal porcine β cells which respond poorly to glucose (19) are also resistant to STZ (20, 21). The sensitivity of a potential source of insulin producing cells for clinical transplantation, neonatal porcine β cells (22, 23) to STZ has not yet been determined. Neonatal porcine islets express GLUT2 mRNA (24) and

display an insulin secretory response to glucose stimulation (25), thus we hypothesize STZ would have a detrimental effect on these β cells.

Islets from neonatal pigs provide an interesting model to study STZ susceptibility in β cells as well as in β cells which develop from precursors. Neonatal porcine islets contain differentiated endocrine cells as well as their precursors which are identified by cytokeratin-7 (CK7) immunoreactivity (25, 26). In addition to the established methods of in vitro maturation (24), an alternative in vivo transplantation model has been shown to be effective for recapitulating the growth and differentiation of islet cells (27). In the time required for normoglycemia to be established in transplanted diabetic recipients, significant differentiation of precursors has been shown to occur (28). The expression of CK7 in precursor cells decreases following in vivo differentiation and is replaced by markers of differentiated endocrine cells (28, 29). Using this model, we examined STZ susceptibility in isolated neonatal porcine β cells and in β cells differentiated in vivo from transplanted precursor cells. Understanding and exploiting selection strategies for the development of β cells which are resistant to diabetogenic toxins, may help in generating β cells which will also resist autoimmune attack in patients with type 1 diabetes.

IV-2 MATERIALS AND METHODS

IV-2*a* Neonatal Porcine and Adult Mouse Islet Isolation and Transplantation

Neonatal porcine islets were obtained from 1- to 2-day-old Duroc pig pancreases (University of Alberta, Edmonton, AB, Canada). Islets were isolated and cultured as previously described by Korbutt et al. (25). Adult mouse pancreatic islets from 6-8 wk old male Balb/c mice (University of Alberta colony) were isolated by collagenase (1.0

mg/ml; Sigma, St Louis, MO, USA) digestion and discontinuous Dextran (Clinical grade; Sigma) gradient purification (30). Immune deficient C57BL/6-rag1$^{tm1/mom}$ (Jackson Laboratory, Bar Harbor, ME) and Balb/c mice were used as recipients of 2000 neonatal porcine or 500 adult mouse islets, respectively. Diabetes was induced by intraperitoneal (i.p.) injection of streptozotocin (STZ; Sigma) dissolved in ice-cold saline (pH 5.5) at a dose of 175 mg/kg for B6 rag$^{-/-}$ and 275 mg/kg for Balb/c mice 2 days before transplantation (31, 32). Animals with a non-fasting blood glucose ≥ 20 mmol/l at the time of transplant were used as recipients. Islets were transplanted beneath the left kidney capsule of anesthetized mice as previously described (25) and monitored for normalization of blood glucose (≤ 10 mmol/l). All mice were housed and fed under specific pathogen-free conditions and were cared for according to the guidelines of the Canadian Council on Animal Care.

IV-2*b* In Vitro and In Vivo β Cell Exposure to STZ

Twenty four hours prior to STZ exposure, neonatal porcine and adult mouse islets were cultured in M199 media (Gibco) supplemented with 10% fetal calf serum (Gibco), 10 mmol/l Hepes and 100 U/ml penicillin and 0.1 mg/ml streptomycin. In vitro exposure of islets to STZ was achieved by adding STZ dissolved in ice-cold saline (pH 5.5) to the medium to a final concentration of 20.0 mmol/l and incubation for 30 min at 37°C. Islets were then washed twice with fresh media and cultured for 48 hrs. Corresponding control neonatal porcine and adult mouse islets received an equal volume of saline alone.

In vivo exposure of mice with neonatal porcine or adult mouse islet grafts to STZ involved a single i.p. injection of STZ dissolved in ice-cold saline (pH 5.5) and

monitoring of blood glucose levels to assess β cell damage. Recipients of syngeneic mouse islets received 275 mg/kg STZ (31, 32) whereas recipients of neonatal porcine islets received either 175 or 475 mg/kg STZ.

IV-2*c* Measurement of STZ Uptake

Streptozotocin uptake into β cells was determined following in vitro and in vivo as previously described (33). STZ was measured spectrophotometrically after neonatal porcine or adult mouse islets were exposed to STZ in vitro for 30 min. Islets were washed twice, sonicated on ice in 0.6 N perchloric acid, then a sample of cell-debris free supernatant was incubated with 6 N HCl and color reagent (0.5% sulfanilic acid and 0.1% N(1-naphthyl)ethylenediamine dihydrochloride (NED) in 30% acetic acid) in a 60°C water bath for 45 min (34). Absorbance was measured at 570 nm. The number of β cells per sample was determined using insulin immunostaining and DNA content (25) and results are expressed as the STZ concentration (μmol/l) per β cell. STZ uptake into β cells following in vivo exposure was determined 25 min after STZ injection. Islet grafts, spleen and kidney were removed from euthanized animals, homogenized and analyzed as described above.

IV-2*d* Islet Cellular Insulin and DNA Content Analysis

Samples for analysis of insulin content were taken in duplicate and measured by radioimmunoassay (Diagnostic Products Corp., Los Angeles, CA). Samples were sonicated in 2 mmol/liter acetic acid containing 0.25% BSA, centrifuged (800 g, 15 min) then supernatants were collected and stored at -20°C until the time of assay.

DNA content was analyzed using PicoGreen, an ultra-sensitive fluorescent nucleic acid stain for double-stranded DNA (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. Duplicate aliquots were washed in citrate buffer (150 mmol/l NaCl, 15 mmol/liter citrate, 3 mmol/liter EDTA, pH 7.4) and stored as cell pellets at -20°C. Cell pellets were resuspended in lysis buffer (10 mmol/liter Tris, 1 mmol/liter EDTA, 0.5% Triton X-100, 4°C, pH 7.5), sonicated, and then analyzed (25).

IV-2*e* Glucose Stimulated-Insulin Secretion

Prior to and 48 hrs after STZ exposure insulin secretory activity was assessed using a static incubation assay (25). Islets were washed twice with Hams F10 media containing 0.5% BSA and 2.8 mmol/l glucose and samples were taken for determination of cellular insulin content. Islets were cultured in 24 well plates, and incubated for 120 min in 1.5 ml of Ham's F10 medium supplemented with 0.5% BSA and either basal (2.8 mmol/l) or stimulatory (20.0 mmol/l) glucose concentrations. At the end of the incubation, supernatants were collected for measurement of insulin release by radioimmunoassay. Insulin secretion was calculated by dividing the insulin released into the supernatant by the cellular insulin content of the islets (percent content).

IV-2*f* Oral Glucose Tolerance Test (OGTT)

OGTTs were performed 3 wks after injection of either STZ or saline into transplanted mice. After an overnight fast (12 hrs), a 50% dextrose solution (Abbott Laboratories, Montéal, Canada) was administered intragastrically at a dose of 3 mg/g

body weight. Blood samples were obtained 0, 15, 30, 60, and 120 min after glucose challenge and analyzed for plasma glucose levels.

IV-2*g* Histological Examination

Neonatal porcine islets were fixed in 4% paraformaldehyde for 30 min then embedded in a 2% low melting point agarose solution and allowed to harden at 4°C before processing, paraffin embedding, and sectioning. Samples of porcine pancreas and islet grafts were immersed in Z-fix solution (Anatech Ltd., Battle Creek, MI) and embedded in paraffin. Sections (5 μm) were immunostained using the immunoperoxidase ABC-DAB method (Avidin-Biotin complex visualized with diaminobenzidine). Endogenous peroxidase was quenched with a 10% H_2O_2 methanol solution. Microwave (1260W) antigen retrieval for GLUT2 and CK7 staining involved 15 min in 10 mmol/l sodium citrate buffer (pH 6.0). Blocking was performed with 20% normal goat serum (Fischer) for 15 min. Primary antibody concentrations were as follows: 1:1000 guinea pig anti-porcine insulin (Dako Diagnostics Canada Inc., Mississauga, Canada), 1:200 rabbit anti-human GLUT2 (Alpha Diagnostics, San Antonio, TX) and 1:25 mouse anti-human CK7 (Dako). Anti-insulin and CK7 antibody incubation was in a humidified chamber for 30 min while GLUT2 antibody incubation was overnight at 4°C in a humidified chamber. Biotinylated goat secondary antibodies (anti-guinea pig, anti-rabbit, and anti-mouse IgG) were obtained from Vector Laboratories (Burlingame, CA) and used at a concentration of 1:200 for 20 min. ABC complex (Vector) incubation time was for 40 min at room temperature and visualized with diaminobenzidine (Biogenex, San Ramon, CA). All tissue sections were counter-stained with Harris' hematoxylin.

IV-2*h* Statistical Analysis

Data are expressed as means ± SEM of n independent experiments. Statistical significance of differences was determined using one-way analysis of variance with SPSS statistical software, version 12.0 for Windows (Chicago, IL). A *P* value of less than 0.05 was considered to be statistically significant.

IV-3 RESULTS

IV-3*a* GLUT2 Expression and STZ Uptake in Neonatal Porcine β Cells

Consecutive sections of 1-3 day old neonatal porcine pancreases were immunostained for insulin and GLUT2 (Fig. 4-1a, b). GLUT2 immunoreactivity appeared colocalized to the insulin immunopositive β cells that were scattered throughout the pancreas. Following collagenase digestion of neonatal porcine pancreases and 7 days culture, aggregates enriched for islet endocrine and CK7-positive precursor cells formed in suspension (25, 29). Staining of consecutive sections of these aggregates also revealed a pattern of GLUT2 immunoreactivity in the insulin-positive β cells (Fig. 4-1c, d). To follow the expression of GLUT2 during the differentiation of β cells, we used an established in vivo model of neonatal porcine islet endocrine cell differentiation from CK7-positive precursor cells (28, 29). In this model, neonatal porcine islets transplanted into diabetic immune-deficient mice develop a significantly increased β cell mass after 6-8 wks (25). Therefore, 2000 neonatal porcine islets were transplanted into STZ induced diabetic B6 $rag^{-/-}$ mice. After 6-8 wks, mice achieved normoglycemia with an average non-fasting blood glucose level of 8.3 ± 1.4 mmol/l, demonstrating that the transplanted islets had developed a sufficient β cell mass to achieve normoglycemia (25).

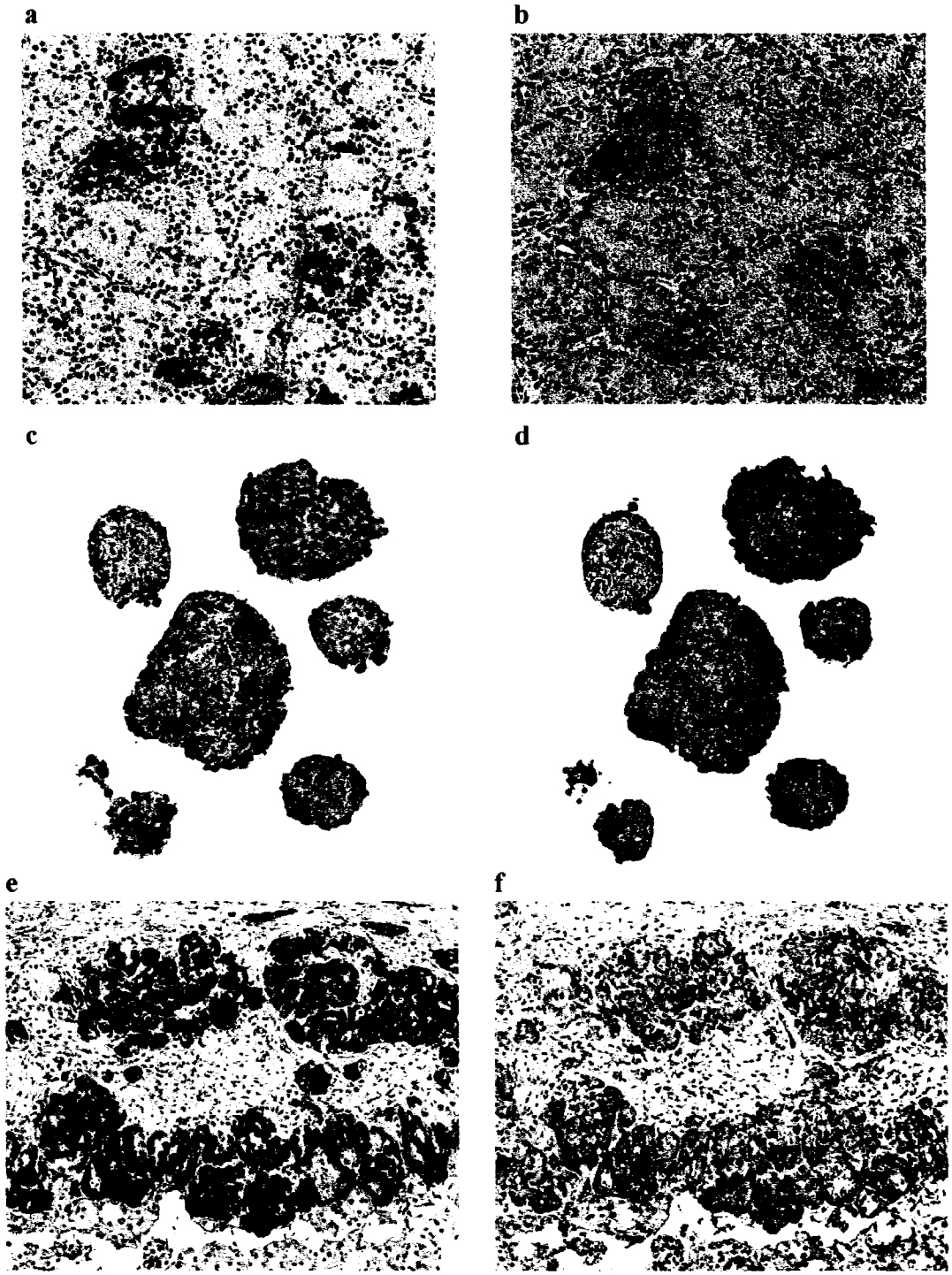


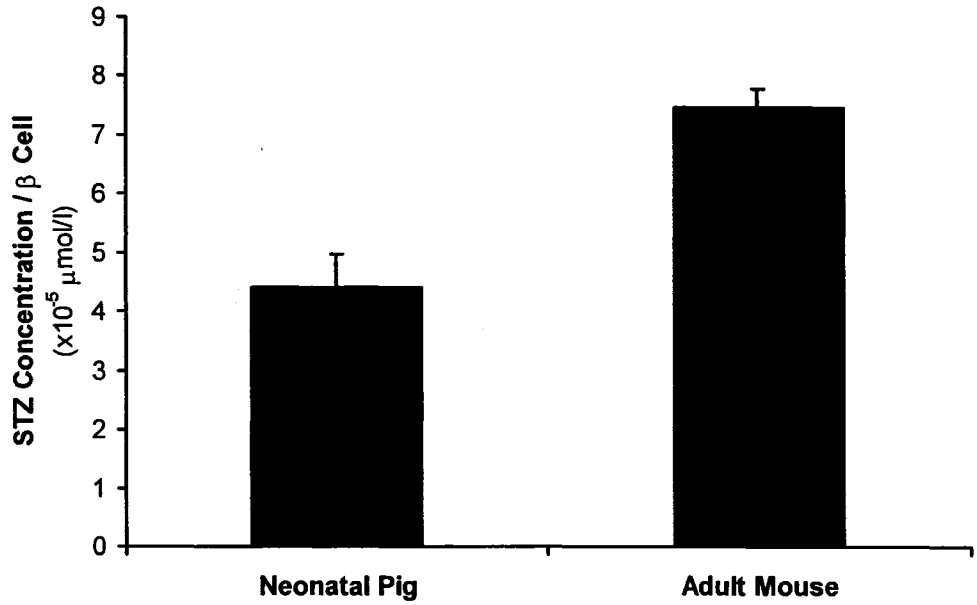
Figure 4-1: Immunohistochemical localization of GLUT2 in neonatal porcine β cells. Consecutive sections of neonatal porcine pancreas (1-3 days old), immunostained for insulin (**a**) or GLUT2 (**b**). Neonatal porcine islets after 7 days in vitro culture of digested pancreases were immunostained for insulin (**c**) or GLUT2 (**d**). Neonatal porcine islet

xenografts harvested from mice 100 days posttransplant and immunostained for insulin (**e**) or GLUT2 (**f**). (x200 magnification).

Insulin immunostaining of harvested grafts revealed an abundance of insulin-positive β cells comprising the majority of large islet-like aggregates (Fig. 4-1e). GLUT2 and insulin staining of islet graft serial sections revealed GLUT2 expression in the β cells (Fig. 4-1f).

Having confirmed GLUT2 expression in neonatal porcine β cells, we measured STZ uptake in β cells after in vitro incubation of neonatal porcine islets with 20.0 mmol/l STZ. Adult mouse islets were used as a positive control. Following a 30 min incubation of islets with STZ (20.0 mmol/l) and adequate washes, islets were analyzed spectophotometrically. Neonatal porcine β cells contained approximately half the concentration of STZ found in adult mouse β cells despite having been exposed to the same concentration of STZ for the same amount of time ($4.4 \pm 0.9 \times 10^{-5}$ vs. $7.5 \pm 0.5 \times 10^{-5}$ μmol/l, respectively; Fig. 4-2a).

a



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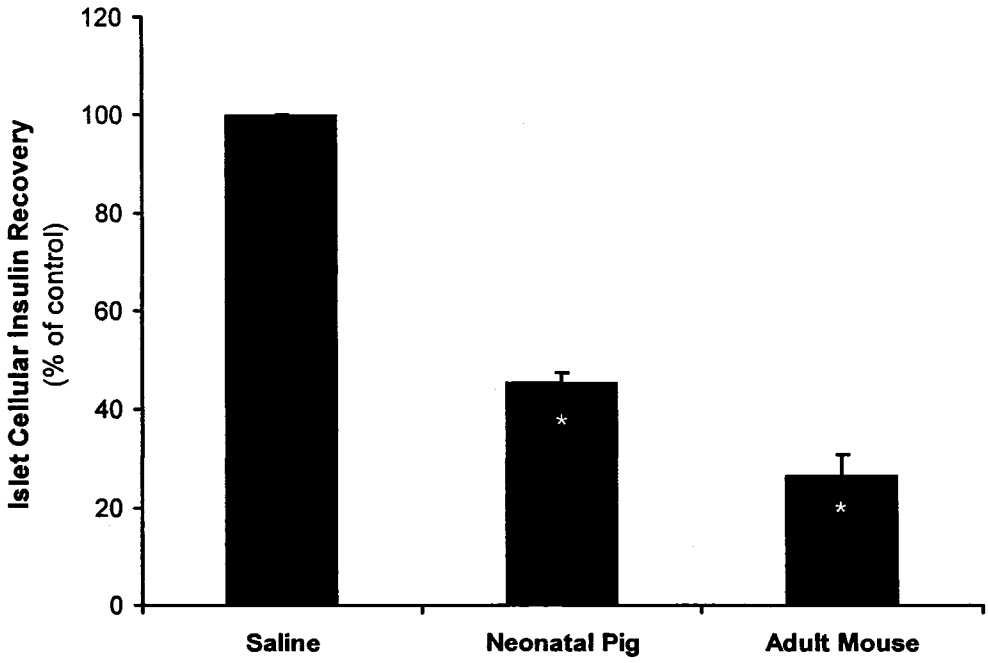

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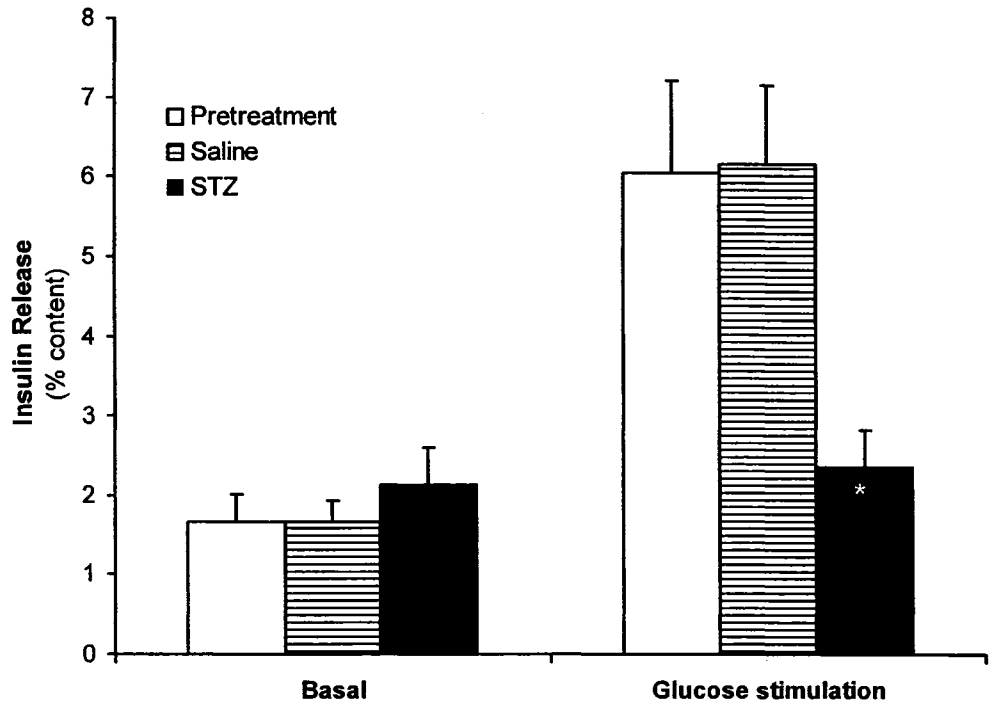

Figure 4-2: STZ uptake in neonatal porcine and adult mouse β cells exposed to STZ (20.0 mmol/l) in vitro (**a**). Effect of STZ on the cellular insulin content of neonatal porcine and adult mouse islets cultured for 48 hrs after 20.0 mmol/l STZ (n=4) (**b**). Results are expressed as percent of saline controls (*P<0.05 vs. saline control). Effect of STZ on neonatal porcine islet glucose stimulated insulin release (**c**). Insulin secretion in response to basal (2.8 mmol/l) and stimulatory (20 mmol/l) glucose before STZ treatment (white bars), after saline (stripes), or STZ (black bars) exposure. *P<0.05 vs. pretreatment and saline treated islets after glucose stimulation.

IV-3*b* Neonatal Porcine β Cell Susceptibility to STZ

The susceptibility of neonatal porcine β cells to STZ was demonstrated after exposure to STZ both in vitro and in vivo. After a 30 min in vitro incubation with 20.0 mmol/l STZ, islets were washed and cultured in fresh M199 media for 48 hrs. The cellular insulin and DNA content of both neonatal porcine and adult mouse islets after STZ exposure were compared to their appropriate corresponding saline treated controls. Recoverable cellular insulin and DNA content of neonatal porcine islets was only 45.7 ±

1.6% and 57.6 ± 4.4%, respectively compared to controls (P<0.05; Fig. 4-2b). Likewise, adult mouse islets contained only 26.7 ± 4.1% and 26.9 ± 6.6% of the cellular insulin and DNA content of saline treated controls, respectively (P<0.05; Fig. 4-2b). The functional viability of neonatal porcine islets was also assessed by measuring glucose stimulated insulin release (25). Neonatal porcine islets treated with saline alone or just prior to STZ treatment showed a 4.3 ± 1.3 and 4.2 ± 1.3-fold increase in insulin release in response to glucose stimulation, respectively (Fig. 4-2c). In contrast, neonatal porcine islets exposed to STZ failed to significantly increase their insulin release in response to a glucose stimulus (20.0 mmol/l) when examined 48 hrs later (1.1 ± 0.1-fold increase, P<0.05). Mouse islets exposed to STZ had undergone such extensive β cell death, that by 48 hrs, no intact β cell aggregates were available to assess glucose stimulated insulin release.

IV-3*c* In Vivo β Cell Differentiation and STZ Sensitivity

To determine the effect of STZ on porcine β cells which have differentiated from precursors exposed to hyperglycemia, recipient mice cured of their diabetes by a neonatal porcine islet xenograft were injected with STZ at day 105 posttransplant (Fig. 4-3a). For controls, recipients of adult mouse islets were injected at a similar time posttransplant. At the time of injection, the average non-fasting blood glucose levels were 7.6 ± 1.8 and 6.4 ± 1.0 mmol/l for recipients of neonatal porcine and adult mouse islets respectively. Following STZ injection at a dose of 275 mg/kg for recipients of adult mouse islets, animals quickly became diabetic with a non-fasting blood glucose level of 27.1 ± 1.7 mmol/l at 2 wks post injection (Fig. 4-3a). Morphological examination of islet grafts harvested from these animals showed extensive β cell destruction (Fig. 4-4a; control graft Fig. 4-4b). In contrast, recipients of neonatal porcine islets remained normoglycemic

following STZ injection at either low (175 mg/kg) or high (475 mg/kg) doses (Fig. 4-3a). Mice were followed for over 40 days after STZ administration, during which time the non-fasting blood glucose levels remained normal at 5.2 ± 0.3, 6.1 ± 0.4 and 5.8 ± 0.9 mmol/l for saline, 175 and 475 mg/kg STZ treated mice by the end of the follow-up period (Fig. 4-3a). Oral glucose tolerance tests performed on these mice demonstrated similar glucose clearance kinetics (Fig. 4-3b), and islet grafts removed from these recipients and immunostained for insulin revealed abundant insulin-positive β cells in both STZ (175 and 475 mg/kg) and saline treated groups (Fig. 4-4c, d). In addition to having confirmed GLUT2 expression in neonatal porcine islet xenografts >100 days after transplantation, STZ uptake into graft β cells was also assessed. Islet xenografts contained 14.0 ± 1.7 μg/ml STZ compared to spleen and kidney STZ levels of 0.4 ± 0.1 and 565.8 ± 91.6 μg/ml, respectively.

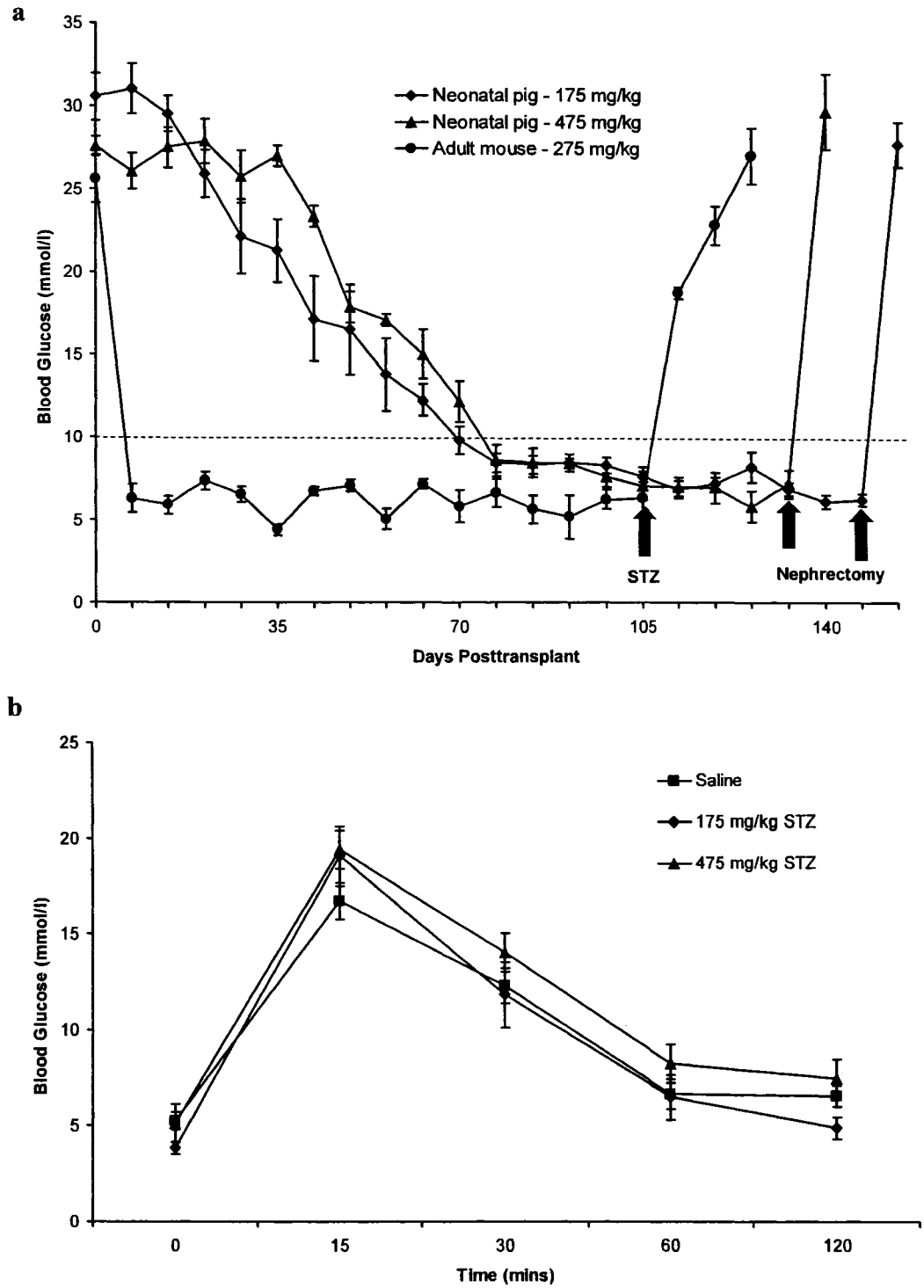

Figure 4-3: Non-fasting blood glucose levels of mice transplanted with either syngeneic adult mouse islets or xenogeneic neonatal porcine islets (**a**). At 105 days posttransplant, recipients of mouse islets were administered 275 mg/kg STZ (circles; n=4), while

recipients of neonatal porcine islets received either 175 mg/kg (diamonds; n=9) or 475 mg/kg (triangles; n=4) STZ. **b** Blood glucose values during oral administration of glucose to mice transplanted with neonatal porcine islets and administered either 175 mg/kg (diamonds; n=5), 475 mg/kg (triangles; n=4) or saline (squares; n=5).

To demonstrate the importance of a hyperglycemic posttransplant environment on the development of STZ resistance, isolated neonatal porcine islets were transplanted into non-diabetic naïve recipients and injected with STZ (175 mg/kg) 100 days posttransplant. Following STZ injection, animals developed diabetes transiently and remained hyperglycemic for 3 wks due to the destruction of β cells in both the mouse pancreas and islet xenograft (Fig. 4-5a). However, by the 4th wk after STZ administration diabetes was eventually reversed. The non-fasting blood glucose levels were reduced to 7.4 ± 2.1 mmol/l and animals remained normoglycemic for up to 2 wks before a nephrectomy was performed resulting in diabetes recurrence. Histological examination of islet xenografts taken before STZ injection (100 days posttransplant) revealed many CK7-positive ductal cells and a marginal β cell mass (Fig. 4-5b, d). In contrast, after STZ treatment and 4 wks of β cell development/diabetes reversal, few CK7-positive cells were observed (Fig. 4-5c), and a marked increase in the β cell mass had occurred (Fig. 4-5e).

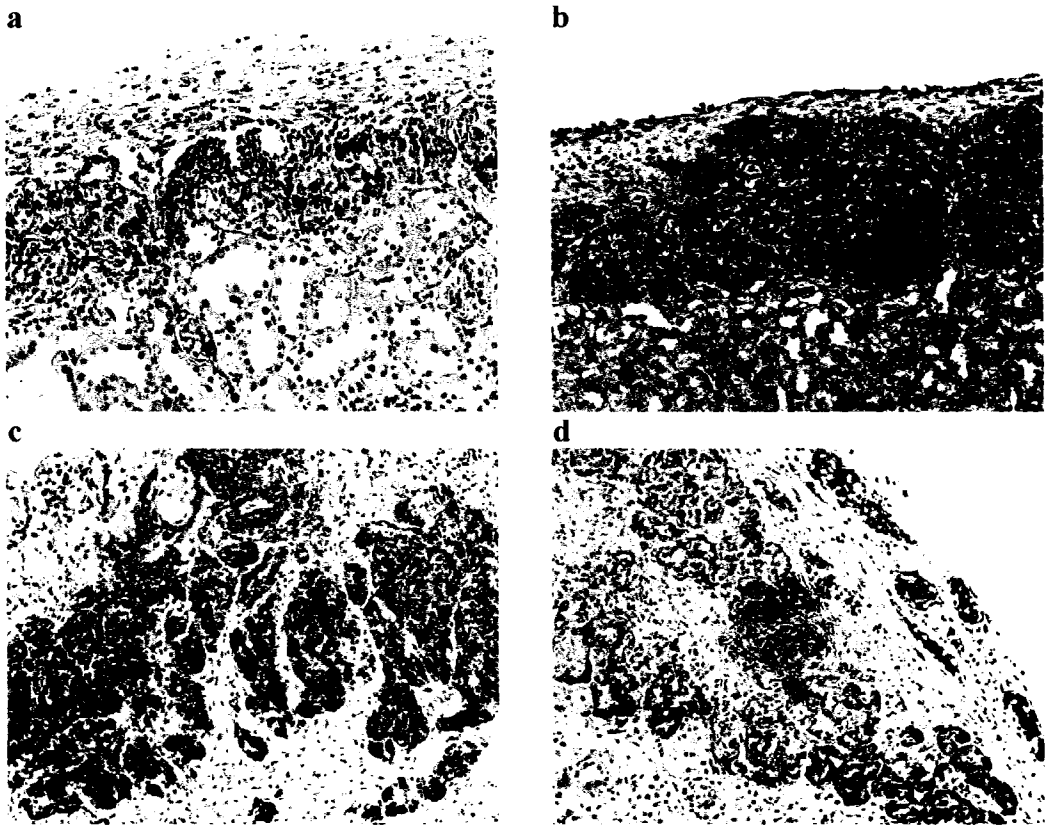

Figure 4-4: Adult mouse syngeneic islet grafts were harvested from mice cured of their diabetes, then later administered 275 mg/kg STZ (**a**) or saline (**b**) and immunostained for insulin. Neonatal porcine islet grafts from diabetic mice immunostained for insulin after injection of 475 mg/kg STZ (**c**) or saline (**d**). (x200 magnification).

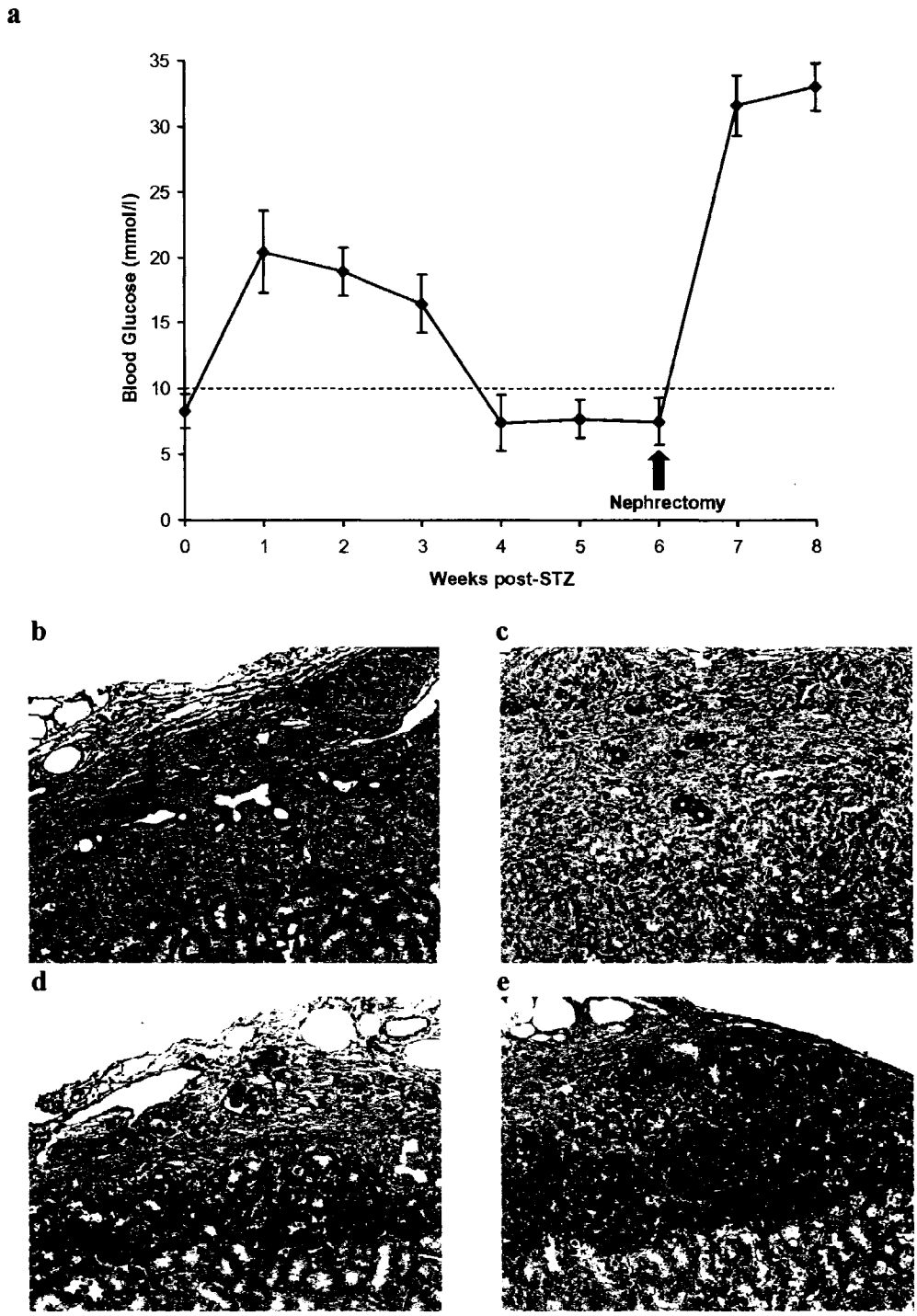

Figure 4-5: Non-diabetic naïve recipients of neonatal porcine islet grafts injected with 175 mg/kg STZ 100 days posttransplant. Animals transiently developed diabetes, but by 4 wks non-fasting blood glucose values had returned to normoglycemic levels (n=4) (**a**). Islet xenografts were harvested from animals before (**b**, **d**) or 6 wks after (**c**, **e**) STZ

administration and immunostained for CK7 (**b, c**) and insulin (**d, e**). (x200 magnification).

IV-4 DISCUSSION

The present study describes the differences in susceptibility to the diabetogenic toxin STZ between pancreatic β cells which develop in situ and β cells which develop from transplanted precursors exposed to hyperglycemia in vivo. Whereas exposure to STZ was capable of damaging β cells isolated from neonatal pigs, STZ had no detrimental effect on β cells which differentiated in vivo from transplanted precursors exposed to hyperglycemia. Adult pigs injected with as little as 100 mg/kg STZ results in permanent and irreversible diabetes (35, 36, 37). The results of this study demonstrate that the development of porcine β cells in a posttransplant hyperglycemic environment results in STZ resistance, despite the fact that the β cells are naturally susceptible to STZ when they develop in their native pancreas.

GLUT2 expression in neonatal porcine islet β cells was detected in situ, following 7 days culture and when examined long-term after transplantation. Unlike in rodent islets (38, 39), in vitro culture or exposure to hyperglycemia in the posttransplant environment did not appear to down-regulate GLUT2 expression in the β cells of neonatal porcine islets. The continued expression of GLUT2 in β cells is necessary for the specific uptake of STZ into β cells (1, 2, 14). Streptozotocin uptake and damage was demonstrated in β cells isolated from cultured neonatal porcine pancreases. In vitro treatment of neonatal porcine islets with STZ significantly reduced the cellular insulin and DNA recoveries as well as the ability of β cells to increase their insulin secretion when challenged with high glucose. Rodent islet toxicity to STZ has been previously demonstrated (40, 41, 42) and

was used as a positive control to assess STZ damage in this study. The concentration of STZ needed to induce significant β cell damage was much higher for neonatal porcine β cells than what has been previously used for adult rodent β cells (20.0 vs. 2.0 mmol/l, respectively; 31, 32). The higher concentration required for neonatal porcine β cells may be because of low GLUT2 protein expression per β cell or because they are developmentally immature and thus differ metabolically in their response to STZ exposure. Boschero *et al.*, reported the flow through the pentose phosphate pathway is higher in neonatal than adult islets (43). This could be responsible for the greater availability of NADH required for DNA repair following injury by STZ. The higher concentration of STZ required to damage neonatal porcine β cells may be due to a greater availability of NADH which is maintained at higher levels than in adult β cells. In addition, STZ has been found to suppress and impair the function of mitochondrial dehydrogenases such as FAD-linked glycerophosphate. Due to the mitochondrial immaturity of neonatal islets compared to adult islets, this may explain the higher dose of STZ required to induce β cell damage (44).

The susceptibility of neonatal porcine β cells to STZ induced damage was no longer evident following transplantation into diabetic recipients and subsequent β cell differentiation from CK7-positive precursors and achievement of normoglycemia (28, 29). Following administration of STZ (175 and 475 mg/kg) these animals remained normoglycemic and moreover also exhibited normal glucose clearance kinetics and normal graft morphology suggesting the xenograft β cells were not adversely affected by STZ, despite measurable uptake of the toxin. In contrast, in diabetic mice transplanted with a syngeneic adult mouse islet transplant, islet graft β cells were rapidly destroyed by

STZ, resulting in a permanent hyperglycemia. Transplantation of neonatal porcine islets into diabetic mice requires 6-8 wks before normoglycemia is achieved, which is necessary for the development of a sufficient β cell mass to develop and reverse diabetes. Previous studies have demonstrated that exposure of islets to high glucose can protect β cells from STZ damage (45, 46) which may be a contributing factor to the resistance of the porcine islet xenografts to STZ in this study. In non-diabetic naïve recipients of neonatal porcine islets administered STZ (175 mg/kg), hyperglycemia was rapidly observed suggesting the β cells of both the islet xenograft and native mouse pancreas were destroyed. These data may indicate that β cells in the neonatal porcine islet grafts did not develop STZ resistance since they were not exposed to hyperglycemia in vivo and hence were destroyed after STZ exposure (175 mg/kg). Another possible explanation is that the graft β cells within non-diabetic naïve recipients were predominantly those present at the time of transplantation (i.e. similar to isolated islets) and did not originate from precursors exposed to hyperglycemia, and were thereby susceptible to STZ damage. Following STZ injection into these recipients, native pancreatic β cells were destroyed and hyperglycemia emerged in these animals which may have triggered CK7-positive cells to differentiate into β cells which resulted in the observed normal blood glucose levels. According to Lukinius *et al.*, fetal porcine endocrine pancreas transplanted into naïve mice recipients follows the same sequential differentiation pattern which occurs in the native pancreas in situ (27). Differentiation of islet cells was found to be genetically determined and independent of the milieu present after transplantation based on the morphology and hormone content of secretory granules. The results of our study agree with those of Lukinius *et al.* since the differentiation of β cells in transplanted neonatal

porcine islets in naïve recipients resulted in STZ susceptibility, which is similar to the susceptibility of β cells found in the native adult porcine pancreas (35, 36, 37). However, when transplanted neonatal porcine islets are exposed to hyperglycemia, precursor differentiation gives rise to β cells which are STZ resistant and thus differ from native pancreatic β cells. Further evidence to support the role of high glucose in the development of STZ resistant β cells from precursors comes from Laybutt *et al.* who recently reported that hyperglycemia causes upregulation of antioxidant and antiapoptotic genes in hypertrophied β cells following partial pancreatectomy resulting in reduced β cell STZ sensitivity (47). The induction of protective genes strongly corresponded to the degree of hyperglycemia in animals since STZ susceptibility in β cells returned when animals were rendered normoglycemic by phlorizin. Exposure of fetal porcine islet precursors to hyperglycemia posttransplant has also been shown to result in the development of β cells which are resistant to other diabetogenic toxins such as the β cell cytotoxin alloxan (48). Fetal porcine islet-like cell clusters which are initially devoid of β cells before transplantation become 80% β cells by 8 wks posttransplant and are resistant to alloxan. Alloxan is a producer of oxygen radicals and reportedly kills cells by both necrosis and apoptosis. Intragraft β cell morphology and function however remained intact suggesting transplanted precursors differentiate into β cells which are resistant to the toxic effects of alloxan and oxygen radicals. Recently, we and others have shown that fetal and neonatal porcine islets express low levels of enzymes responsible for nitric oxide production (iNOS) and high levels of antiapoptotic genes (XIAP), respectively, in contrast to adult porcine islets which express high levels of iNOS and no XIAP (G.S. Korbutt, unpublished observation, 49).

The results of this study suggest β cell differentiation from transplanted precursors exposed to hyperglycemia in vivo results in enhanced protection against cell death. The use of high glucose in protocols to differentiate stem cells into β cells is common and may or may not result in populations of differentiated β cells which are resistant to diabetogenic toxins. The development of cytotoxin resistant β cells may be more clinically useful for transplantation therapy in type 1 diabetic patients (50). Improved cellular defense mechanisms to protect against β cell cytotoxicity may be useful in protecting transplanted β cells from cytokine or free radical induced damage caused by autoimmune or xenogeneic attack.

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CHAPTER FIVE

GENERAL DISCUSSION AND SUMMARY

V-1 GENERAL DISCUSSION

The number of people suffering from diabetes mellitus is expected to reach 366 million worldwide by the year 2025, a significant increase from 30 million only 40 years earlier in 1985. For widespread application of β cell replacement therapy to treat the majority of type 1 diabetic patients, two major challenges need to be first overcome. First, an unlimited supply of insulin-producing cells that does not rely on scarce brain-dead organ donors needs to be found. Second, newer less toxic and more "islet-friendly" anti-rejection regimens which do not impair islet vascularization, proliferation and function need to be discovered to ensure long-term islet survival and function. The currently used protocol of immunosuppression for allogeneic islet transplantation, which includes tacrolimus and sirolimus, results in insulin independence rates of 70% at one year, however, by 5 years only 10% remain insulin independent (1).

Recent milestone studies have demonstrated the feasibility of using xenogeneic insulin-producing cells to cure diabetes. These studies used islets isolated from non-genetically modified wildtype pigs of either neonatal or adult ages with a combination of immune-suppressing drugs for transplantation into diabetic monkeys (2, 3). The encouraging results obtained in monkeys, which were cured for several months, suggest that the islet supply problem may be solved in a foreseeable future using xenogeneic porcine islets. Sufficient progress in the field of experimental islet xenotransplantation in pre-clinical non-human primate models will be required before commencement of human clinical trials. Few groups have reported clinical trials of islet xenotransplantation [reviewed in (4)] with disappointing results because of a lack of specific pre-clinical studies in non-human primates by these groups.

Several studies have demonstrated insulin-producing islets from fetal, neonatal and adult pig pancreases can normalize diabetes in animal models (2, 3, 5). Although the yield and purity of β cells is markedly lower in islets from perinatal animals, grafts of these islets are capable of significant growth after transplantation, unlike adult islets (6, 7). Neonatal porcine islets isolated from 1-3 day old animals contain a heterogeneous mixture of hormone-producing and undifferentiated epithelial cells capable of insulin secretion and growth potential. The precursors in neonatal islets are considered ductal epithelial in nature because they express cytokeratin proteins. In islet grafts isolated from adult pancreases, ductal cells have been shown to be important for the long-term metabolic success of the graft (8). However, the differentiation potential of adult pancreatic ductal cells is limited compared to perinatal ductal cells (9, 10). In islets isolated from perinatal animals, the precursor cells substantially contribute to the β cell mass after transplantation (6, 11). These cells may also be manipulated in vitro for example to expand the endocrine cell mass as well as reduce tissue immunogenicity.

In Chapter 2, neonatal islet precursors were genetically engineered to over-express the developmental transcription factor ngn3 with the aim of increasing the endocrine cell mass in these islets. Ectopic expression of ngn3 was found to activate glucagon gene and protein expression. The increased proportion of α cells rather than β cells is similar to the situation seen in embryonic cells which over-express ngn3 (12, 13). The lack of β cell development in neonatal porcine pancreatic precursors which express ngn3 highlights the need to properly recapitulate embryonic pancreas development in these cells with several transcription factors other than just ngn3 to ensure the proper development of all islet cells. A reduction in the proportion of proliferating cells in grafts

expressing ngn3 after transplantation was observed which also may have contributed to the insufficient β cell mass. The proliferation of pancreatic progenitor cells is important to expand a particular subtype of pancreatic cells before terminal differentiation. According to Jensen et al., rodent ngn3^{+} cells proliferate (Ki67-positive), whereas human ngn3 expressing cells do not (BrdU and Ki67-negative) (14, 15). Taken together, these studies suggest that the proliferation of pancreatic progenitor cells differs in small and large mammals. Since porcine pancreas development is more similar to human pancreatic development than rodents (16), ectopic over-expression of ngn3 in porcine pancreatic precursor cells may inhibit cell proliferation by a similar mechanism to that which occurs in human pancreas progenitors. The difference in species dependent progenitor proliferation may reflect the differences in timing of embryological development.

In Chapter 3, the effect of prolonged in vitro exposure to high glucose on neonatal porcine islet function, growth and survival was examined. It has been repeatedly demonstrated that adult β cells are particularly susceptible to prolonged exposure to high glucose both in vitro and in vivo. One advantage to using islets from young animals for transplantation was demonstrated by the lack of an effect of high glucose on cell death as measured by low rates of β cell apoptosis. This may be because of higher expression and activity levels of antioxidant enzymes such as glutathione peroxidase which has been shown to protect β cells from glucose toxicity and is down-regulated in islets from diabetic mice (17, 18).

Exposure to high glucose can enhance β cell maturation and differentiation from precursors in prenatal islets isolated from rodents (19), but not humans (20). In neonatal

porcine islet grafts exposed to hyperglycemia, β cell proliferation, neogenesis and maturation all contribute to the development of a metabolically adequate porcine β cell graft which is capable of reversing diabetes (6). To examine the effects of high glucose on neonatal porcine islet function and survival, we incubated neonatal porcine islets in high glucose (28.0 mmol/l) media for 1 week (Chapter 3). Interestingly, neonatal porcine β cells maintained in vitro glucose responsiveness and increased in proportion to the number of CK7-positive precursors after exposure to high glucose.

In contrast to human islets, programmed cell death was not initiated in the majority of neonatal porcine β cells (48% vs. 5% Tunel-positive) after prolonged exposure to high glucose. The isolation procedure likely causes non-specific cell death in many of the islets of any species. A recent study found pretransplant culture of islets greatly improves the transplantation outcome of islet grafts compared to freshly isolated islets, by allowing the 'unhealthy' and damaged islets to die in culture. Using islets isolated from adult pigs, 7-14 days culture resulted in diabetes reversal in all nude mice (13/13) compared to grafts of freshly isolated islets which reversed diabetes in only 1/18 mice. In the original Edmonton protocol, islets were not initially cultured in vitro, however, recent clinical islet programs have begun culturing islets for 48-72 hrs (21). In addition to the increased rate of diabetes reversal, culture also minimizes the time for normoglycemia to become established in recipients of either adult or neonatal islets. However, with the case of neonatal porcine islets, simply increasing the number of β cells does not fully result in a metabolically responsive graft since between 6-8 x 10^5 perinatal porcine β cells are incapable of immediately reversing diabetes in rodents (6, 22). Immature β cells undergo functional maturation which can be assessed by an increase in

insulin content and cell volume following transplantation. The use of high glucose culture may aid in partially enhancing the functional maturation of β cells prior to transplantation so that less time is required for the reversal of diabetes after implantation.

Another unique feature of high glucose induced β cell differentiation is the development of enhanced resistance mechanisms to protect against cell death caused by diabetogenic cytotoxins (Chapter 4). Recent evidence suggests environmental toxins activate autoimmune mechanisms in genetically susceptible individuals which can contribute to type 1 diabetes onset (Table 5-1). Diabetogenic toxins such as *N*-nitroso compounds are found in preserved meats, beer, regional drinking water supplies, and rodenticides which have means of entering the human food chain. A product of the streptomyces species, which produces streptozotocin, also produces bafilomycin and concanamycins, toxic macrolide antibiotics with many uses in cell biology (23). Bafilomycin is a highly specific inhibitor of vacuolar ATPase, but can also impair glucose tolerance (23). The diabetogenic effect of bafilomycin has also been demonstrated in the NOD mouse model of autoimmune diabetes in which there was an acceleration of diabetes onset (median age 21 weeks versus 29 weeks for controls) and an increase in diabetes incidence (100% vs. 65% in controls) (24). Interestingly, streptomyces species can infect tuberous vegetables such as potatoes and sugar beets and thus accumulate in the food chain. Indeed entry into the human diet has occurred as the rise in the use of processed potato products in developed countries correlates with the incidence of type 1 diabetes in recent decades (25).

The low expression and activity levels of antioxidant enzymes as well as the reliance on aerobic glycolysis for energy needs in β cells renders them susceptible to

cytotoxic injury (26). The reliance of β cells on aerobic glycolysis means NAD levels are low relative to other cell types and hence are susceptible to NAD depletion. DNA damage and strand breakage caused by alkylating agents such as streptozotocin, activate the DNA repair enzyme poly(ADP-ribose) polymerase (PARP) which utilizes NAD and critically depletes the β cell resulting in cell death. In addition, streptozotocin generates reactive oxygen species such as hydroxyl radicals which are crucial effectors in β cell damage (27). The ability of β cells, which develop from neonatal porcine precursors, to resist streptozotocin mediated cell death may have implications in the ability of these cells to survive cytotoxic injury caused by free radicals and hence function long-term after transplantation.

Table 5-1: Diabetogenic toxins

Toxin	Source	Species demonstrated	Comment
N-nitroso compounds	Food, water (nitrates)	Human/rodent	Damages DNA
Vacor	Rodenticide	Human	Inhibits mitochondria
Cycasin	Cycad plants	Human	Damages DNA
Streptozotocin	Streptomyces	Many including rodents	Damages DNA
Alloxan	Oxidised uric acid	Many including rodents	Generates free radicals
Bafilomycin	Streptomyces in vegetables	Mouse	Disturbs islet development

[Adapted from (25)].

V-2 SUMMARY

The endocrine pancreatic stem cell although difficult to identify in the adult is clearly identifiable in the neonatal islet. Genetic manipulation of these precursor cells can result in endocrine cell differentiation. Islet cell development neonatal porcine islet precursors appears similar to both rodent and human pancreas development. The

inability of $ngn3^+$ neonatal islet precursor cells to proliferate is similar to human $ngn3^+$ cells, while the ability of high glucose to induce β cell differentiation is similar to the rodent situation. These unique characteristics make porcine islets attractive for both basic islet research and potential clinical usage.

The results of these studies may also have important implications for clinical islet transplantation and provide further proof of principle evidence in support of the use of xenogeneic porcine islets. Fetal porcine islets which have been shown to resist the detrimental effects of high glucose (28), streptozotocin (29), and cytokines (30) showed signs of survival in type 1 diabetic recipients on maintenance immunosuppression for a kidney transplant (31). Similarly, neonatal porcine islets, which are advantageous over fetal porcine islets because they are glucose responsive, have also been shown to resist the detrimental effects of high glucose (Chapter 3), streptozotocin (Chapter 4) and cytokines (J. Torsen, MSc thesis, University of Alberta, 2001); indeed these islets can survive and function for prolonged periods in diabetic non-human primates immunosuppressed with a costimulation blockade-based regimen (3).

Neonatal porcine islets possess excellent growth and functional capabilities and are thus under consideration as an alternative source of insulin-producing cells for clinical islet transplantation. Neonatal porcine islet xenografts may survive better than adult grafts in the immediate posttransplant environment and function long-term. Their ability to do so will be as beneficial to the field of islet transplantation as them providing an unlimited supply of insulin-producing cells.

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