University of Alberta

CHARACTERIZATION OF NEONATAL PORCINE ISLETS AS A TISSUE SOURCE FOR ISLET TRANSPLANTATION

by

Tanya Michelle Binette

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of the requirements for the degree of Doctor of Philosophy

in Experimental Surgery

Department of Surgery

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This text is dedicated to the memory of my Baba, Pauline Stechishin, who always supported me, no matter how crazy she thought my pursuits were.

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LIST OF ABBREVIATIONS

ABC	avidin-biotin complex
AdPdx-1	adenovirus containing mouse Pdx-1
ANOVA	analysis of variance
BTC	betacellulin
CK	cytokeratins
COII	cytochrome oxidase II
DAB	3,3-diaminobenzidinetetrahydrochloride
DCCT	Diabetes Control and Complications Trial
EC	encapsulated
ES	embryonic stem (cells)
FACS	fluorescence activated cell sorter
FBS	fetal bovine serum
FCS	fetal calf serum
FDA	US Food and Drug Administration
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FITC	fluorescein isothiocyanate
Galα(1,3)Gal	galactose-α(1,3)-galactose
GDM	gestational diabetes mellitus
GFP	green fluorescent protein
GLP	glucagon-like peptide
HBSS	Hank's balanced salt solution

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HGF/SF	Hepatocyte growth factor/scatter factor
IAPP	islet amyloid polypeptide
ICC	islet-like cell cluster
IDDM	insulin-dependent diabetes mellitus
IDX-1	islet/duodenum homeobox-1
IHH	indian hedgehog
i.p.	intraperitoneal
IPF-1	insulin promoter factor-1
IUF-1	insulin upstream factor-1
k.c.	kidney capsule
kD	kilo Daltons
MODY	maturity-onset diabetes of the young
NHP	non-human primate
NIDDM	non-insulin-dependent diabetes mellitus
NGN3	neurogenin 3
Non-EC	non-encapsulated
NPI	neonatal porcine islets
OGTT	oral glucose tolerance test
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC1/3	Prohormone convertase 1/3
PC2	Prohormone convertase 2
PCR	polymerase chain reaction

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PDX-1	pancreatic and duodenal homeobox gene-1
PERV	porcine endogenous retrovirus
RIA	radioimmunoassay
RT-PCR	reverse transcription polymerase chain reaction
SDS	sodium dodecyl sulfate
SEM	standard error of mean
SHH	sonic hedgehog
SI	stimulation index
STF-1	somatostatin transactivating factor-1
Stz	Streptozotocin
XNA	xenoreactive natural antibodies

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CHAPTER 1

GENERAL INTRODUCTION

1.1 THE PANCREAS

1.1.1 Development

The pancreas develops from a dorsal and a ventral bud of the gut endoderm; each develop both endocrine and exocrine tissue independently, and meet upon rotation of the duodenum, allowing the two buds to fuse and form the entire organ (1). The pancreas is composed of distinct tissue types with specific roles: the exocrine cells secrete digestive enzymes, the ductal cells form ducts through which digestive enzymes and bicarbonate buffer are transported to the intestine, and the endocrine cells make endocrine hormones to regulate glucose homeostasis which are secreted directly into the bloodstream. The endocrine cells make up micro-organs termed the Islets of Langerhans within the pancreas. There are four major types of endocrine cells: insulin-producing β-cells, glucagon-producing α-cells, somatostatin-producing δ-cells, and pancreatic polypeptide-producing PP-cells. During embryogenesis, all pancreatic cells differentiate from the endodermal bud through a process called "branching morphogenesis" of ductal structures labeled by cytokeratins (CK) (2;3). Studies involving lineage tracing have indicated that exocrine, endocrine (4) and ductal (5) cells are derived from a common PDX-1 expressing precursor. It is thought the endocrine precursors then make up a sub-population within the ducts, which express NGN3 (5). Exocrine cells lose the PDX-1 phenotype, beginning to express p48, and finally amylase (6). Maintenance of PDX-1 and subsequent expression of NGN3 and later NEUROD define the endocrine cells (6).

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It has been observed during development, that some endocrine cells immunostain double-positive for two hormones and progressively each cell is restricted to express only one (7-10). However, Herrera recently designed experiments that clearly indicate that β-cells do not activate the glucagon promoter at all during embryonic development and α-cells similarly do not turn on the insulin promoter (11). He suggests, as does Jensen (6), that dual hormone expression is not involved in islet cell development. Both α- and β-cells begin to express PAX6, shortly after which α-cells begin to produce glucagon and stop expressing PDX-1. β-cells continue to express PDX-1 and PAX6 while also expressing PAX4 and finally insulin. Somatostatin-producing δ-cells continue to express PDX-1 (6). The individual roles of microenvironment, growth factors, and transcription factors on pancreatic development are discussed in further detail below.

1.1.1.1 Microenvironment

Signaling between tissues in embryonic development determine organ differentiation, spatially and temporally. The Hedgehog family of inducing factors was first identified in Drosophila as a mutation of the gene which resulted in the larvae looking like hedgehogs (12). The Sonic hedgehog protein (SHH) is provided by the notochord, and induces intracellular patterning.

It was observed that direct contact between the notochord and the dorsal endoderm resulted in a commitment to formation of the pancreas (13;14). SHH expression was absent in the pancreatic epithelium, and this was attributed to the notochord – endoderm interaction repressing Shh expression in the endoderm while in contact (15;16). Hebrok et al. identified activin-βB as the signal released from the notochord which represses Shh (16). At the time of dorsal aorta fusion, the notochord is

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separated from the endoderm, and SHH expression is observed near the pancreas, however not in regions where pancreatic genes are expressed (17). A clear boundary is observed separating the SHH expressing stomach and duodenum from the SHH-negative pancreas (15;16). This pattern remains throughout the development of the pancreas (18;19).

Isolated chick endodermal epithelium, with no notochord contact and thus no repression of SHH, had no PDX-1 or insulin expression. If SHH was inhibited by either subsequent co-culture with notochord or the addition of an anti-SHH antibody, PDX-1 and insulin expression were restored (16). Apelqvist et al. created a transgenic mouse which expressed Shh behind the Pdx-1 promoter so that SHH would be expressed in the pancreatic epithelium. A change in pancreatic morphology resulted, with pancreatic mesoderm developing into smooth muscle. Insulin and glucagon-positive cells were present, however no islets had formed. Two intestine-like appendages and the duodenum appeared where the pancreas developed in normal mice (15). In a Shh knockout mouse, the mass of the pancreas is the same as that of a wild type littermate, however the mutant embryonic development is severely compromised, and other organs of the intestinal tract are reduced in size (18). SHH is expressed in the endoderm outside of the pancreas, perhaps defining and limiting the pancreas to within a certain area.

Gittes et al. illustrated that, by dissecting epithelium away from its surrounding mesenchyme, islets may form with no other stimulus, however basement membrane containing laminin-1 must be supplied for ductal structure development, and mesenchyme is required for the development of the acinar pancreas (20;21). It would also appear that laminin-containing basement membrane is required at a specific time for

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duct formation (22), indicating temporal sensitivity for various signals or tissue interactions.

Another interaction with a neighboring tissue affecting pancreatic development, is that seen with the endothelium. Lammert et al. observed that pancreatic growth is initiated at points where the endodermal epithelium comes in contact with the endothelium of major blood vessels (23). PDX-1 and insulin expression were also regionalized to where the pancreatic endoderm contacted blood vessels during development. It had previously been shown that islets express vascular endothelial growth factor (VEGF), and that pancreatic endothelial cells have VEGF receptors (24). Experimentally, overexpression of VEGF in islet cells resulted in a hypervascularized pancreas as well as islet hyperplasia and a decrease in acinar area, indicating a role of this interaction in proper pancreatic development (23). In addition, by dissecting the aorta out of Xenopus, embryos indicated no expression of endocrine pancreatic genes although the notochord was present (23). These results indicate, that in addition to signaling between the mesoderm, ectoderm, and notochord, endothelial cells also play a role in pancreatic development.

1.1.1.2 Growth Factors

Many growth factors have been associated with islet development. Below several growth factors are described, however this field is relatively unexplored, and this list will likely expand dramatically in the future as understanding of pancreatic development increases.

Growth hormone (GH), prolactin (PRL), and placental lactogen (PL) have all been shown to stimulate insulin release and β-cell proliferation in rat, mouse, and human

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islets (25-28). PRL has also been shown to increase glucose sensing in neonatal rat islets (29). Use of porcine GH, PRL, and PL in culture with NPIs, however, has no effect (unpublished observations and (30)).

Hepatocyte growth factor/scatter factor (HGF-SF) is expressed in mesenchymal cells which are in contact with the epithelium of developing organs (31). HGF/SF has been shown to increase β-cell proliferation in fetal and adult human islets (32-34). Lefebvre et al. observed proliferation of ductal cells, but not β-cells when isolated adult human islets were cultured with HGF/SF in combination with an extracellular matrix (35). Lopez-Avalos et al. did not observe any proliferation of NPI when culturing with HGF/SF and extracellular matrix, however human recombinant HGF/SF was used, and it was hypothesized that there may be species differences in growth factors (30).

Betacellulin (BTC) is a member of the epidermal growth factor (EGF) family. It was identified originally in a mouse pancreatic tumor (36), and has since been shown to be expressed in the normal human small intestine and pancreas (37). BTC, in combination with another growth factor, Activin A, has been shown to convert an exocrine cell line (rat AR42J cells) into insulin-secreting cells (38;39), indicating that BTC may be important in the differentiation of non-endocrine cells into β-cells. BTC has been shown to increase β-cell volume both in mice with alloxan-induced diabetes (40) and with Activin A in neonatal mice following streptozotocin treatment (41). Using a 90% pancreatectomy model in rats to induce pancreas regeneration, Li et al. showed that BTC administration increased β-cell mass and plasma insulin levels, as well as proliferating β-cells (42). Activin A has been detected in fetal rat pancreas (43), and through the above studies has been implicated in endocrine cell development.

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Fibroblast growth factors (FGF) and their receptors (FGFR) have been shown to be expressed in mouse pancreas, and FGF signaling shown to be involved in pancreas development (44). Interference with FGFR1c in mice resulted in diabetes which was attributed to low levels of glucose transporter 2 (Glut2) as well as deficiencies in prohormone convertases 1/3 and 2, which resulted in high levels of unprocessed proinsulin (44). FGF10, a ligand for FGFR2b, is secreted by the mesenchyme transiently during mouse embryonic development from embryonic day (e) 10.5 to e11.5 (45). In FGF10 knockout mice, the early dorsal and ventral buds of the pancreas are seen, but there is a reduction in Pdx-1-positive epithelial progenitor cells resulting in arrested branching morphogenesis of the pancreas (45). Bhushan et al. hypothesize from their experiments that the role of FGF10 in development is to control the population of pancreatic epithelial progenitor cells (45). This observation has been confirmed in two studies which overexpress FGF10 in this population of cells, behind the Pdx-1 promoter (46;47). In both studies, overexpression of FGF10 resulted in hyperplasia of the pancreas due to an increase in the population of pancreatic epithelial cells. Notch signaling was activated in the presence of FGF10, impairing neurogenin 3 (ngn3) expression in the epithelium (46;47). FGF10 appears to be responsible for maintenance of a pancreatic progenitor state, blocking differentiation.

1.1.1.3 Transcription Factor Involvement

Many transcription factors have been implicated in pancreatic and islet development. These transcription factors are involved in cascades of signaling. Timing of signaling in pancreatic and islet development has been determined using knockout and overexpression studies. Although these models of development do reveal information as

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to the role of various factors, they do not always reflect natural development due to potential interference with patterning signals. Lineage tracing has become a valuable tool when attempting to define progenitor cells, and the fate of specific cell types, however "leakiness" of cre recombinase techniques used in the system of tracing has been suggested (48), indicating that caution must be taken when drawing broad conclusions from these studies. Below, several signals involved in pancreatic and islet development are discussed, however this is not an exhaustive review, only an outline of the more common and well-known factors at the time of writing which are of relevance to this manuscript.

1.1.1.3.1 Homeodomain Factors

A homeodomain factor is characterized as an approximately 60 amino acid protein, containing mainly basic residues and a helix-turn-helix DNA binding site. They are encoded by a homeobox, which is so named due to its identification in homeotic genes, which are genes specifying what body part develops, and where within the organism it develops. Although not all homeodomain transcription factors are homeotic, many do have a role in development (12). Several well characterized homeodomain transcription factors have a role in pancreas and islet development, including PDX, HB9, NKX2.2 and NKX6.1.

1.1.1.3.1.1 PDX-1

In 1989, Wright et al. reported a homeobox in Xenopus, XlHbox8, which was expressed in the endoderm and persisted in epithelial cells of the pancreas and duodenum of mature frogs (49). The mammalian homologue was isolated independently by several

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laboratories (50-52). This novel transcription factor was given several names: insulin promoter factor 1 (IPF1) (50), somatostatin transactivating factor-1 (STF-1) (51), islet/duodenum homeobox-1 (IDX-1) (52), insulin upstream factor-1 (IUF-1) (53), but was renamed pancreatic and duodenal homeobox gene-1 (PDX-1) by the International Committee of Standardized Genetic Nomenclature for Mice reflecting its expression in both the pancreas and duodenum (54). PDX-1 functions in the regionalization of the gut endoderm, forming the beginnings of the pancreas, and in β-cell maturation. Homozygous pdx-$1^{-/-}$ mice are born without a pancreas and do not survive postnatally (54-56). Stoffers et al. described a clinical case of a female born without a pancreas (57). A frame shift mutation was located in the pdx-1 gene, resulting in the production of a truncated, non-functional, protein (16 kD instead of 42-43 kD). A less severe pdx-1 mutation is associated with one form of Maturity Onset Diabetes of the Young (MODY) (58).

PDX-1 is first detected at embryonic day 8.5 in the mouse endoderm of the dorsal region of the gut, later becoming widespread in endoderm of the developing duodenum and pancreas (59). By embryonic day 11.5, many cells adjacent to the pancreatic duct are PDX-1-positive, extending to most of the ductal epithelium two days later (59). When islets begin to form, around embryonic day 16.5, less PDX-1 is observed in the ducts, and the majority of PDX-1 expressing cells are in the islets. Peshavaria et al. detected PDX-1 only in β- and δ-cells of the adult pancreas (60).

When pancreatic formation in the pdx-1 null knockout mouse is examined, the buds of the developing pancreas begin to form, and a small, irregularly formed ductal tree is observed (54;56). Some early insulin and glucagon containing cells are observed (56),

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indicating that PDX-1 is not required for initiation of pancreatic development, however it is required for pancreatic commitment (50;54;56;59). Ahlgren et al. used a cre/lox system to turn Pdx-1 off in mature β-cells specifically (61). The number of islets remained the same in these animals, however the number of β-cells was reduced by 40%, suggesting that PDX-1 is critical for maintaining β-cell mass (61). PDX-1 has been shown to bind to the promoters of several endocrine and β-cell specific genes: somatostatin, insulin, Glut2, IAPP, and glucokinase (50;51;62-65), explaining the need for PDX-1 to maintain β-cells. Overexpression and ectopic expression of PDX-1 have been proposed as a means of creating expanded pancreatic tissue, this will be discussed further in Chapter 4 of this manuscript.

1.1.1.3.1.2 HB9

HB9 is coded by a homeobox gene, HLXB9, and has a role early in the dorsal and ventral pancreatic bud development from embryonic day 8 in the mouse. It is not detected between embryonic days 12.5 and 17.5, after which its expression is restricted to β-cells (66;67). In a HB9 null mutant mouse, the dorsal lobe of the pancreas does not develop, however the ventral bud is present but contains small islets with an unorganized morphology and 65% fewer β-cells than in the normal mouse (66;67). Thus, HB9 has a role in pancreatic development, to initiate dorsal bud formation, and then later in β-cell differentiation.

1.1.1.3.1.3 NKX 2.2

NKX2.2 appears to have multiple roles in islet development. It is first detected in the pancreatic buds at embryonic day 9.5 in the majority of the endothelial cells (68). By

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embryonic day 12.5, its expression becomes restricted, and NKX2.2 is seen in a population of endocrine precursor cells also expressing NGN3 (68). Finally, NKX2.2 is expressed late in development in all islet cells except δ-cells. Nkx2.2$^{-/-}$ mice are lacking β-cells, and have fewer α- and PP-cells than usual (69), likely due to a disruption of the precursor population which would normally express NKX2.2. Homozygous knockouts survive gestation, but die shortly after birth due to hyperglycemia (69). Replacing the β-cell population are cells expressing normal levels of the β-cell markers islet amyloid polypeptide (IAPP) and prohormone convertase 1/3, but not glucose transporter 2 (Glut 2) or glucokinase. It would appear from this observation that NKX2.2 is necessary for the final differentiation of β-cells (68;69). Recently, this population of incompletely differentiated β-cells has been further described to contain ghrelin, an appetite-promoting peptide which is normally found in the stomach (70). A small number of ghrelin-positive cells were found in the normal mouse pancreas and, thus, were defined as a fifth type of islet cell, the epsilon cell (70).

1.1.1.3.1.4 NKX6.1

Similar to NKX2.2, NKX6.1 is detected at three time points during pancreatic and islet development. It is first seen at embryonic day 10.5, expressed in most pancreatic epithelial cells. At embryonic day 12.5, NKX6.1 is co-localized with both NGN3 and PDX-1. NKX6.1 may have a role in these precursor cells, perhaps in either proliferation, or differentiation (71). Finally, NKX6.1 is expressed in only β-cells in the adult pancreas (71-74). NKX6.1 homozygous null mutant mice embryos are comparable to wild type embryos before embryonic day 12.5, both have some insulin-positive cells (71). However, by embryonic day 13, mutant embryos do not have the exponential increase of

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insulin-positive cells that the wild type embryos do. Almost no β-cells are detected at embryonic day 18.5 in the mutants. It is hypothesized that NKX6.1 has a role in β-cell precursor proliferation, as this lack of β-cells is not replaced by undifferentiated β-cells as seen in the NKX2.2$^{-/-}$ mice (69), or by an expansion of another population as seen in PAX4$^{-/-}$ mice (75), nor is it explained by an increase in apoptosis (71). The few β-cells that are present have the β-cell markers IAPP, PC1/3 and 2, PDX-1, and NKX2.2 (71).

NKX2.2 / NKX6.1 double knockout mutants were studied, and phenotypically these were identical to the NKX2.2 knockout mice (71;75). This, along with the fact that NKX6.1 is not expressed in NKX2.2$^{-/-}$ mutants, led to the conclusion that in β-cell development, NKX6.1 is downstream of NKX2.2 (71).

1.1.1.3.2 Pax protein factors

The Pax proteins contain a 6 α-helical DNA-binding domain which is also known as a paired domain. Often they also contain a homeobox domain. These factors are named for their discovery in Drosophila: the paired protein (12). Two Pax genes, Pax 4 and Pax 6, are expressed early in pancreas development (76). Double knockout mice lacking both Pax 6 and Pax 4 do not develop any mature endocrine cells, however the exocrine pancreas remains unaffected and some cells expressing Pdx-1 are observed (77).

1.1.1.3.2.1 PAX 6

Pax 6 is expressed in the early pancreas, at embryonic day 9.5 (78), in endocrine cells only. In early pancreatic development, Pax 6 is expressed in glucagon-positive cells predominently, and in later stages it is seen in all four endocrine cell types (77;78). Pax 6 binds to the insulin, glucagon, and somatostatin promoters to initiate gene transcription

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(78). Mutant $Pax6^{-/-}$ mice have reduced numbers of all endocrine cells, but particularly α-cells. Unlike the NKX2.2 mutant mice, there was no observed population of partially differentiated cells, which may indicate that Pax6 has a role in early pancreatic gene expression, thus cells do not differentiate at all in the mutant pancreas at a point where PAX6 is required. Islets from $Pax6^{-/-}$ mice were disorganized, that is they did not have the usual rodent islet structure with the β-cells in the core and α-cells on the outside of the islet (77;78).

1.1.1.3.2.2 PAX 4

Pax 4 gene expression is first noted at embryonic day 9.5 in the developing mouse pancreas. In Pax $4^{-/-}$ mice, there are no differentiated β- or δ-cells, but α-cells are present at much higher levels than usual (75). The pancreas of the mutant animals appeared normal, however due to the lack of β-cells, these mice died shortly after birth, likely due to diabetes. PDX-1 positive cells are present in the early pancreas of these mice, as are early glucagon and insulin-positive cells, thus these do not have a requirement for Pax 4 binding for initial pancreatic development. Pax 4 is proposed to have a role suppressing differentiation of α-cells while allowing β-cells to differentiate, thus when not present to suppress, the default differentiation of early endocrine cells is towards an α-cell fate (79). Recent studies have confirmed that Pax 4 is involved in transcriptional repression and that it binds to the glucagon, insulin, and IAPP promoters (80;81). Pax 4 inhibits Pax 6 mediated insulin gene transcription in non-endocrine and α-cells via competition or protein-protein interactions. Pax 6 and Pax 4 are co-expressed in β-cells until birth, at which point Pax 4 expression is stopped (79), likely removing the repressive effect on Pax 6 insulin gene binding (80).

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1.1.1.3.3 Basic helix-loop-helix factors (bHLH)

These transcription factors are characterized by their formation of heterodimers of a basic DNA-binding region and a hydrophobic helix-loop-helix region. One of the members of the dimer is ubiquitous in all tissues of the organism, and the other portion is tissue specific. Repressor proteins may be present which form inactive dimers (12).

1.1.1.3.3.1 Neurogenin 3 (NGN3)

Ngn3 is expressed transiently in early pancreatic cells, but not in differentiated endocrine cells. It is detected in the mouse at embryonic day 9.5, is most prevalent at embryonic day 15.5, and is not detectable in the neonatal or adult pancreas (6;82-84). Gu et al. showed using lineage tracing, that ngn3 cells are endocrine precursor cells for all four islet endocrine cell types (5). NGN3-positive cells are able to proliferate as indicated by double-staining for Ki-67 (6). PDX-1 / NGN3 double-positive cells were detected by immunostaining, however NGN3 has not been seen to co-localize with any endocrine hormones, indicating it is a signal for early development, prior to initiation of hormone transcription (6;83). In ngn3 knockout mice, no endocrine cells develop, and the mice die shortly after birth due to hyperglycemia (83). The gross morphology of the pancreas observed in the neonatal ngn3$^{-/-}$ mice seems normal. Cells expressing PDX-1, NKX2.2, and NKX6.1 were observed early in the mutant pancreas (embryonic day 10.5), but no NeuroD, Pax4, or Pax 6 was detected which places ngn3 temporally between these signals in pancreas development (83). PDX-1 is observed in the early cells of the pancreas epithelium which become the exocrine, ductal, and endocrine cells. Ngn3 appears to be the next major "switch" dictating endocrine fate to the PDX-1 pancreatic precursor population, and, thus, indicates the endocrine precursor population.

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1.1.1.3.3.2 BETA2 / NeuroD (NeuroD)

NEUROD forms a heterodimer with E47 (the ubiquitous bHLH factor), forming a complex which initiates insulin gene transcription (85-88). NEUROD is detected in islet endocrine cells, the intestine, and the brain. It acts in the brain to cause neurons to differentiate (89), and may have a similar role in islets. Unlike NGN3, NEUROD does not co-localize with Ki-67, indicating that NEUROD cells are not proliferating (6). NEUROD is seen as early as embryonic day 9.5 in the mouse (6), and may be activated by NGN3 as NGN3 overexpression studies have resulted in ectopic NEUROD expression (90).

In the NEUROD knockout mouse, islet cell differentiation is achieved, however numbers of β-, α-, and δ-cells are decreased by 75%, 40%, and 20%, respectively (91). In addition, increased apoptosis of islet cells was observed, indicating that NEUROD's role in islet cell differentiation may have more to do with maintenance of cells, which in its absence results in deletion of differentiated cells. The exocrine portion of the pancreas is also affected in mutant mice due to a loss of cell polarity (91).

1.1.2 Islet cell turnover and pancreatic stem cells

There is evidence of endocrine cell population turnover in the mature pancreas. Finegood et al. developed a mathematical model which proposed that there is a balance between β-cell division, growth and death, and estimated the dynamics of this process (92). It was determined that an average β-cell life span was between one and three months, which would indicate dynamic cell turnover throughout life. Also observed was the ability of the mature pancreas to regenerate after damage induced by partial pancreatectomy (42), chemical destruction of islets with streptozotocin or alloxan

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(40;93), exposure to periods of hyperglycemia (94;95), cellophane wrapping of the head of the pancreas (96), or ductal ligation (97). This would indicate the existence of a pancreatic precursor cell. The identity of this putative precursor has not been determined. Evidence of regeneration warrants working with the remaining pancreatic tissue post-islet isolation, such as the ductal or exocrine tissue, as well as with expansion of both adult and immature islets themselves.

A recent report indicated that adult β-cells are formed through proliferation, or self-duplication, rather than from a pancreatic precursor cell (98). Transgenic mice were used to label β-cells with a tamoxifen-inducible Cre/lox system to create a pulse, thus labeling β-cells at that time point only. It was found that, up to 1 year later, the same percentage of β-cells were labeled as had been at the pulse time point. Because the labeled population had not been diluted by unlabeled β-cells, it was concluded that β-cells are derived from the replication of pre-existing β-cells (98). Although this data is convincing, it does not preclude the existence of a pancreatic precursor cell. Inconsistent numbers of β-cells were labeled using their system, and much of the conclusion drawn was based on only 30% of cells being labeled. Because their system used the insulin promoter to express their tag, any precursor cells which express insulin would also be labeled as β-cells, thus mistaking differentiating cells for proliferating cells.

1.2 DIABETES MELLITUS

Diabetes Mellitus is a disorder where the body cannot utilize glucose due to inadequate production of insulin or inability to use insulin properly, resulting in hyperglycemia, or high blood glucose (99). Currently there are two million people in Canada with diabetes, and this number is expected to rise to three million by the year

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2010 (100). Of these, approximately 10% have type 1 diabetes, formerly called juvenile or insulin-dependent diabetes. In type 1 diabetes, the insulin-producing β-cells in the islets of Langerhans are destroyed by an autoimmune reaction (101). The cause of this attack is not precisely known, however there is evidence indicating that genetic predisposition (102;103) plays a role, however in identical twins, there is only a 40% concordance rate for type 1 diabetes, indicating that factors other than genetics do play a role in disease manifestation (104). Environmental factors, such as diet or viruses, may be triggers of diabetes (105-107). Type 2 diabetes is characterized by inadequate insulin secretion, or by insulin resistance whereby the body cannot use the insulin produced (108). A third type of diabetes, gestational diabetes mellitus (GDM) occurs in 2 to 4% of pregnancies. GDM is due to insulin resistance caused by gestational hormones, and the inability of the body to increase insulin production to counter this (109). Symptoms of GDM disappear after the birth of the child, however its occurrence may indicate a risk of the mother developing diabetes (usually type 2, but rarely type 1). Maturity onset diabetes of the young (MODY) is classified as a form of type 2 diabetes. Several genetic defects have been identified which result in MODY. This chapter will focus discussion on type 1 diabetes.

In 1993, the Diabetes Control and Complications Trial (DCCT) indicated that fluctuations in blood glucose were responsible for the long-term complications of diabetes, and that this could be overcome by intensive insulin therapy in order to maintain blood glucose concentrations close to the normal range (110). The long-term complications are a result of glucose-based substances building up on small blood vessel walls (99) as a result of the transient periods of hyperglycemia (99). This glucose build-

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up causes circulation problems and can manifest into complications such as kidney failure, heart failure, blindness, and necessary amputation of limbs.

1.3 TREATMENTS FOR DIABETES

1.3.1 Pre-Insulin Discovery

"I've never seen a living creature as thin as he was, except pictures of victims of famine or concentration camps" (111). This quote was reported to Michael Bliss in 1980 in a personal interview with Stella Clutton, who 59 years before was a secretary at the diabetic clinic at the Toronto General Hospital, referring to a young boy who was brought in after being treated for his diabetes by following a diet of 450 calories a day (111). Prior to the discovery and purification of insulin, the only known treatment for type 1 diabetes was essentially a starvation diet, only allowing carbohydrate to be consumed until glucose appeared in the urine.

1.3.2 Exogenous Insulin

In 1922, at the University of Toronto, insulin was discovered (111). A teenager named Leonard Thompson was the first recipient of an insulin injection, and in 1923 insulin became commercially and widely available (112). Bovine or porcine insulin was extracted and used for treatment, until 1963 when insulin became the first protein to be chemically synthesized (112). Animal insulin caused some minor adverse reactions post-injection (skin rashes). In 1978, human insulin was manufactured and large scale production began (112). Subcutaneous insulin injection became the conventional treatment for type 1 diabetes, and largely prevented the metabolic complications of the disease, such as diabetic ketoacidosis and resulting coma.

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1.3.3 Long-acting insulin and insulin pumps

Recent developments in insulin production and insulin pumps have greatly improved the lives of people afflicted with diabetes. Even with combinations of long and short-acting insulin, tight blood glucose regulation requires planning ahead and allotting for meals and exercise. An insulin pump allows the user the flexibility of injecting fractions of insulin units at a time, allowing for very fine control of blood glucose, however the need to test blood glucose levels, approximately six times a day for someone with type 1 diabetes, does not change. Developments in high technology fields, such as nanotechnology, keep the hopes for a "closed-loop system" high, that is an insulin pump-type device that also constantly monitors blood glucose levels, and responds immediately to correct them. In the near future, it is not unreasonable to hope for a semi-manual "closed-loop system" which will sense the blood glucose of the user, sound an alarm when specified levels are reached, and prompt the user to correct by pressing a button to administer the correct amount of insulin. Although this type of technology will prove useful to the majority of the population affected by diabetes, brittle diabetes will likely still be very hard to treat.

Subcutaneous insulin injection remains problematic for very tight regulation of blood glucose, as the insulin goes into the systemic circulation, rather then into the portal vein as occurs physiologically. This is relevant as the liver extracts a significant amount of insulin normally, thus the liver will be exposed to less insulin, and peripheral tissues to more insulin than in a normal state (113). Transplantation of insulin-producing tissue, in a way that restores physiological balance is an alternate means of treating diabetes.

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1.3.4 Pancreas Transplantation

Transplanting insulin-producing tissue may replace the need for daily insulin injections. Although exogenous insulin therapy restores the regular lifestyle of the majority of individuals with type 1 diabetes, the search for a more permanent and controlled physiological method of restoring glucose homeostasis is on-going.

The first whole pancreas transplantation was in 1966, and normoglycemia was achieved for several weeks before the pancreas and the kidney it was transplanted with were both rejected (114), proving the principal of transplantation of insulin-producing tissue. During the 1970's, few pancreas transplants were attempted without much success (115). By 2001, the International Pancreas Transplant Registry (IPTR) reported a worldwide total of 18,909 pancreas transplants, with 1792 performed in the year of 2001 alone (116). The dramatic increase in whole pancreas transplantation may be attributed to improvement of the very complicated surgical techniques required. Many complications were encountered in early transplants due to the exocrine pancreas, and the need to drain the digestive enzymes. By 1982, drainage into the bladder was considered safe, thus allowing for more pancreas transplants (117). Another reason for the increase in transplant success was the introduction of the immunosuppressant cyclosporine in the 1980's, and the advent of new immunosuppressant drugs has further improved pancreas transplant outcomes. A third reason for increased numbers of transplants coincides with the establishment of the United Network for Organ Sharing (UNOS) which regulated organ procurement and applications for transplants in the U.S.A. (118). Primarily, pancreas transplantation is performed on uremic diabetic recipients of kidneys due to the fact that immunosuppression is required for the kidney transplant anyway. There is some

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indication that a pancreas transplant in later stages of diabetes can have a positive effect on neuropathy and nephropathy (119). Despite the success of pancreas transplantation, it remains a complicated surgical procedure which is associated with morbidity and some mortality (120). Due to the surgery and complications associated with chronic immunosuppression, pancreas transplantation is a practical option only when in conjunction with renal transplant.

1.3.5 Islet Transplantation

Although recipients of an islet allograft require chronic immunosuppression, the surgical procedure is much simpler than pancreas transplantation, thus making islet transplantation a more attractive means of replacing insulin-producing tissue.

1.3.5.1 History of Islet Transplantation

The Islets of Langerhans, so named by Paul Langerhans who identified these "island" structures within the pancreas described in his thesis (121), make up only 2% of the pancreas (122). Minkowski showed, in 1892, that transplanting fragments of autologous pancreas back into a pancreatectomized dog could reverse diabetes (123). Sobolev, in 1902, suggested the separation of the islets from the exocrine pancreas for transplantation, although this idea was not followed up (124). Once insulin had been isolated, much work went into improvement of exogenous insulin, and the attention was drawn away from islet transplantation until 1965, when Moskalewski first isolated islets using the enzyme collagenase to digest chopped up guinea pig pancreas (125). Two years later, Lacy and Kostianovsky added intraductal distension to the islet isolation, which disrupted exocrine tissue and made the subsequent mechanical and enzymatic

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digestion more efficient. This was followed by the use of sucrose gradient centrifugation to separate islets from exocrine remnants (126). Ficoll later replace sucrose for density gradient separation as it provided a better osmotic environment for the islets (127;128).

Once islets were effectively being isolated, progress in experimental islet transplantation followed. In 1970, Younoszai et al. transplanted islets into diabetic rats (chemically induced) and achieved normoglycemia temporarily (129). Ballinger and Lacy had the first successful experimental islet transplantation results after they had transplanted 400 to 600 islets either intraperitonially (i.p.) or into the thigh muscle of rats (130). In 1973, Kemp et al. demonstrated the efficacy of the liver as a site of islet transplantation. In the i.p. site, 800 islets had only partially normalized blood glucose previously, however 800 islets were transplanted into the liver via the portal vein and diabetes was cured (131).

Success in the small animal indicated the need for trial in large animals before islet transplantation could be initiated in the clinic. Techniques used to isolate rodent islets did not work with a more fibrous pancreas as seen in larger animals, particularly in humans. Mirkovitch and Campiche were successful in isolating dog islets by removing the density gradient step of the rodent islet isolation protocol, as well as adding a requirement for the collagenase digestion step to take place at 37°C in a shaking water bath. Transplantation of their isolated islets, into the spleen, cured diabetes in 20 of the 25 dogs (132). Transplantation into the portal vein of the dog liver revealed some complications associated with less pure preparations (133;134), which disappeared when contaminating exocrine tissue was removed from the grafts (135;136).

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Knowledge gained from the large animal model was transferred to the clinic, and between 1974 and 1996, 305 adult islet allografts were performed worldwide, including at the University of Alberta (137). One week of insulin independence was seen in 33 out of the 305 recipients. Small numbers of recipients (13/305) stayed off insulin for more than one year, including one transplant that was successful for more than 5 years (138).

1.3.5.2 Current Success in Islet Transplantation

In 2000, years of islet transplantation research, led by Dr. Ray Rajotte and other persistent researchers, came to fruition. The use of a unique glucocorticoid-free immunosuppression regime, combined with improvements in islet isolation techniques and increased mass of transplanted islets, produced a 100% success rate in 7 patients who, post-transplantation, remained normoglycemic without exogenous insulin for a minimum of 1 year (139). In 2004, the number of islet recipients has increased to 300 worldwide with the implementation of multi-centre clinical trials (140;141). Varying success rates have resulted due to several experimental protocols (reviewed in (142)). One relevant improvement on the original protocol is the success of single-donor transplants, as opposed to the two or three donor pancreas requirement of the original protocol for a sufficient islet mass (142;143).

The most current update as to success of islet transplantation in Edmonton, reports a total of 54 procedures on 30 recipients (144). Detailed follow up of 15 subjects was included. On these, 12 were insulin independent at one year (80%). Some complications of the procedure and side effects of the immunosuppressive therapy were noted, however overall it was concluded that the procedure is a good option for people with severe problems maintaining normoglycemia.

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1.3.5.3 Limitations of Islet Transplantation

1.3.5.3.1 Immunosuppression

Recipients of islet transplants trade their daily requirement of exogenous insulin for a daily requirement for immunosuppressive drugs. These include sirolimus, tacrolimus, and daclizumab (139). Patients were also treated with antibiotics and antivirals to prophylactically prevent infections. No patients have developed malignancy or a life-threatening infection thus far, however several complications have manifested which are attributed to the immunosuppressive drugs. These commonly included recurring mouth ulcers, diarrhea, edema of the legs, anemia, and fatigue. Rare, but more serious complaints included temporary neutropenia, skin infection, urinary tract infection, and interstitial pneumonitis, some cases requiring discontinuation of the immunosuppressive drugs (145;146). In this context, islet transplantation is a valuable treatment for type 1 diabetes only when the negative effects of immunosuppression outweigh the risks associated with exogenous insulin maintenance of diabetes.

Strategies to overcome the need for immunosuppressive therapy for islet transplantation include induction of tolerance (145) (reviewed in (147;148), co-transplantation of islets with sertoli cells (149-153), and immunoisolation techniques (154-156) (reviewed in (157)). Until immunosuppressive drugs can be eliminated, islet transplantation will not be an appropriate treatment for the population it could help most: children or adolescents, where immediate tight regulation of blood glucose fluctuations will prevent secondary complications of diabetes (110)

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1.3.5.3.2 Tissue Supply

Recipients of a successful islet transplant required 10,000 islet equivalents per kilogram body weight, which often required a minimum of two donor pancreases (139;158). Obtaining enough cadaveric organs to satisfy the need for transplant requirements is a common theme in transplantation, and islet transplantation is no exception, in fact the inadequate tissue supply is further amplified by the need for multiple pancreases to isolate enough islets for one recipient. Many tissue sources are under investigation to solve this problem, including induction of embryonic or adult stem cells to differentiate into islets, β-cell lines, transdifferentiation of other terminally differentiated cell types, and xenogeneic sources (reviewed in (159)). Before ES cells can realistically be manipulated into insulin-producing β-cells, a clearer understanding of the signals involved in β-cell development must be obtained. In addition, ES cell-derivatives are potentially tumorigenic, thus caution must be taken before clinical applications are a reality (160). The pancreas itself might contain adult stem cells, or precursor cells. Similarly to using ES cells, a clear understanding of development of β-cells will be required to push these cells towards a β-cell fate, however it may be that these cells have the correct machinery in place, and may need fewer signals to achieve this end. Transdifferentiation of closely related tissues have been proposed for obtaining an insulin-producing cell population, utilizing culture conditions or genetic manipulation to convert tissue types. Examples include manipulation of pancreatic ductal tissue (161;162), and hepatocytes (163-165). Xenogenic tissue sources for transplantation, cellular and whole organ, would truly solve the tissue supply problem and is discussed in detail below.

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1.4 XENOTRANSPLANTATION

Xenos is the Greek root for "foreign" or "strange" (166), thus xenotransplantation is the term for transplantation of foreign tissue, referring to tissue from other species. Having been attempted in the form of blood transfusions, skin transplantation, and whole organ transplants over at least the past two centuries, xenotransplantation is not a new concept (166). Increased understanding of the human immune system and the various checks and balances in place, which react to reject foreign tissues, brings researchers one step closer to being able to prevent these reactions, and allow engraftment of foreign tissues. Advances in immunosuppressive drugs and transgenic animal technology to create pigs which express human proteins, may contribute to accelerating the process. Xenozoonisis, or infections transmitted across species, remain a concern, and more research into modes of transmission and potential for infection will bring us one step closer to being able to safely use xenogenic organs to relieve the demand for transplantable tissue.

1.4.1 Xenogeneic Sources of Tissue

Several sources of insulin-producing tissue are being considered for xenotransplantation. Non-human primates (NHP) would seem a logical source for xenogeneic tissue, due to the genetic similarities to humans. Unfortunately, the NHPs that are most similar to humans in size are generally endangered species, and are much too expensive to breed specifically for xenotransplantation purposes. Other NHPs are simply too small to supply organs suitable for most human recipients, which would not be a problem for islet transplantation, but has diverted much attention of xenotransplantation researchers away from this source. Another major argument against

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using NHPs is the similarity between NHP pathogens and human pathogens, making the risk of cross-species infection too high (166). Porcine (167-171) and bovine (172) islets, as wells as fish-Brockman bodies (173;174) are the major candidates.

1.4.1.1 Porcine Tissue

Pigs are most likely to provide a solution to the organ supply problem. Pigs breed rapidly, have large litter sizes, can be housed in a pathogen-free environment, are inexpensive, and widely ethically acceptable as a source of food and other products. In addition, porcine organs are similar in size to human organs and have many morphological and physiological similarities to humans. Porcine insulin has been used safely as a treatment for type 1 diabetes, and differs from human insulin by only one amino acid. In addition, the set point of porcine glucose levels is similar to that in humans (discussed in (175)), which represents the ratio of glucokinase to hexokinase in β-cells, determining rate of insulin release in response to glucose (176).

1.4.1.1.1 Adult Porcine Islets

To prove the potential of adult porcine islets as a source of transplantable insulin-producing tissue, autotransplantation of isolated islets into pancreatectomized pigs has been attempted several times since 1974 (177), with only minimal success (178;179). Difficulties obtaining a pure enough islet preparation (179) as well as the fragility of adult porcine islets (171) were likely the main reasons for lack of transplant function.

Ricordi et al. published an isolation protocol for adult porcine islets based on rodent islet isolation protocols, yielding approximately 79,000 islets per pancreas (180). The same group, using techniques adapted from those used to isolated human islets (181),

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were able to isolate 690,000 islets per adult porcine pancreas, however after purifying this tissue, recovery dropped to 255,000 islets (171). Adult porcine islets have proved to be difficult to isolate due to the lack of a peri-islet capsule (182). This results in rapid dissociation of islets into single cells during pancreas digestion and subsequent loss of viability (180). Tissue culture of islets post-isolation may allow for alteration of immunogenicity, or simply pooling of tissue and purity checking, however loss of tissue during culture has been reported post-isolation of adult porcine islets (183).

Overall, inconsistencies in the number of islets isolated, the purity of the preparations, survival of islets in culture, and ability to respond to a glucose challenge make adult porcine islets a less desirable source of xenotransplantable tissue (171;183;184). These inconsistencies have been attributed to age (185), breed (182;186;187), and quality of organs (188).

1.4.1.1.2 Fetal Porcine Islets

Collagenase digestion of fetal porcine islets results in an abundant yield of viable islet-like cell clusters (ICCs) that can restore normoglycemia in diabetic nude mice two months post-transplantation (167). The cells isolated survive well in culture and the isolation protocol is much less labor intensive than that to isolate adult porcine islets (167). Transplantation of fetal porcine islets into patients with type 1 diabetes has been performed (168). Porcine C-peptide was detected in the urine of four out of ten recipients up to 400 days post-transplant, indicating that in these cases porcine tissue survived in a human (168). All patients remained healthy throughout the procedure and experienced no adverse effects.

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Fetal pancreatic β-cells isolated from rat (189-192), pig (167), and human (193) respond poorly to glucose, however this is rapidly converted to an adult-like pattern after birth (189;191), thus attention has been turned to neonatal tissue.

1.4.1.1.3 Neonatal Porcine Islets (NPIs)

The use of fetal vs. neonatal porcine islet donors is debatable, both sources having the capacity to grow and mature. With rapid restoration of normoglycemia in islet recipients being the ultimate goal of transplantation, the advantage of NPIs being more mature and glucose responsive will be great. The response of NPIs to glucose stimulation in vitro exceeds that of fetal porcine islets (167;194). In addition, the discussion of the effect of microenvironment on pancreas development above indicates that natural pancreatic development may be desirable for as long as possible before islet isolation and further manipulation.

Viable NPIs are successfully isolated in large numbers by culturing collagenase digested pancreas for 9 days (194). Transplantation of 2,000 NPIs can restore diabetic immunodeficient mice to a normoglycemic state within 6-8 weeks post-transplantation (194-197). Examination of the cellular insulin contents of the harvested grafted kidneys showed an increase in insulin content 5 – 20 times that of the day of transplant (198).

Unlike adult islets, NPIs are composed of only 40-45% endocrine cells and also contain about 50% ductal cells (194). It is this population of ductal cells, identified by cytokeratin 7 (CK7) immunoreactivity, (197;199) that is thought to contain precursor cells in the pancreas. This assumption is based on several observations. Our group, (200) and other researchers (196), have noted a dramatic decrease in the number of CK7 cells in grafts recovered from normalized diabetic mice. This is associated with an

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increase in the number of insulin-positive cells, indicating differentiation of the ductal precursor cells into ß-cells. When observing NPIs engrafted under the kidney capsule of immunocompromised mice one week post-transplant, insulin-positive cells are not organized into islets, but are found as single cells or clusters of cells, generally associated with ducts (194;200). The phenomena of islet endocrine precursor cells budding off the pancreatic ducts has previously been described (199) and has also been observed in transplanted NPIs. In NPIs matured in vivo (50 days post-transplant), the insulin-positive cells become organized into islets, and the proportion of CK7-positive cells has decreased (200). Once diabetes has been ameliorated in immunocompromised mice 5-8 weeks post-transplantation, NPI grafts are predominantly composed of insulin-positive cells (196;197;200), while few, if any, CK7-positive cells are observed at this time (196;200). This increase in ß-cell mass has been attributed to both proliferation of ß-cells as well as differentiation of precursor (ductal) cells into ß-cells (196;197).

Between 30,000 and 50,000 NPIs may be obtained from a single piglet pancreas (194;197;201), requiring between 8 and 10 piglets to provide enough tissue for transplantation to an adult human (500 mg insulin required per recipient, with each pancreas providing 50 mg of insulin or more (202)). Because litters often number 15 or more piglets, each litter would be able to supply enough tissue to transplant two (or more) humans (202).

Porcine tissue should theoretically mimic human pancreatic islet development, thus NPIs may be utilized as a model for studying human islet proliferation and neogenesis, in addition to use as a xenotransplantable tissue source. Knowledge gained through studies of islet developmental biology will prove invaluable whether starting

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with embryonic stem cells or adult precursor cells as sources of transplantable insulin-producing tissue.

1.4.2 Barriers to Xenotransplantation

Immunological complications of xenotransplantation, and especially the potential for cross-species transmission of known, or unknown, diseases, have led the US Food and Drug Administration (FDA) to develop a Xenotransplantation Action Plan which oversees any clinical xenotransplantation trials in the U.S.A. (these include any cells or tissue transplants directly from a nonhuman animal source, or which have been exposed ex vivo to any nonhuman animal cells, tissues or organs (203)). Researchers have proposed a moratorium on xenotransplantation which most countries have adhered to, and will continue to, until basic research has extensively addressed these concerns (204).

1.4.2.1 Immunological Barriers

Porcine to human xenotransplantation is an example of a discordant xenograft, which implies that the recipient of the graft has pre-formed antibodies against the donor tissue. Humans have xenoreactive natural antibodies (XNA) to several porcine antigens, however more than 80% of these recognize the sugar moiety galactose-α(1,3)-galactose (Galα(1,3)Gal) (205). Galα(1,3)Gal is also expressed on the cell wall of bacteria, thus Galα(1,3)Gal antibodies develop in humans shortly after birth once the gastrointestinal tract is colonized (206). Human Galα(1,3)Gal XNA bind to Galα(1,3)Gal on porcine tissue and activate the complement cascade which destroys the foreign tissue (207-209). This immunological response is called hyperacute xenograft rejection (HAR) due to the rapid destruction of the grafted tissue. NPIs are susceptible to XNA-mediated destruction

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(210), however the presence of Galα(1,3)Gal on NPIs is age dependent and disappears with tissue maturation (200). Thus maturation of NPIs in combination with other strategies to avoid HAR (depletion of XNA in recipients (211), or removal of the Galα(1,3)Gal epitope on tissue for transplantation (212)) will allow researchers to surpass this one mechanism of the immune system. As mentioned above, Galα(1,3)Gal XNA make up about 80% of the XNA repertoire, leaving another 20% to respond to the presence of foreign tissue. Deletion of the recipient complement system prolongs graft survival, however the graft is still lost (213). Natural killer (NK) cells (214) and T-cells, via direct and indirect pathways, also play a role in xenograft rejection. Immunologists are confident that achieving tolerance to xenogenic grafts is possible, and strategies are currently being investigated to overcome all immunological barriers (215).

1.4.2.2 Xenozoonosis

Xenozoonosis, or xeonosis, refers to transfer of infection through transplantation of xenogenic tissues or organs (204). Such an infection could be caused by bacteria or a virus harbored in the transplanted tissue. Many microorganisms endogenous to the host species may not manifest into disease, however once in humans, may cause a new disease. This risk of transmission may be increased if the xenotransplant recipient undergoes immunosuppressive therapy, or if the potential pathogen is not recognized by the recipient's immune system (216). Of particular concern, when considering porcine tissue xenotransplantation, is porcine endogenous retrovirus (PERV). PERV is closely related to the mouse and feline leukemia viruses which do cause leukemia in an infected host (217). In vitro studies have demonstrated that human cell lines (218;219) and primary human cells (220-222) may be infected with PERV, however there has been no

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convincing in vivo evidence reported of productive PERV infection (223-227). Other porcine pathogens are of concern, however many may be eliminated by breeding pigs in a pathogen-free environment. It may not be possible to remove an endogenous retrovirus from pigs, thus concern is focused on PERV.

1.5 OBJECTIVES OF THIS STUDY

The main objective of this thesis is to further characterize NPIs as a source of xenotransplantable insulin-producing tissue. In addition, we hope that information provided on the expansion and maturation of NPIs may be transferable to human tissue, thus aiding in the field of islet allotransplantation. Considerable work has been done by our group previously, characterizing the isolation and content of NPIs (113;194;198), as well as the immune response the human body is expected to initiate upon encountering NPIs (195;200;210;215).

Attempts to enhance maturation of fetal or neonatal islet tissue have met with modest success (30;32;228;229). In Chapter 2, a model of in vitro maturation of NPIs is described which results in a much faster correction of hyperglycemia in mice. An in depth analysis of in vivo maturation of NPIs is described in Chapter 3, addressing the morphological changes in the grafts as well as PAX6 and NKX2.2 protein expression in matured grafts, and expanding on previous work looking at in vivo maturation of NPIs (196;197). Knowledge of what happens during in vivo maturation of NPIs in a diabetic recipient will help us understand optimal transplant conditions once xenotransplantation of NPIs becomes a clinical reality. Several examples of ectopic expression of PDX-1 have indicated that liver, intestinal, and pancreatic cells can express β-cell specific genes (230-233). We wanted to replicate this enhancement of insulin-producing tissue in NPIs,

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attempting to exploit their ability to mature by transducing the precursors available with an adenoviral construct. Studies presented in Chapter 4 of this text indicate that adenoviral transfection of NPIs to overexpress PDX-1 does not lead to enhanced maturation of the tissue. This study does not eliminate the possibility of incompatibility between mouse PDX-1 and the porcine DNA binding domains, however due to studies claiming the need for partner proteins to allow for PDX-1 binding, as well as those indicating sequestering of the transcription factor in the cytoplasm (164;234), design of future experiments will benefit from these findings.

Chapter 5 addresses the need to gather insight into the potential for PERV transmission through NPI transplantation. Conflicting evidence as to whether PERV will be a concern in transplantation or not is presented, as is the potential benefit of encapsulation of transplanted tissue. Prevention of cell-to-cell contact between graft and host may play a role in prevention of PERV transmission.

Overall the studies presented in this manuscript address two major concerns when considering NPIs for transplantation as a treatment for diabetes. First, the immaturity of NPIs must be overcome in order to provide an adequate β-cell mass to correct hyperglycemia immediately upon transplantation. The slow return of NPI transplanted diabetic mice to euglygemia is reflective of the many progenitor cells transplanted in NPIs, which provide the graft with the capacity to grow and mature. By understanding the processes involved in islet maturation, both in vitro and in vivo (as addressed in Chapter 2 and 3, respectively), we may be able to provide signals or conditions which encourage maturation and expansion of the tissue, resulting in an abundant and effective source of transplantable insulin-producing tissue. Although Pdx-1 ectopic- or over-

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expression leading to conversion of progenitors and non-β-cells into insulin-producing cells has been demonstrated (230;232;233;235;236), in our hands the Pdx-1 signal alone does not accelerate NPI maturation (shown in Chapter 4). This finding is in agreement with some others, and indicates the likely need for additional factors to be provided (164;237;238). Second, the potential for PERV transmission must be thoroughly addressed in animal models of islet transplantation in order for porcine xenotransplantation clinical trials to move forward (presented in Chapter 5). The knowledge accrued from these studies provides in vitro and in vivo models with which researchers may further understanding of islet development, insight into the consortium of signals necessary to mature islets in vitro, and risks associated with PERV infection post-NPI transplantation.

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CHAPTER 2

IN VITRO FUNCTIONAL MATURATION OF NEONATAL PORCINE ISLETS

2.1 INTRODUCTION

With the recent success of islet transplantation as a treatment for Type I diabetes comes the need for a greater supply of insulin-producing tissue in order to make this therapy available to more eligible recipients (1-3). Likely sources for abundant transplantable islet tissue include stem cells, non-endocrine portions of the human pancreas, and xenotransplantable tissue (reviewed in (4)).

As compared to adult porcine islets which are difficult to isolate and do not survive well in culture, and fetal porcine islet cell clusters (ICC) which do not respond to glucose stimulation, neonatal porcine islets (NPI) present the most likely source of xenotransplantable islet tissue. NPI are easy to isolate, robust in culture, respond to glucose stimulation, and have the capacity to grow and differentiate due to a high percentage of ductal cells (5-9). Transplantation of 2,000 NPI was able to restore diabetic (alloxan induced) nude mice to a normoglycemic state within 6-8 weeks post transplantation (10). A delay before diabetes is cured in mice post-NPI transplantation has been observed consistently when studying this tissue source (8;9;11;12). It is thought that within this lag time, during which the diabetic recipients survive, the NPI mature and ß-cell mass increases through proliferation and neogenesis (9) to a critical level.

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Reduction or elimination of this delay time may be beneficial for xenotransplantation of NPI.

In order to harness the potential of precursors, or transdifferentiation of non-endocrine tissue, understanding of developmental biology of endocrine cells and the cells from which they are derived is mandatory. Evidence continues to point to a population of cells within ductal epithelium as the pancreatic precursor cell. In vivo models of pancreatic regeneration after inflicting an insult on the organ have shown embryonic-like re-population of both acinar and endocrine cells via expansion of ductal cells, followed by the appearance of endocrine and exocrine cells either associated with, or budding off of ducts (13-15). NPI, composed of 25–40% ductal cells (as indicated by cytokeratin 7 (CK7) immunostaining (16;17), data not shown), provide an ideal model to experiment with different culture media or transfections of pancreatic tissue with the purpose to expand precursor/endocrine populations. Results from such experimnets may be extrapolated to human tissue.

In 1995, Ao et. al (18) found that transplantation of alginate microencapsulated canine islets caused a more rapid return to normoglycemia in diabetic nude mice than a transplant containing the same number of non-encapsulated islets. In a similar study, the same group found that alginate microencapsulation of canine islets improved islet survival in long-term culture (for up to three weeks) (19). Although originally a method of immunoisolation for cell transplantation, alginate microencapsulation also has an effect on the quality of islets in long-term culture, as well as on decreasing the length of time required to cure diabetes when encapsulated islets are transplanted into diabetic mice. This may indicate improvement of overall islet health due to the presence of an

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extracellular matrix (ECM). Provision of an ECM has been associated with activation of genes, leading to maturation of tissues, such as mammary glands in mice (reviewed in (20)). The effect of ECM on the growth of neonatal rat islet cells (21) and fetal human islets, especially with the addition of growth factors such as hepatocyte growth factor/scatter factor (HGF/SF), (22) has proven to be favorable. Beattie et al (22) indicated that, in addition to growth factors and additives to the media, cell-to-cell contact in an islet-type aggregation is required for islet-specific gene expression leading to islet development. Adult human islets grown in a 3D culture in Matrigel (rich in ECM components and growth factors) plus serum resulted in expansion of ductal epithelial population resembling embryonic and regenerating pancreas, where endocrine cells were observed budding off of ductal cysts (11). The combination of an ECM and the correct growth factors has had a profound effect on islet development. Other groups working with NPI have attempted to address the delay in NPI maturation by culturing with various growth factors (12) and providing an ECM (8;12). Although modest improvements in insulin content and survival of cells were observed, this did not translate into functional maturation as, once transplanted, these NPI did not shorten the period of time required to cure diabetes. Understanding of the importance of mesenchymal-epithelial interactions during development has led to the identification of other factors relevant to pancreas development, such as fibroblast growth factor 10 (FGF10) (23-25). Autologous serum, isolated from piglets at the time of islet isolation, may contain such secreted factors necessary for islet development.

We believe that neonatal porcine islets will provide a valuable model to study islet development due to many of the same properties that make NPI an attractive source

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of xenotransplantable tissue: reproducibility of the isolation procedure, the large number of ductal, or potential precursor cells, the capacity for the tissue to mature, and the physiological similarities between the pig and the human. We hypothesized that alginate microcapsules as an extracellular matrix around NPI and growth factors existing in autologous serum would provide a method to increase functional maturation of NPI. This would not only result in shortening the lag time seen before correction of diabetes post-transplantation, but would also provide an ideal in vitro model to study islet development. Thus, several morphological and molecular aspects of NPI development as well as function as a transplant were followed in order to evaluate the suitability of this in vitro maturation protocol as a model to study islet development.

2.2 RESEARCH DESIGN AND METHODS

2.2.1 Preparation of neonatal porcine islets and serum

All animal protocols used in this study follow guidelines set out by the Canadian Council for Animal Care (CCAC). The method used to isolate NPI has previously been described (5). Briefly, donor pancreases were obtained from Landrace-Yorkshire neonatal pigs (1-3 days old, 1.5-2.0 kg body weight) of either sex. Piglets were anesthetized with halothane and subjected to laparotomy and complete exsanguination. Pancreases were removed, cut into small pieces, and digested in 2.5 mg/mL collagenase (Sigma, Oakville, ON). After filtration through a nylon screen (500 μm), the tissue was cultured for 6 days in HAM's F10 medium (Gibco, Burlington, ON) containing 10mM glucose, 50 μm isobutalmethylxanthine (IBMX; ICN Biomedicals, Montreal, PQ), 0.5%

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bovine serum albumin (BSA) (fraction V, radioimmunoassay grade, Sigma), 2mM L-glutamine, 10 mM nicotinamide (BDH Biochemical, Poole, England), 100 U/mL penicillin, and 100 μg/mL streptomycin. Blood collected from piglets during surgery was aliquotted into Vacutainer® SST® Gel and Clot Activator tubes (Becton Dickinson, Franklin Lakes, NJ), incubated for 20 minutes and centrifuged at 650 *g* for 15 minutes for serum removal. Neonatal porcine serum was heat inactivated at 58°C for 30 minutes and stored at -20°C until use.

2.2.2 Encapsulation and culture of NPI

For alginate microencapsulation, NPI were washed with HBSS (Sigma) without calcium or magnesium, supplemented with 10 mmol/L HEPES. Islets were resuspended in 0.5mL HBSS and 0.5mL of 1.5% (w/v) highly purified MVG alginate (Pronova Biomedical, Oslo, Norway) dissolved in HBSS. The resulting islet/alginate mixture was vortexed and transferred into a 1mL syringe. Microcapsules (350 – 450 μm in diameter) were formed by passing the alginate/islet suspension through an electrostatic generator followed by collection in a 120mmol/L $CaCl_2$ (10mmol/L HEPES, 0.01% Tween 20) solution. The capsules were washed by gravity sedimentation in HAM's F10 medium. Day 0 samples were taken immediately after the encapsulation procedure. Encapsulated NPI were cultured in HAM's F10 medium alone, or supplemented with 10% autologous neonatal porcine serum (prepared as described above). Unencapsulated NPI cultured in HAM's F10 plus 10% autologous serum formed large clumps in culture, and subsequently necrosis of NPI was seen likely due to inability of nutrients to diffuse and/or hypoxia, thus this group was not included in this study.

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Media was changed every second day for the duration of the culture period. For sampling, alginate capsules were dissolved by incubating the encapsulated islets in calcium-free medium for 15 minutes at 37°C. Samples were taken at days 0, 2, 4, and 6 post-encapsulation and culture.

2.2.3 Hormone and DNA content analysis of NPI

All measurements were assessed from duplicate aliquots of islet cell suspensions. Hormone content was measured after extraction in 2 mmol/L acetic acid containing 0.25% BSA. Samples were sonicated in acetic acid, centrifuged (800g, 15 minutes), then supernatants were collected and stored at -20°C until assayed for hormone content by radioimmunoassay (porcine insulin: Linco Research, Inc., St. Charles, MO; glucagon: Diagnostic Products Corp., Los Angeles, CA). For DNA content, 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. Before being assayed, cell pellets were placed in 1000 μL of lysis buffer (10 mmol/L Tris, 1 mmol/L EDTA, 0.5% Triton X-100, 4°C, pH 7.40), sonicated, diluted 1:40 then a further 1:20, and 100 μL was aliquoted into a 96-well plate with 100 μL Pico Green reagent (5 μL/mL in lysis buffer) (Molecular Probes, Inc., Eugene, OR). Fluorescence was measured at 485nm excitation / 527nm emission of samples in duplicate. Samples were run in parallel with and diluted in proportion to a 9-point (0-1000 ng/mL) standard curve, which was generated using a Molecular Probes standard kit.

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2.2.4 Immunhistochemical analysis of cell populations and NPI morphology

For determination of populations of cells, samples were taken at time points throughout the culture period, and NPI were dissociated into single cells by gentle agitation in calcium-free medium supplemented with 1mM EGTA and 0.5% BSA for 7 minutes before addition of 25 μg/mL trypsin (Boehringer Mannheim, Laval, PQ, Canada) and 4 μg/mL DNase (Boehringer Mannheim) followed by further pipetting for 4 minutes. After washing cells with phosphate-buffered saline, they were placed on Histobond slides (Marienfeld, Germany) and fixed in Bouin's solution for 12 minutes before storage at 4°C in 70% ethanol. To observe islet morphology, intact NPI were embedded in paraffin and 3μm sections cut for immunohistochemistry. Primary antibodies used were: guinea-pig anti-insulin 1:1000 (Dako Diagnostics, Mississauga, ON, Canada), rabbit anti-glucagon 1:100 (Dako), mouse anti-CK7 1:200 (Dako), and rabbit anti-PDX-1 253 1:1000 (kind gift from Dr. J.F. Habener, Massachusetts General Hospital, Boston, MA). Microwave antigen retrieval was performed for 6 x 5 seconds in 10mM sodium citrate on high power (Sanyo, 1260W) prior to CK7 and PDX-1 staining. Following quenching with a 20% H_2O_2 /80% methanol solution, dissociated cells or 3μm sections were blocked with 20% normal goat serum (Vector Laboratories, Burlingame, CA) for 15 minutes followed by a 30 minute incubation with primary antibody. Slides were washed and incubated with the corresponding biotinylated secondary antibody for 20 minutes (1:200; Vector Laboratories). The avidin-biotin complex/horseradish peroxidase (Vector Laboratories) detection method was used and developed with 3,3-diaminobenzidinetetrahydrochloride (DAB) (BioGenex, San Ramon, CA) to produce a brown colour.

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For the purpose of double-staining for insulin and CK7, indirect immunofluorescence used Cy3-labelled goat anti-mouse and FITC-labeled goat anti-guinea pig (Jackson ImmunoResearch Laboratories, Inc., Bio/Can Scientific, Mississauga, ON, Canada). Pictures were acquired of identical fields using two filters, one to detect light emitted in the FITC range, and one in the Cy3 range in order to differentiate between bleeding through of the relatively brighter Cy3 dye seen when using a dual band filter. Analysis of the single-stained fields, side-by-side, allowed for quantitation of single- and double-positive cells.

2.2.5 Detection of PDX-1 and NKX2.2 protein in cultured NPI by Western blot

NPI samples collected at each time point were lysed in 100 μL of ice-cold RIPA buffer for 30 minutes at 4°C (150 mM NaCl, 20 mM Tris-Cl, 1 mM EDTA, pH 7.5, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1mM DTT, and 0.1% protease inhibitor cocktail [Sigma-Aldrich Canada Ltd.]). The lysates were spun at 12,000 *g* for 5 minutes at 4°C. The supernatants were removed and assayed for total protein content using the Bradford method (BioRad, Mississauga, ON, Canada). Ten micrograms of cell lysate protein was fractioned on 10% polyacrylamide gels containing SDS in a minigel apparatus (BioRad). After the gel separation, proteins were transferred to Nitrocellulose membranes (Micron Separations Inc., Westborough, MA) at 10 mAmps for 16 hours at 4°C in buffer containing 20% (v/v) methanol, 48 mM Tris base, 39 mM glycine, and 0.037% SDS at pH 8.3. Nitrocellulose membranes were then incubated in blocking buffer containing 136 mM NaCl, 20 mM Tris, pH 7.6, and 5% skim milk powder for 1 hour at room temperature with gentle shaking. Membranes were washed three times for 10 minutes in PBS-T (0.1%) buffer

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(137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, pH 7.5 and 0.1% Tween-20). Fresh blocking solution was then applied containing 1:10,000 dilution of PDX-1 253 rabbit with shaking for 2 hours. Membranes were washed as before, followed by a second antibody to rabbit immunoglobulin G coupled to horseradish peroxidase at 1:500 dilution (Amersham Biosciences, Inc., Baie d'Urfé, PQ, Canada) for 1 hour at room temperature, followed by 3 additional 10 minute washes. The blots were developed with an enhanced chemiluminescence Western blotting detection kit (Amersham Biosciences, Inc.). The same blots were stripped in 62.5 mM Tris-Cl pH 6.7, 2% SDS, and 100 mM 2-mercaptoethanol (Sigma) for 30 minutes at 50°C with occasional agitation, rinsed in PBS-T (0.1%) buffer and re-probed as described above with a 1:1000 dilution of NKX2.2 rabbit antibody (generously provided by Dr. T.M. Jessell, Columbia University, New York, NY).

2.2.6 RNA isolation for Northern blotting and RT-PCR

All samples were removed under aseptic conditions utilizing RNase/DNase free reagents and instruments to prevent cross-contamination. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The RNA pellet was dissolved in 50 μL DNase/RNase free water (Sigma) and the concentration measured spectrophotometrically. A 50 μL reverse transcription (RT) reaction of 1 μg of RNA was performed using MultiScribe™ Reverse Transcriptase (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The samples were placed in a thermal cycler for 10 minutes at 25°C, 30 minutes at 48°C, and 5 minutes at 95°C for reverse transcription.

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2.2.7 Detection of preproinsulin mRNA in cultured NPI by Northern blot

Two micrograms of total RNA from NPI samples was denatured and electrophoresed on a 1% agarose 5.4% formaldehyde gel. The integrity of the extracted RNA was determined by the visualization of 28S and 18S ribosomal RNA bands with ethidium bromide staining. After electrophoresis at 3V/cm, the RNA was transferred from the gel to a positively charged Nylon membrane (Micron Separations Inc.) by capillary action. Hybridization was then performed using a 1017 bp human insulin genomic DNA fragment that was PCR amplified with human specific primers (sense primer: 5'-CCAGCCGCAGCCTTTGTGA-3'; antisense primer: 5'-GGTACAGCATTGTTCCACAATG-3') and T-A cloned into pGEM-T vector (Promega, Madison, WI). The 1017 bp fragment was isolated by digestion with NcoI and NotI, and radiolabled with [α-^{32}P]dCTP, using a commercial kit (Random Primers DNA Labeling System, Invitrogen). The blots were pre-hybridized for 2 hours at 60°C in 7% SDS, 0.5 M Na_2PO_4, 1 mM EDTA, 1% BSA, followed by hybridization with the probe at 60°C for 16 hours in 7% SDS, 0.5 M Na_2PO_4, 1 mM EDTA, 1% BSA. Blots were washed three times at room temperature in 0.1X SSC and 0.1% SDS for 5 minutes each. Autoradiograms were developed after exposure to X-ray film at -80°C, using an intensifying screen.

2.2.8 Quantitative RT-PCR analysis of gene expression

Two methods of quantitative RT-PCR were used; SYBR® green detection of double stranded DNA and real time TaqMan using FAM/TAMRA probes. All porcine specific primers and probes were designed using Primer Express software, version 2.0 (Applied Biosystems), according to the guidelines set out by the manufacturer. All PCR

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reactions were completed in an ABI Prism 7700 Sequence Detection System (Applied Biosystems). Day 0 and day 6 ECser^{+} samples were analyzed and compared to determine the change in gene expression in the NPI samples which were functionally mature.

SYBR® green I dye (SYBR® green RT-PCR reagents kit, Applied Biosystems) was used to quantitate levels of Hypoxia Inducible Factor-1 (HIF-1), Connective Tissue Growth Factor (CTGF), thymosine, and ß-actin. The sequences of the gene-specific primers are shown in Table 2-1. Direct detection of PCR product was monitored by measuring the increase in fluorescence caused by the binding of SYBR® green dye to double stranded DNA. Absence of non-specific or genomic amplification was assessed by including a non-template control and minus RT controls. The fluorescent signal in each sample was normalized to a corresponding ß-actin signal (endogenous control).

Real time TaqMan was used to quantitate levels of Pdx-1, preproinsulin, proglucagon, NeuroD, and GAPDH. Primers and probes used are outlined in Table 2-1. All probes carried a 5' FAM reporter dye and a 3' TAMRA quencher dye. Each TaqMan PCR assay was completed twice, and samples were analyzed in triplicate for each target gene in a 96-well optical plate. A comparative threshold method and the standard curve method were used to calculate the amount of cDNA according to the method described in Perkin Elmer ABI Prism 7700 User Bulletin no. 2, 1997. The estimated amount of gene of interest was normalized to the amount of porcine GAPDH to compensate for differences in the quantity of cDNA per sample as well as for any differences in reverse transcription. Briefly, 1 µL of cDNA was used in a 50 µL reaction containing final concentrations of 1x TaqMan Universal PCR Master Mix (Applied Biosystems), 300 nM of each of the forward and reverse primers, and 150 nM FAM-TAMRA probe. The

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thermal cycling conditions used were as specified by the manufacturer in the TaqMan Universal PCR Master Mix protocol (Applied Biosystems).

Fold changes in gene expression were calculated by using the comparative CT method with values of the baseline (day 0) samples used as the endogenous reference (fold change = 1).

Table 2-1. Primers and probes used in two methods of quantitative RT-PCR.

Method		Forward Primer	TaqMan probe	Reverse Primer
SYBR green	HIF-1	GGTTTAGGTTTTGCCCAGAATTC		GGCAGCCCAAGGCTTTGT
	CTGF	AGTTCATCCATCAATACAGGTTGTTT		CCACATTTCCTACCTACAAATCAGACT
	Thymosine	CGGTCCCAGCCTAGCTGTAG		TGGCTTGTCACTCATCTTGACTAAA
	ß-actin	CTCTTCCAGCCCTCCTTCCT		CGCACTTCATGATCGAGTTGA
TaqMan	Pdx-1	GAGGAGAACAAGCGGACGC	CCGCCTACACTCGCGCGCA	CGGCCTAGAGATGTATTTGTTGAA
	Proinsulin	TGGCATCGTGGAGCAGTG	TGCACCAGCATCTGTTCCCTCTACCA	GGCCTAGTTGCAGTAGTTCTCCAG
	Proglucagon	GCTGCCAAGGAATTCATTGC	TGAAAGGCCGAGGAAGGCGAGATT	TCTTCGACAATGGTAACTTCCTCTG
	Neuro D	CAGCCCTTCCTTCGATGGA	AGCCCGCCGCTCAGCATCAA	TCGTGTTTGAAAGAGAAGTTGCC
	GAPDH	GATTTGGCCGCATCGG	CGCCTGGTCACCAGGGCTGCT	CAATGTCCACTTTGCCAGAGTTAA

Where no probe sequence is noted, SYBR green method was used which does not require a probe.

2.2.9 Transplantation of NPI into diabetic SCID mice

Diabetes was induced using 225mg/kg body weight Streptozotocin (Sigma) injected intraperitonially (i.p.) in 6-8 week old CB17 SCID beige mice (Taconic Farms, Germantown, NY). All diabetic recipients had a minimum blood glucose level of 20 mmol/L prior to transplantation. Blood glucose levels were monitored weekly using a OneTouch Ultra blood glucose monitor (Lifescan, Milpitas, CA). Three groups of diabetic SCID mice received NPI transplants: 1) 2000 alginate microencapsulated NPI which had been cultured in the absence of autologous serum for 6 days (ECser$^-$) were transplanted intraperitonially (i.p.), n=12, 2) 2000 alginate microencapsulated islets which had been cultured with autologous serum for 6 days (EC ser$^+$) were transplanted

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i.p., n=12, 3) 2000 NPI EC ser^{+}, however in this group, prior to transplantation, the capsules were dissolved and the islets transplanted under the left kidney capsule, n=10.

2.2.10 Statistical Analysis

Data are expressed as means±SEM of (n) independent observations. Statistical significance of differences for hormone and DNA content, immunohistochemical, blotting, and glycemia data was calculated by a one-way ANOVA using Scheffé's post hoc test, $p<0.05$ was considered significant. A Student's unpaired T-test was used to determine statistical significance of difference for all quantitative RT-PCR results, $p<0.05$ was considered significant.

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2.3 RESULTS

2.3.1 Hormone and DNA content of encapsulated NPI cultured with and without autologous serum

Initial experiments to determine whether the addition of an alginate microcapsule and/or autologous serum would hasten the functional maturation of NPI by increasing insulin content and number of β-cells revealed the success of this technique. Non-encapsulated NPI (non-EC) cultured for 8 days in Ham's F10 media, without the addition of autologous serum, had a slightly elevated insulin content compared to the beginning of the experiment, measuring 148.2±25.0% of the initial insulin value (Table 2-2). Glucagon and DNA content (an index of cell number) decreased during the culture period, to 44.8±8.3% and 73.5±6.9% respectively, of the values of the starting tissue (Table 2-2). Without the addition of autologous serum to the culture media, alginate microencapsulated islets (EC ser^-) also show an increase in the insulin content of 156.0±10.0%, and a decrease in both the glucagon and DNA content, 89.9±11.9% and 86.6±11.0% respectively. When autologous serum is added to the culture media (EC ser^+), significant increases in insulin, glucagon, and DNA content of the samples are noted. The insulin content increased to 416.0±36.9% of the original content, glucagon content increased to 146.1±11.9%, and DNA content increased to 140.3±25.2% (Table 2-2, all significant).

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Table 2-2. Effect of microencapsulation and autologous serum on cellular insulin, glucagon, and DNA content of neonatal porcine islet cell aggregates after 8 days in culture.

Condition		% of original tissue		
		Insulin	Glucagon	DNA
Non-encapsulated		148.2±25.0 (11)	44.8±8.3 (11)	73.5±6.9 (11)
Encapsulated				
	no serum	156.0±10.1 (19)	89.9±11.9 (17)	86.6±11.0 (17)
	plus serum	416.0±36.9 (28)‡ **	146.1±11.9 (17)‡ *	140.3±25.2 (17)† *

Values are means±SEM of (n) independent experiments. In each experiment, NPIs were prepared from 2 pancreases. Statistical significance of differences was calculated by one-way ANOVA. †p<0.05, ‡p<0.0001 vs. non-encapsulated , *p<0.05, ??p<0.0001 vs. encapsulated no serum samples.

2.3.2 Insulin and glucagon cell populations post encapsulation and culture

A time course was used to observe changes in the NPI population during the observed maturation. Throughout the time course, the percentage of cells staining positively for insulin increased in both the samples cultured without serum, and in the samples cultured with autologous serum (Table 2-3). At the start of the time course, 26.3±1.4% of the cells were insulin-positive. By day 6 of culture, the EC ser$^-$ sample contained 34.3±2.0% insulin-positive cells, whereas the day 6 EC ser$^+$ sample had a slightly higher proportion of insulin-positive cells (38.8±1.5%, Table 2-3). These two samples taken at day 6 were not significantly different, however significance was noted between the day 0 and the day 6 EC ser$^+$ samples. Similarly, the proportion of cells staining positive for glucagon increased throughout the time course in EC ser$^-$ as well as the EC ser$^+$ samples. At the start of the experiment, 16.2±1.7% of the NPI cells were glucagon-positive, and at day 6 EC ser$^-$, 31.6±2.7% of the cells were glucagon-positive, a slightly higher number than in the EC ser$^+$ group (29.5±1.5%, Table 2-3). Due to these results in conjunction with the hormone and DNA content results, it was determined that

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Table 2-3. Immunohistochemical determination of populations of cells prior to and post-alginate microencapsulation and culture with (ser^{+}) or without (ser^{-}) autologous serum.

Condition		% positive cells			
		Insulin	Glucagon	CK7	CK7 + Insulin
Day 0		26.3±1.4 (19)**	16.2±1.7 (16)*	35.6±9.4 (4)	6.58±1.5 (8)~
EC ser -					
	Day 2	26.7±2.1 (11)**	26.3±3.8 (11)	39.8±7.2 (4)	ND
	Day 4	30.0±1.4 (12)	26.1±2.3 (12)	37.3±4.6 (4)	ND
	Day 6	34.3±2.0 (15)	31.6±2.7(16)	36.6±6.7 (4)	ND
EC ser +					
	Day 2	30.1±1.8 (15)~	21.1±2.6 (12)	43.5±5.8 (4)	11.5±3.1 (5)
	Day 4	35.1±1.9 (15)	26.2±2.3 (12)	38.0±3.4 (4)	17.9±4.1 (6)
	Day 6	38.8±1.5 (19)	29.5±1.3 (16)	23.7±1.7 (4)	21.1±2.3 (8)

Values are means±SEM of (n) independent experiments. ND indicates not determined.
At least 500 cells per sample were counted to determine percentage positive.
Statistical significance of differences was calculated by ANOVAusing Scheffe post hoc test.
~p<0.05, *p<0.01, **p<0.005 vs. Day 6 EC ser+ sample.

the best in vitro conditions involved culturing the encapsulated islets for 6 days in HAM's F10 supplemented with 10% autologous serum.

2.3.3 Insulin and CK7 co-expression

Due to the observed increase in the percentage of cells staining positively for insulin in the day 6 EC ser^{+} samples, the aliquots of cells were double-immunostained for insulin and CK7 to determine if the ductal cells were differentiating into ß-cells. CK7 is commonly used as a marker for ductal cells (16) and ductal cells are thought to be stem cells in the pancreas (16;26;27). At the beginning of the time course, 35.6±9.4% of the cells were cytokeratin 7 (CK7)-positive. Throughout the time course, no significant change in the proportion of CK7-positive cells was noticed in any of the conditions. At day 0, very few cells expressed both insulin and CK7, however by day 6 EC ser^{+}, many cells were co-expressing these two markers (Table 2-3). At day 0, 6.58±1.5% of the cells were double-positive for insulin and CK7. After 2 days EC ser^{+}, 11.5±3.1% of cells were

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double-positive, 17.9±4.1% after 4 days culture, and finally 21.1±2.3% of the cells were double-positive at day 6 EC ser^+ culture (Table 2-3).

2.3.4 Morphological analysis of NPI post-encapsulation and culture

Staining for insulin at day 0 and day 6 EC ser^+ revealed a qualitative view of the increase of insulin protein during NPI maturation (Figure 2-1 A, day 0, Figure 2-1 D, day 6 EC ser^+). PDX-1 protein was seen throughout the NPIs during the time course. Figures 2-1 B, C, E, and F show NPIs samples at days 0, 2, 4, and 6 from the EC ser^+ group, respectively.

2.3.5 PDX-1 immunostaining during NPI culture with autologous serum post-encapsulation

As compared to PDX-1 immunostaining in the rat pancreas (Figure 2-2 A,B), the neonatal porcine pancreas shows a lack of maturity as the PDX-1 positive cells are scattered throughout the section (Figure 2-2 C,D) as opposed to in organized islets. Also seen in the neonatal porcine pancreas are ductal cells staining positively for PDX-1. A dramatic example implicating these PDX-1 positive ductal cells in islet maturation is seen in Figure 2-3. After culture of EC ser^+ NPI, an islet budding-structure is clearly associated with a duct (Figure 2-3). Many PDX-1 positive cells are seen within the NPI post-EC ser^+ culture.

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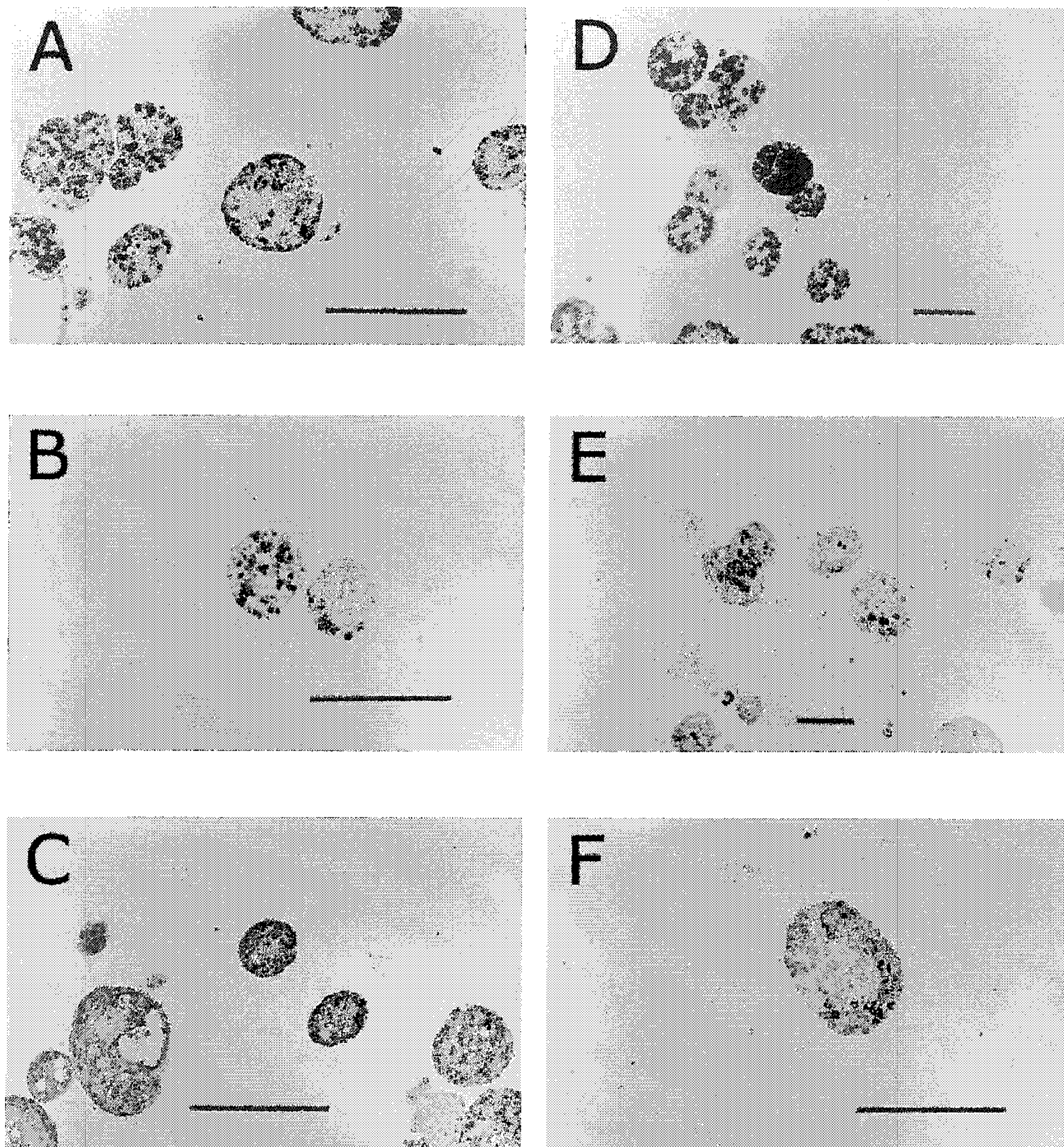

Figure 2-1. Immunohistochemical analysis of morphometry of neonatal porcine islets post-alginate microencapsulation and culture with autologous serum. An increase in the number of cells immunostaining positively for insulin is seen from day 0 (A) to day six EC ser^+ (B). PDX-1 staining is shown throughout the culture period, samples are shown stained for PDX-1 at day 0 (C), day 2 (D), day 4 (E), and day 6 (F) EC ser^+. Scale bars represent 100μm.

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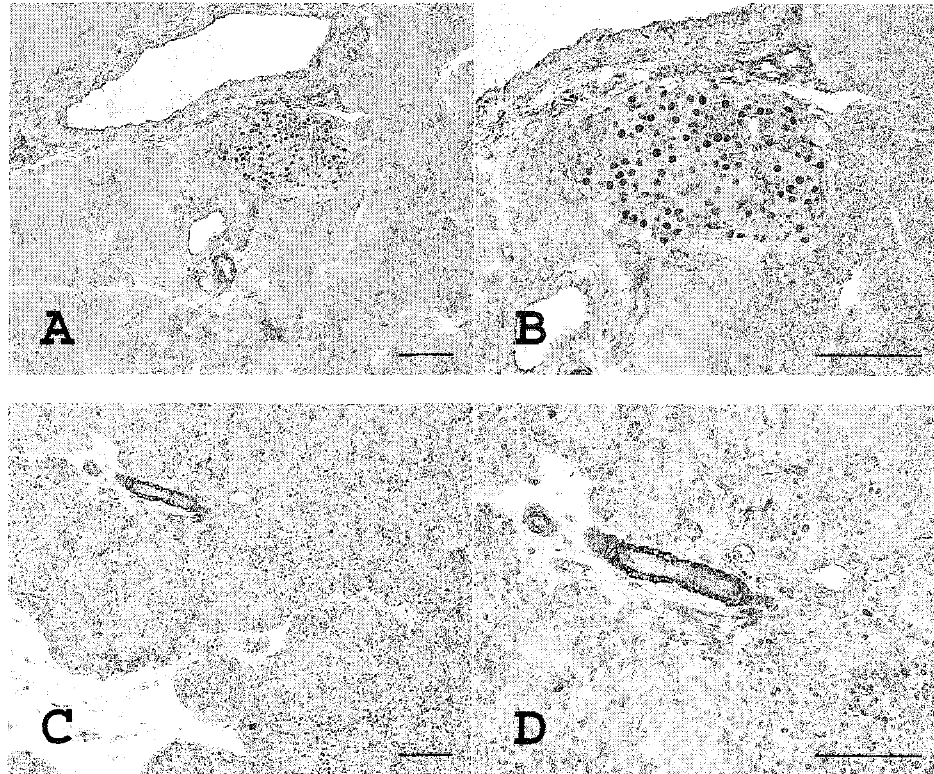

Figure 2-2. PDX-1 antibody immunohistochemically stains pancreatic sections. PDX-1 clearly stains cells in both the rat (A, B) and neonatal porcine (C, D) pancreas. In the rat, staining is mainly localized in the mature islets and appears to be nuclear. In the immature porcine pancreas, endocrine cells are not arranged in islets always, and consequently positive cells are seen scattered throughout the section. Cells within ducts appear to be PDX-1 positive. Scale bars represent 50 μm.

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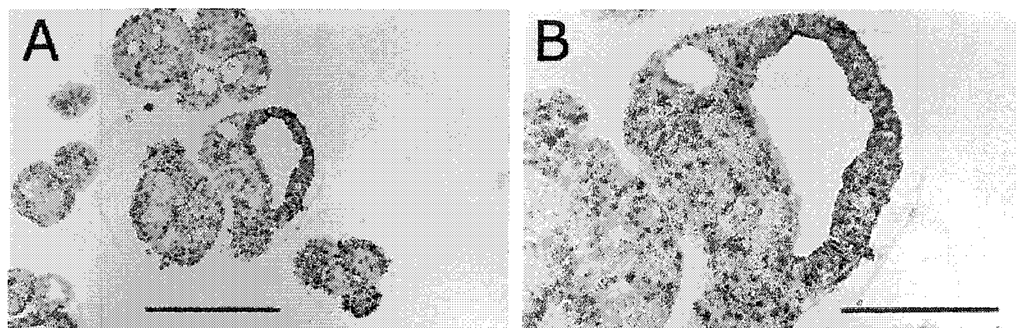

Figure 2-3. Light micrographs of PDX-1 immunostaining of day 6 ECser$^+$ neonatal porcine islets. After alginate microencapsulation of neonatal porcine islets and culture in autologous serum, a duct-like structure is seen budding off of an islet (A: low magnification and B: higher magnification). Many cells within the islet are positive for PDX-1 protein, as are the majority of the cells in the duct. Scale bars represent 100 μm (A) and 50 μm (B).

2.3.6 PDX-1 and Nkx2.2 protein expression

Western blot analysis of PDX-1 protein expression throughout the maturation time course reveals an apparent increase in PDX-1 protein. When quantitated using densitometry, all samples showed an increased protein expression, with the day 6 EC ser$^+$ sample being the highest with a 1.94±0.57 fold increase compared to day 0 (Figure 2-4 A). Nkx2.2 protein expression showed a similar result, with most time points showing increased expression compared to day 0. Day 6 samples, both EC ser$^+$ and ECser$^-$, showed the greatest increase in Nkx2.2 expression (1.76±0.33 and 1.81±0.23 fold, respectively compared to day 0, Figure 2-4 B). EC ser$^+$ and ECser$^-$ samples showed similar values at the time points examined pertaining to Nkx2.2 expression.

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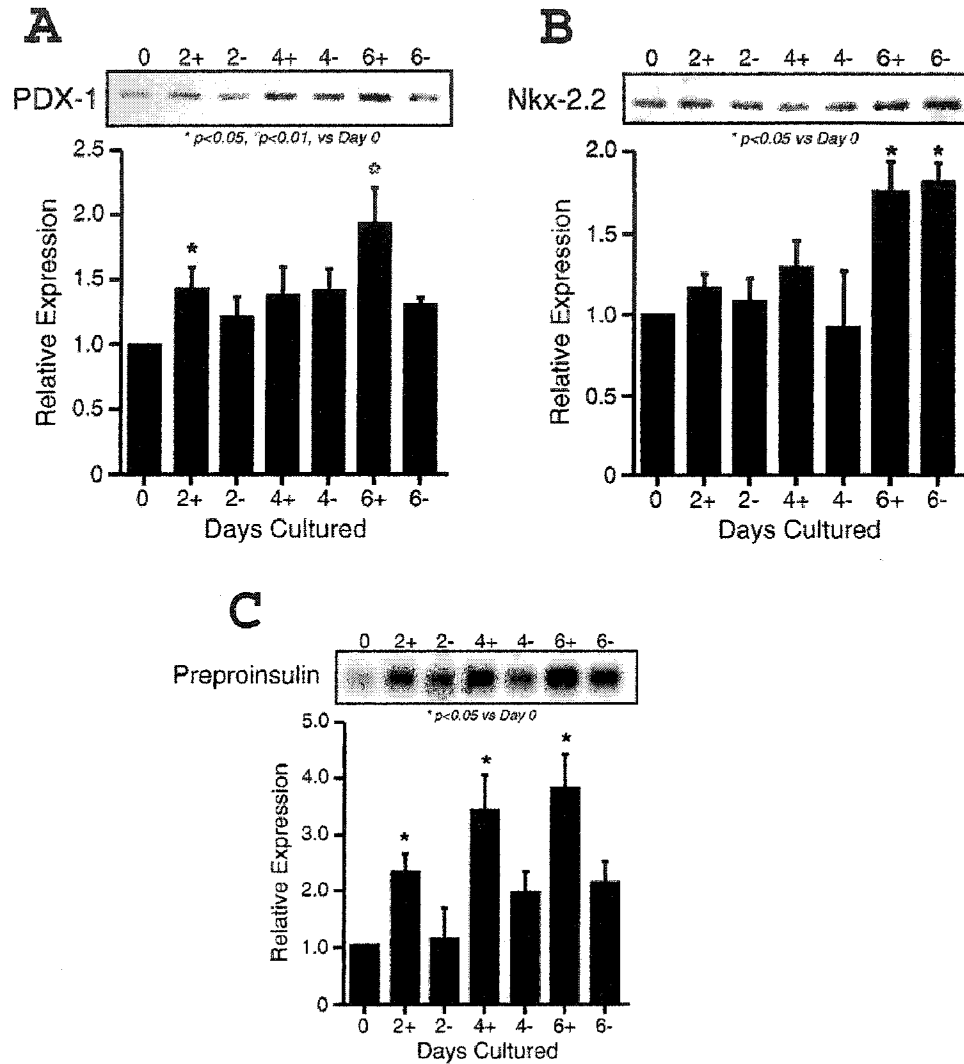

Figure 2-4. Culture of encapsulated neonatal porcine islets with autologous serum increases PDX-1 and NKX2.2 protein expression as well as proinsulin mRNA expression. (A) Representative Western blot for PDX-1 in encapsulated (EC) NPI cultured from 0 to 6 days in the presence (+) or absence (-) of autologous serum. (B) Representative Western blot for NKX2.2 in EC NPI cultured from 0 to 6 days + and – autologous serum. (C) Representative Northern blot for proinsulin mRNA in EC NPI cultured from 0 to 6 days + and – autologous serum. All expression levels were quantitated relative to day 0 as determined from densiometric values derived from 4 separate experiments. Statistical significance of difference was determined by one-way ANOVA. Black star: $p<0.05$, white star: $p<0.01$ vs. Day 0.

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2.3.7 Preproinsulin mRNA expression post-encapsulation and culture

Preproinsulin mRNA expression increases gradually over the time course of NPI encapsulation and culture with autologous serum. When the day 0 sample is normalized to 1, day 2 EC ser^+ increases 1.13±0.98 fold, day 4 EC ser^+ increases 1.95±0.63 fold, and day 6 EC ser^+ increases 2.13±0.65 fold (Figure 2-4 C). The preproinsulin message levels are all higher when the encapsulated NPI are cultured in autologous serum; 2.3±0.52 fold after 2 days, 3.41±1.08 fold after 4 days, and 3.81±1.04 fold after 6 days.

2.3.8 HIF, thymosine, CTGF, preproinsulin, proglucagon, Pdx-1 and NeuroD gene expression in NPI post-encapsulation and culture

To further understanding, on a molecular level, of changes during the maturation process, we looked at relative gene expression of islet specific genes, as well as genes which were up- or down-regulated when cDNA libraries of day 0 and day 6 EC ser^+ NPI were analyzed. Using non-quantitative methods of library screening, differences in clone numbers of some genes were apparent between day 0 and day 6 EC ser^+ NPI (data not shown). Specifically increases were seen in clone numbers of hypoxia inducible factor (HIF) and thymosine beta, and a decrease was seen in connective tissue growth factor (CTGF). Hypoxia of islets has been associated with alginate microencapsulation (28), however, using quantitative RT-PCR techniques, HIF mRNA levels were unchanged (day 6 EC ser^+ were 1.47±0.1 fold of day 0 mRNA levels) (Table 2-4). CTGF is a cytokine, which induces fibrosis in injury is also involved in development and differentiation of many tissue types (29). CTGF mRNA levels were found to be significantly reduced in day 6 EC ser^+ samples compared to day 0 samples (0.26±0.05 fold, Table 2-4).

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Table 2-4. Results of quantitative RT-PCR. Day 6 EC ser$^+$ NPI mRNA expression levels are shown as a fold change compared to corresponding baseline (day 0) sample levels.

	Fold Change Expression
HIF-1	1.47±0.1
CTGF	0.26±0.1*
Thymosine	2.32±0.2*
Preproinsulin	7.91±1.2*
Proglucagon	1.64±0.2
Pdx-1	1.55±0.3
NeuroD/Beta2	1.23±0.1

Values are expressed as mean±SE, n=4. Statistical significance of differences was determined by an unpaired Student's t-test.
*$p<0.001$ vs Day 0 levels.

Thymosine is involved in differentiation and actin polymerization of cells, thus in cell restructuring (30). Thymosine mRNA was up-regulated in day 6 EC ser$^+$ samples (2.32±0.18 fold when compared to day 0 levels, Table 2-4). Further quantitative RT-PCR was completed, looking at genes specific to islet function and development. Preproinsulin mRNA expression levels increased 7.91±1.2 fold in the day 6 EC ser$^+$ samples relative to day 0 samples (Table 2-4). Proglucagon, Pdx-1, and Neuro D/Beta 2 mRNA levels were all relatively unchanged in the EC ser$^+$ NPI samples (1.64±0.2, 1.55±0.3, and 1.23±0.1 fold of day 0 levels, respectively, Table 2-4).

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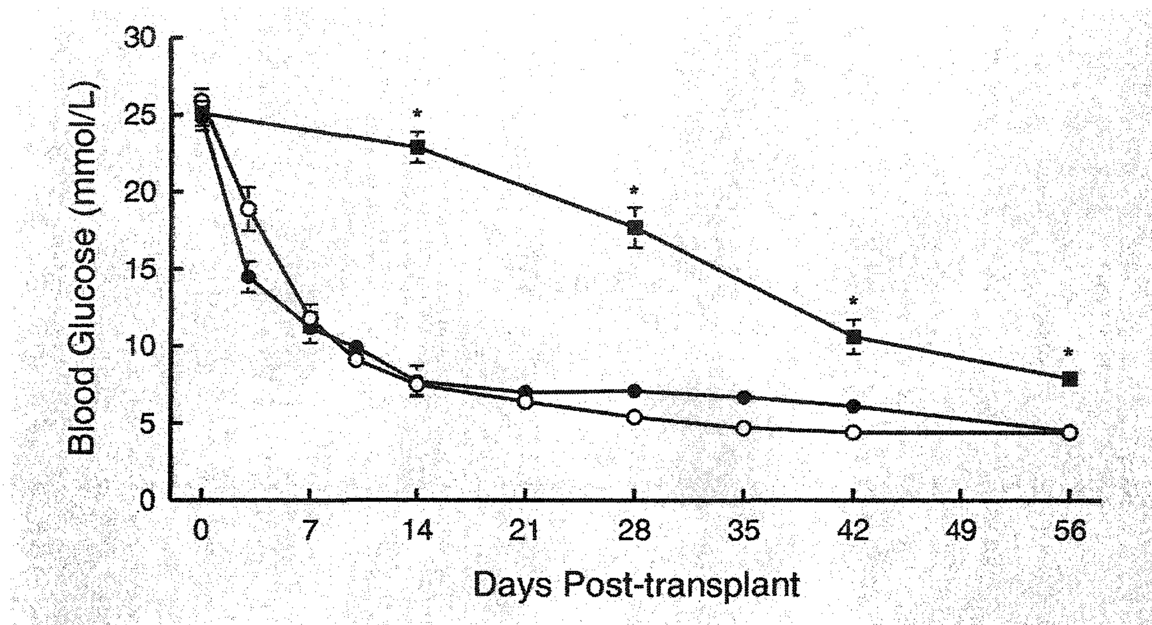

Figure 2-5. Culture of encapsulated neonatal porcine islets in autologous serum improves islet function and cures diabetes effectively when transplanted into diabetic mice. Blood glucose values in streptozotocin induced diabetic SCID mice transplanted with 2000 NPI, (■, n=12) day 6 EC ser$^-$ intraperitonially (i.p.), (●, n=12) day 6 EC ser$^+$ i.p., or (○, n=10) day 6 EC ser$^+$ transplanted under the kidney capsule after dissolving the alginate capsules. Values are mean ± SEM. Statistical significance of difference was determined by one-way ANOVA using Scheffé's post hoc test. *$p<0.0005$

2.3.9 In vivo functional analysis of NPI encapsulation and culture

Three groups of diabetic SCID mice received NPI transplants. The first group, having received an intraperitonial (i.p.) transplant of 2000 encapsulated NPI at day 6 EC ser$^-$. These animals became normoglycemic at 47.1±2.8 days post-transplant (as defined by blood glucose <10mmol/L, Figure 2-5). A second group received 2000 encapsulated NPI i.p. at day 6 EC ser$^+$. These animals became normoglycemic 10.2±1.0 days post-transplantation (Figure 2-5). This was markedly faster than the first group of animals. The third group of animals received a transplant under the kidney capsule of 2000 NPI,

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which had been in EC ser^+ for 6 days, however prior to transplant, the alginate capsules were dissolved and NPI were transplanted under the kidney capsule. This group behaved very similarly to the second group and became normoglycemic 10.7±1.0 days post transplantation (Figure 2-5).

2.4 DISCUSSION

Islet transplantation as a therapy for Type I diabetes is advantageous, as the recipient achieves glucose homeostasis without the fluctuations associated with taking exogenous insulin. At least two donor pancreases are often required to supply the 10 000 islet equivalents per kilogram body weight required for a successful islet transplant (1-3). Thus, there is demand for an abundant source of transplantable islet tissue, which may be solved by the derivation of islet tissue from stem cells or non-endocrine pancreatic tissue, or by using xenotransplantable islet tissue. We believe that NPI will provide a potential source of xenotransplantable tissue, and with the capacity to grow and differentiate. NPI are also an excellent model for the study of islet development.

This study investigated the use of autologous serum to accelerate the functional maturation of alginate microencapsulated NPI, such that they more rapidly reach a critical mass of ß-cells in order to correct diabetes in mice. We found that our preliminary studies looking at the culture of NPI either 1) without encapsulation in HAM's F10 without serum (non-EC), 2) with encapsulation in HAM's F10 without serum (EC ser^-), or 3) with encapsulation plus 10% autologous serum in HAM's F10 (EC ser^+) for 8 days revealed an obvious advantage using the combination of encapsulation plus serum (Table 2-2). Insulin content in the EC ser^+ group had increased 416±36.9%

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from the starting tissue, whereas both the non-EC and the EC ser$^-$ groups only increased moderately (148±25.0% and 156±10.1%, respectively, Table 2-2). Glucagon content was 146±11.9% of the starting tissue with the addition of the alginate capsule (EC ser$^+$), while the EC ser$^-$ and non-EC conditions both dropped in glucagon content. DNA content also showed a decrease in both the EC ser$^-$ and non-EC conditions, however the EC ser$^+$ group increased to 140±25.2% of the starting tissue. Previous studies adding serum to the culture medium of non EC NPI resulted in clumping and poor survival of the tissue (unpublished observations and (31)), thus this condition was not included in this study.

Our alginate microcapsules provide an advantageous culture condition for NPI, especially when combined with autologous serum in the media. We have cultured our EC NPI in the presence of FCS and determined that 10% autologous serum was most favorable (data not shown). This is in contrast to previous studies where NPI were cultured in autologous serum versus FCS (8) which found that culture in FCS provided better results. However, these studies were performed on non-encapsulated NPI, and as previous studies have indicated that the addition of serum is not optimal for culture of non-EC NPI, it may be difficult, in these conditions, to determine which serum type is truly optimal. We (18;18) and others (21;22) have reported successful culture of islets with an extracellular matrix, however, it has been shown that NPI immobilized in a solid alginate gel do increase in insulin content, but do not indicate any functional advantage over non-alginate cultured islets when transplanted into diabetic mice (8). By encapsulating one or two islets per microcapsule and allowing the capsules to freely float in media, NPI may have better exposure to oxygen as well as nutrients, thus preventing hypoxia and maintaining health.

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To observe the effect of EC ser^+ on NPI, we observed changes versus the EC ser^- group using a time course. Day 0 samples were taken after the NPI had been encapsulated, the capsules were dissolved immediately and samples taken. The EC islets were split into the two groups: ser^+ and ser^- and samples were taken at days 2, 4, and 6 of the culture period. As shown in Table 2-3, immunohistochemically, the number of cells staining positively for insulin increased in both the ser^+ and ser^- groups, thus indicating some advantage provided by the ECM. However, the addition of serum to the culture media did bring about increases at all time points observed over the ser^- group. Similarly, the number of cells staining positively for glucagon increased over the time course. In this instance, the addition of serum to the media resulted in a slight decrease in the number of glucagon positive cells. In both the case of insulin and glucagon, the hormone contents of the EC ser^+ group were significantly higher than those of the EC ser^- groups which was not caused simply by an increase in the number of ß- or α-cells. Thus this increase in hormone content must be reflecting more granulated endocrine cells in the EC ser^+ group, which may come with maturation of the islets. Cytokeratin 7 (CK7) has been shown previously to be a marker for ductal cells (16), particularly in NPI (17). At day 0, 36.6±1.5% of the cells were CK7-positive ductal cells, which are hypothesized to be pancreatic precursor cells. This population of cells remained fairly consistent throughout the time course, with a small decrease in the CK7 population by day 6 in the EC ser^+ group. This is in contrast to data previously shown by our group (32) which had indicated a steady decrease of the CK7 population over time in culture. This inconsistency may be due to the heterogenous nature of the donor piglets used to procure the NPIs. To observe the role of CK7 cells as precursors for ß-cells in EC ser^+ culture of

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NPI, samples were double stained at days 0, 2, 4, and 6 and the number of cells staining positively for both insulin and CK7 were quantitated. At day 0, very few of the cells present were double-positive (6.58±1.5%, Table 2-3). The number of double-positives progressively increased, and a significant increase was observed on day 6 EC ser^+ (21.1±2.3%, Table 2-3). It has been observed that the percentage of NPI expressing both insulin and CK7 does increase after isolation (17), and by adulthood, these double-positive cells are no longer observed (17), (G. Rayat, personal communication). It is possible that these ductal precursor cells may be maturing and becoming ß- cells.

NPI recovered throughout the 6-day time course were embedded in paraffin and sectioned for morphometrical and immunohistochemical analysis. Insulin staining at day 0 (Figure 2-1 A) and day 6 EC ser^+ (Figure 2-1 D) qualitatively appear to reflect the increase in the number of cells staining positively for insulin (Table 2-3) as well in insulin content (Table 2-2). Pancreas duodenal homeobox 1 (PDX-1) plays crucial roles in both pancreatic development as well as maintenance of the ß-cell phenotype and regulation of insulin and other ß-cell genes (reviewed by Asizawa et.al. (33)). Immunohistochemical analysis of PDX-1 expression in NPI from the EC ser^+ group was observed throughout the time course (Figure 2-1 B, E, C, and F). PDX-1 protein was present in NPI throughout the time course. Compared to PDX-1 expression in the rat pancreas (Figure 2-2 A, B) which appears to be mainly nuclear and localized in islet cells, the neonatal porcine pancreas contains scattered cells which are PDX-1 positive, as well as dark PDX-1 positive cells in the ducts where the protein appears to be cytoplasmic (Figure 2-2 C, D). This may be indicative of the immature nature of the neonatal pancreas as many of the cells, which do stain positively for PDX-1, are likely

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precursor cells or immature ß-cells. Observations of islet cells budding off of ducts are not uncommon when observing fetal human pancreatic cells in vitro (11;34;35), in vivo models of regeneration (14), expansion of adult human ductal tissue in vitro (36), and even when studying sections of human pancreas (37). These structures seem to indicate some recapitulation of embryonic pancreas development, and, thus, neogenesis of islet cells. At day 6, in the EC ser$^+$ group, such a structure was observed (Figure 2-3). When stained for PDX-1 protein, many of the cells in both the duct and the islet were positive, which may indicate that many of these are differentiating ß-cells.

Protein was extracted from samples taken throughout the time course from both the EC ser– group and the EC ser$^+$ group at days 0, 2, 4, and 6 for Western blot analysis. Levels of PDX-1 protein, as determined from densitometric values relative to day 0, were significantly greater at day 2 and day 6 in the EC ser$^+$ group (Figure 2-4 A). It is thought that PDX-1 is not necessary for initiation of ductal cell differentiation in a partial pancreatectomy model, however it is up-regulated in the transition of ductal to ß-cells (38). The slightly higher levels of PDX-1 protein observed at these two time points do correlate to slight increases in the number of ß-cells present at these time points (Table 2-3), however increases in ß-cells are seen in other groups which do not express significantly higher amounts of PDX-1 protein. As the Western blot is quantitating total protein present, increases may also reflect ductal cells, which express PDX-1 but are not yet expressing insulin, or δ-cells present, which are also known to express PDX-1 (39). Quantitative RT-PCR was used to determine relative levels of Pdx-1 mRNA present at day 6 EC ser$^+$. No upregulation of Pdx-1 expression was observed, indicating that PDX-

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1 amounts present were adequate for the observed differentiation, or that an increase in PDX-1 mRNA occurred at another time point.

NKX2.2 is a transcription factor required for the terminal differentiation of ß-cells (40). In EC NPI cultured with or without autologous serum, an increase in NKX2.2 protein is seen at day 6 of culture (Figure 2-4 B). An increase in the number of ß-cells present at day 6 was observed in both groups (Table 2-3) which may correspond with terminal differentiation of ß-cells.

Preproinsulin mRNA expression was observed using Northern blotting throughout the time course (Figure 2-4 C) as well as quantitative RT-PC R to determine relative expression at day 6 EC ser^+ (Table 2-4). Levels of preproinsulin at days 2, 4, and 6 were significantly higher than day 0 in the EC ser^+ group (Figure 2-4 C). Slight increases in preproinsulin were seen in the EC ser^- group. These results seem to correlate with insulin content levels measured in the two groups (Table 2-2). Quantitative RT-PCR also found a significant increase in the levels of preproinsulin levels at day 6 EC ser^+ (Table 2-4) which were 7.91±1.2 fold the levels at day 0.

Quantitative RT-PCR (qRT-PCR) was also used to determine relative expression of other islet-related genes in the day 6 EC ser^+ samples as compared to day 0: proglucagon and NeuroD/Beta2. No difference in the expression of either of these genes was observed on day 6 EC ser^+. In the case of proglucagon, this may be because the number of glucagon-positive cells had increased by day 2 when NPI were cultured EC with or without autologous serum, thus samples analyzed at day 6 were too late to see an increase in gene expression. NeuroD/Beta 2 is a transcription factor required for normal development of the pancreas (41). It is expressed early in endocrine cell development,

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which again may explain why no increase in expression was seen in day 6 EC ser$^+$ samples, as the expression may have already been down-regulated. When cDNA libraries from day 0 and day 6 EC ser$^+$ samples were screened, three genes were identified to have differences in number of clones (data not shown). Hypoxia inducing factor-1 (HIF-1), which appeared to be induced with encapsulation and culture in the library screening, did not show increased expression when analyzed by qRT-PCR. Although hypoxia has been associated with cell encapsulation (28), this is dependent on the size of the microcapsules used and may have been prevented due to the capsule preparation of the samples measured for qRT-PCR. Similarly, thymosine was seen to be induced in the library but no difference was noted from the qRT-PCR results (Table 2-4). Connective tissue growth factor (CTGF) was seen to be down-regulated in the day 6 EC ser$^+$ library, and qRT-PCR showed a significant decrease in expression (0.26 ± 0.1 fold of day 0 values). CTGF has a role in differentiation and development, and is expressed in the mesenchyme (29). Its down-regulation in EC ser$^+$ NPI may reflect the initial permissive requirements for mesenchymal stimuli associated with pancreatic development decreasing with maturity (42).

Other studies intending to mature NPI failed to decrease time required to cure diabetes post-transplantation (8;12). Using our alginate microencapsulation plus culture in HAM's F10 supplemented with 10% neonatal porcine serum, we saw a significant increase in the functional maturity of our NPI indicated by a rapid correction of diabetes in SCID mice (Figure 2-5). Three groups of animals were transplanted, the first group with day 6 EC ser$^-$ NPI intraperitonially (i.p.), the second group with day 6 EC ser$^+$ NPI i.p., and the third group with day 6 EC ser$^+$ NPI, but with the capsules dissolved and the

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NPI transplanted under the kidney capsule. The first group remained significantly more hyperglycemic than the two groups receiving EC ser^{+} islets throughout the experiment, only becoming normoglycemic (as defined by a blood glucose of <10 mmol/mL) at 47.1±2.8 days post-transplantation. Both groups receiving EC ser^{+} in vitro matured NPI normalized quickly (10.1±1.0 days and 10.7±1.0 days for i.p. and kidney capsule transplants, respectively) (Figure 2-5). The main difference between this study and previous studies is the means of providing an extracellular matrix. It appears that a free-floating microcapsule aids functional maturation more than an alginate gel containing NPI layered on a tissue culture plate (8). In addition, it would appear that length of time in culture may have an affect of the health of the NPI. Longer culture periods (up to three weeks) are associated with loss of response to glucose (unpublished observations and (8)).

Research into the in vitro expansion of human tissue in order to provide increased numbers of transplantable islets has shown some promise, however more insight into culture conditions or manipulation of the proper starting tissue is necessary. Studies investigating the origin of pancreatic cancer have led to the hypothesis that in vivo transdifferentiation of exocrine to ductal tissue may occur (43), leading to the formation of tumors which are of ductal origin. Exocrine to ductal phenotype transdifferentiation has been shown in vitro (43;44), as has expansion of ductal tissue derived from exocrine pancreas (45). When considered with recent evidence of ductal cells differentiating in vitro into islets (36), it appears that non-endocrine human pancreatic tissue, which is normally discarded after an islet isolation, may be a potential source of expanded, if not unlimited, transplantable insulin-producing tissue.

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NPI provide both a promising source of transplantable islet tissue as well as a model with which to study islet development and culture or molecular manipulations which may expand other potential tissue sources. We have shown that alginate microencapsulation and culture of NPIs with autologous serum functionally matures the tissue, allowing for much improved results post-transplantation into diabetic mice. In summary, in vitro culture of alginate microencapsulated NPI with autologous serum emphasizes the potential of this tissue source as a model of in vitro islet development. NPI may, either directly or indirectly through knowledge gained with this model, help to relieve the demand for tissue for islet transplantation.

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CHAPTER 3

IN VIVO MATURATION OF NEONATAL PORCINE ISLETS

3.1 INTRODUCTION

Recent success in clinical islet transplantation has increased the demand for transplantable insulin-producing tissue (1-4). Attention has turned to alternative tissue sources. Use of stem cells, both embryonic stem cells and pancreatic adult precursor cells, has been proposed, and promising research is being conducted evaluating these sources (reviewed in (5)). Islet sources from other species are also being considered. The pig is a prime candidate for xenotransplantable tissue as pigs may be raised in a pathogen-free environment and are physiologically quite similar to humans with regard to size of organs and metabolic demands.

The capacity of neonatal porcine islet (NPI) tissue to mature has been documented previously (6-9). The increase in β-cell mass observed once NPI are engrafted is due to neogenesis of β-cells from a population of precursor cells in the immature tissue (6;7;9;10). The precursor population is likely the ductal cells, or a subpopulation of ductal cells. Cytokeratin 7 (CK7) has been established as a marker of ductal cells (7;11). NPIs appear as clusters pre-transplantation, composed of CK7-positive cells and scattered endocrine cells, which become organized grafts populated mainly by insulin-positive cells, once matured in vivo (6-8). Numerous CK7 cells express insulin in maturing NPIs. These double-positive cells are likely protodifferentiated cells, which are becoming β-cells (7;8) (Chapter 2 of this manuscript).

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After NPIs have been transplanted into diabetic mice, a lag time is observed, while the β-cell mass increases, before animals become euglycemic. During this period, enough insulin is provided by the graft to keep the animal alive, but 6 weeks or longer is required for enough insulin to be produced to provide euglycemia to the recipient (6-8). By gaining an understanding of the maturation process of NPIs in vivo, we may be able to supply factors or signals resulting in accelerated NPI maturation.

An increase in cellular insulin content of NPI grafts, once hyperglycemia in mice is corrected, of 20 to 30 fold is seen (6-8), indicating the potential for NPIs to mature or replicate in vivo. In vivo maturation may also provide a model which can be used to study islet development. Lukinius and Korsgren paralleled in vivo development of fetal porcine islet clusters transplanted under the kidney capsule of mice to naïve maturation of the same tissue in porcine embryos (12). It was shown at all time points observed, that in vivo maturation mirrored differentiation and maturation of the naïve tissue, indicating transplantation and in vivo maturation of NPIs is a good model of neonatal islet development.

High glucose has been shown to stimulate β-cell proliferation of fetal and neonatal tissues (8;9;13-15). This is consistent with pregnancy, where increased metabolic demand results in hyperglycemia which is corrected by an increase in β-cell mass for the duration of the pregnancy (16). These considerations, in addition to re-creating a realistic scenario for NPI transplantation (into a recipient with type 1 diabetes), were taken into account when streptozotocin-induced diabetic mice were chosen to be recipients of NPI grafts for this study.

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The aims of this study were to establish a model of NPI in vivo maturation with which to study islet development, and to observe NPI maturation with the idea that we will one day be transplanting NPIs to treat diabetes, and thus need to understand the behavior of this tissue in vivo.

3.2 RESEARCH DESIGN AND METHODS

3.2.1 Preparation and transplantation of neonatal porcine islets

All animal protocols used in this study follow guidelines set out by the Canadian Council for Animal Care (CCAC). The method used to isolate NPI has previously been described (6). Briefly, donor pancreases were obtained from Landrace-Yorkshire neonatal pigs (1-3 days old, 1.5-2.0 kg body weight) of either sex. Piglets were anesthetized with halothane and subjected to laparotomy and complete exsanguination. Pancreases were removed, cut into small pieces, and digested in 2.5 mg/mL collagenase (Sigma, Oakville, ON). After filtration through a nylon screen (500 µm), the tissue was cultured for 6 days in HAM's F10 medium (Gibco, Burlington, ON) containing 10mM glucose, 50 µm isobutalmethylxanthine (IBMX; ICN Biomedicals, Montreal, PQ), 0.5% bovine serum albumin (BSA) (fraction V, radioimmunoassay grade, Sigma), 2mM L-glutamine, 10 mM nicotinamide (BDH Biochemical, Poole, England), 100 U/mL penicillin, and 100 µg/mL streptomycin.

Diabetes was induced using 225mg/kg body weight Streptozotocin (Stz.) (Sigma) injected intraperitonially (i.p.) in 6-8 week old CB17 SCID beige mice (Taconic Farms, Germantown, NY) or 175mg/kg Stz. i.p in C57BL/6 $Rag^{-/-}$ knockout mice (purchased

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from Dr. Colin Anderson, University of Alberta, Edmonton, AB, Canada). All diabetic recipients had a minimum blood glucose level of 20 mmol/L prior to transplantation. Blood glucose levels were monitored weekly using a OneTouch Ultra blood glucose monitor (Lifescan, Milpitas, CA). Between 3500 and 5500 NPI per transplant were implanted under the renal capsule of halothane anaesthetized mice (determined as 18 μg of DNA per 2000 NPI (6)). Transplanted mice were killed on days 0, 3, 6, 12, 24, and 48 post-transplantation. Graft bearing kidneys were removed, and grafts were excised and snap-frozen for insulin or protein extraction, mashed and suspended in 1 mL Trizol (Invitrogen, Carlsbad, CA) for RNA extraction, or placed in Z-fix (Anatech Ltd., Battle Creek, MI) for histological examination.

3.2.2 Insulin and DNA content analysis

Insulin content was measured after extraction in 2 mmol/L acetic acid containing 0.25% BSA. Duplicate cellular (pre-transplant) samples were sonicated in acetic acid, centrifuged (800*g*, 15 minutes), then supernatants were collected and stored at -20°C until assayed for porcine insulin content by radioimmunoassay (RIA) (Linco Research, Inc., St. Charles, MO). Grafts were homogenized and then sonicated at 4°C in 10 mL acetic acid. After incubating for 2 hours at 4°C, tissue homogenates were re-sonicated, followed by centrifugation (8000g, 20 minutes) and collection of supernatant. Pellets were further extracted as above, and a second supernatant sample was collected and added to the first. Duplicate samples were measured by RIA.

For DNA content, all measurements were assessed from duplicate aliquots of islet cell suspensions. Samples 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. Before being

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assayed, cell pellets were placed in 1000 μL of lysis buffer (10 mmol/L Tris, 1 mmol/L EDTA, 0.5% Triton X-100, 4°C, pH 7.40), sonicated, diluted 1:40 then a further 1:20, and 100 μL was aliquoted into a 96-well plate with 100 μL Pico Green reagent (5 μL/mL in lysis buffer) (Molecular Probes, Inc., Eugene, OR). Fluorescence was measured at 485nm excitation / 527nm emission of samples in duplicate. Samples were run in parallel with, and diluted in proportion to a 9-point (0-1000 ng/mL) standard curve, which was generated using a Molecular Probes standard kit.

3.2.3 Immunohistochemical analysis, quantitation of insulin-positive area

Pre-transplantation samples were dissociated into single cells by gentle agitation in calcium-free medium supplemented with 1mM EGTA and 0.5% BSA for 7 minutes before addition of 25 μg/mL trypsin (Boehringer Mannheim, Laval, PQ, Canada) and 4 μg/mL DNase (Boehringer Mannheim) followed by further pipetting for 4 minutes. After washing cells with phosphate-buffered saline, they were placed on Histobond slides (Marienfeld, Germany) and fixed in Bouin's solution for 12 minutes before storage at 4°C in 70% ethanol.

NPI grafts were processed, and embedded in paraffin. Graphs were sectioned for quantitation of the insulin-positive graft area as outlined in Appendix B. Briefly, the entire graft was sectioned (at 4μm), and a representative sample of sections, from throughout the graft, was designated a complete series for quantitation.

Tissue sections were deparaffinized and rehydrated for immunohistochemistry. Samples were stained for insulin using a guinea-pig anti-insulin antibody (1:1000; Dako, Mississauga, ON). Slides were then incubated with biotinylated goat anti-guinea-pig IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA). The avidin-biotin

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complex/horseradish peroxidase (Vector Laboratories) method of detection was used and developed with 3,3-diaminobenzidinetetrahydrochloride (DAB) (BioGenex, San Ramon, CA) to produce a brown colour. Graft sections were counterstained using hemotoxylin (Zymed Laboratories Inc., San Francisco, CA) and eosin Y solution (Sigma).

Quantitation of grafts proceeded as described in Appendix B, using photographs of non-overlapping fields of view of stained sections (taken on an AxioCam, using AxioVision, all on a Zeiss Axioskop 2-Plus microscope, Carl Zeiss Canada, Calgary, AB, Canada), and MetaMorph® Software (Universal Imaging Corporation™, Downingtown, PA) for integrated morphometry analysis. Insulin-positive area was expressed as a percentage of total graft area.

3.2.4 Protein extraction and western blotting

Grafts collected at each time point were mashed and lysed in 100 μL of ice-cold RIPA buffer for 30 minutes at 4°C (150 mM NaCl, 20 mM Tris-Cl, 1 mM EDTA, pH 7.5, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1mM DTT, and 0.1% protease inhibitor cocktail [Sigma-Aldrich Canada Ltd.]). Lysates were spun at 12,000 *g* for 5 minutes at 4°C. Supernatants were removed and assayed for total protein content using the Bradford method (BioRad, Mississauga, ON, Canada). Ten micrograms of cell lysate protein was fractioned on 10% polyacrylamide gels containing SDS in a minigel apparatus (BioRad). After the gel separation, proteins were transferred to Immun-Blot™ PVDF membrane (BioRad) at 10 mAmps for 16 hours at 4°C in buffer containing 20% (v/v) methanol, 48 mM Tris base, 39 mM glycine, and 0.037% SDS at pH 8.3. PVDF membranes were then incubated in blocking buffer containing 136 mM NaCl, 20 mM Tris, pH 7.6, and 5%

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skim milk powder for 1 hour at room temperature with gentle shaking. Membranes were washed three times for 10 minutes in PBS-T (0.1%) buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, pH 7.5 and 0.1% Tween-20). Fresh blocking solution was then applied containing 1:200 dilution of PAX6 antisera (Covance Research Products, Inc., Berkeley, CA), or 1:50 dilution of NKX2.2 antisera (Developmental Studies Hybridoma Bank, Iowa City, IA) with shaking for 2 hours. Membranes were washed as before, followed by a second antibody to rabbit IgG (for PAX6) or mouse IgG (for NKX2.2) coupled to horseradish peroxidase at 1:5000 dilution (Amersham Biosciences, Inc., Baie d'Urfé, PQ, Canada) for 1 hour at room temperature, followed by 3 additional 10 minute washes. The blots were developed with an enhanced chemiluminescence Western blotting detection kit (Amersham Biosciences, Inc).

3.2.5 RNA extraction and RT-PCR

All samples were removed under aseptic conditions utilizing RNase/Dnase free reagents and instruments to prevent cross-contamination. Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's protocol. The RNA pellet was dissolved in 50 µL DNase/RNase free water (Sigma) and the concentration measured spectrophotometrically. A 50 µL reverse transcription (RT) reaction of 1 µg of RNA was performed using MultiScribe™ Reverse Transcriptase (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The samples were placed in a thermal cycler for 10 minutes at 25°C, 30 minutes at 48°C, and 5 minutes at 95°C for reverse transcription.

Primers used are outlined in Table 3-1, and were designed using Primer 3 software (17), except those for glucagon and Nkx6.1 (18). The starting template was 1

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μL of RT reaction in a 50 μL reaction volume (1X PCR buffer, 2mM $MgCl_2$, 300 nM of each primer, 200 μm dNTP, 1.5 U Taq DNA Polymerase (Invitrogen)). PCR was performed using the Gene Amp PCR System 9700 (Applied Biosysems, Foster City, CA) using the following conditions: 25 cycles at 94°C denaturing for 30 seconds, 56°C annealing for 30 seconds, and 72°C extension for 30 seconds.

Table 3-1. Porcine specific primers used for RT-PCR.

Primer		**Sequence**								**Amplified Product (bp)**
Insulin	Forward	GCT	TCT	TCT	ACA	CGC	CCA	AG		150
	Reverse	CCA	GCT	GGT	AGA	GGG	AAC	AG		
Glucagon	Forward	GAC	AAG	CGC	CAC	TCA	CAG			227
	Reverse	TTC	ACC	AGC	CAA	GCA	ATG			
Pdx-1	Forward	CGA	GGA	GAA	CAA	GCG	AC			119
	Reverse	ACA	TGA	CAG	CCA	GCT	CCA	C		
Nkx6.1	Forward	TTT	GTT	GGA	CAA	AGA	CGG	G		165
	Reverse	AGA	CTC	TTG	ACC	TGA	CTC	TCT	G	
NeuroD	Forward	CTC	GGA	CTT	TTC	TGC	CTG	AG		149
	Reverse	GTG	GAA	GAC	ATG	GGA	GCT	GT		
Somatostatin	Forward	TGG	AGC	CTG	AAG	ATT	TGT	CCC		122
	Reverse	GAA	ATT	CTT	GCA	GCC	AGC	TTT	G	
PP	Forward	CCG	CAG	ATA	CAT	CAA	CAT	GC		232
	Reverse	ATT	TAG	CCT	GTT	TGG	GAG	CA		
β actin	Forward	TGT	ATT	CCC	CTC	CAT	CGT	G		
	Reverse	GGA	TCT	TCA	TGA	GGT	AGT	CTG	TC	500

3.2.6 Statistical analysis

Data are expressed as means±SEM of (n) independent observations. Statistical significance of differences was calculated using an unpaired Student's t-test, $p<0.05$ was considered significant.

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3.3 RESULTS

3.3.1 Blood glucose after transplantation

The blood glucose levels of animals transplanted with 3500-5500 NPIs are shown in Figure 3-1. Transplants were performed using pooled NPI tissue from 3-4 sibling piglets. Transplant recipients from each set of pooled tissue were allocated for day 0, 3, 6, 12, 24, and 48 post-transplant sampling to establish a time course of NPI in vivo development (time course groups). For analysis purposes, samples were always compared within time course groups. Blood glucose analysis of time course groups revealed two clear groups which will be referred to from this point as Group 1 – Normalized and Group 2 – Not normalized. In Group 1, transplanted NPIs had restored normoglycemia by day 48, when the final time course samples were taken. In Group 2, on day 48, animals were still hyperglycemic (defined as >10mmol/L blood glucose). Statistically significant differences in blood glucose were noted at day 6, day 12, day 24, and day 48 post-transplantation (day 6 $p<0.05$, all others $p<0.001$). As animals were killed in order to sample NPI grafts throughout the time course, the numbers of animals in both groups changed at each time point. In Group 1, 47 mice received NPI transplants, and one was sacrificed immediately. Eight animals were sacrificed to take day 3 post-transplant samples, leaving 38 animals on day 6. On day 6, nine animals were sacrificed for time point samples, thus leaving 29 mice on day 12. Nine animals were sacrificed for samples on each of days 12 and 24, thus on day 24, n= 20 mice, and on day 48, n=11 animals, all of which were sacrificed for day 48 samples. In Group 2, 59 animals were transplanted and three mice were sacrificed for day 0 samples, leaving 56 mice until day 6. On day 6, 8 mice were sacrificed, leaving 48 animals until day 12. Eleven mice were

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sacrificed on day 12, and 16 on day 24, thus n=37 mice were measured on day 24 post-transplant, and n=21 on day 48, all of which were sacrificed for samples as dya 48 was the last timepoint analyzed. In group 1, SCID mice were transplant recipients. In group 2 however, 50% of recipients were SCID mice, and 50% were C57BL/6 Rag knockout mice as the mouse model utilized by the laboratory changed at this time.

Differences between NPI tissue transplanted into Group 1 and Group 2 mice are noted in Table 3-2. No significant difference between the amount of insulin per transplant was noted (2709.81 ± 89.1 ng and 2567.18 ± 60.2 ng per transplant in Group 1 and 2, respectively), however a significant difference in DNA of NPI tissue per transplant was noted (50420.92 ± 1528.5 ng and 33223.83 ± 783.7 ng per transplant in Group 1 and 2, respectively). Overall, a higher percentage of the transplanted NPIs stained positively for insulin in the Group 2 transplants (20.85 ± 0.5%, compared to 15.33 ± 0.3% in the Group 1 transplants). Due to more DNA per transplant in the Group 1 transplants, calculation of the number of β-cells per transplant resulted in a higher number of β-cells transplanted into Group 1 recipients (Table 3-2). One other difference between Group 1 and Group 2 was the lot of streptozotocin used to induce diabetes as a new lot was purchased during the course of the experiments.

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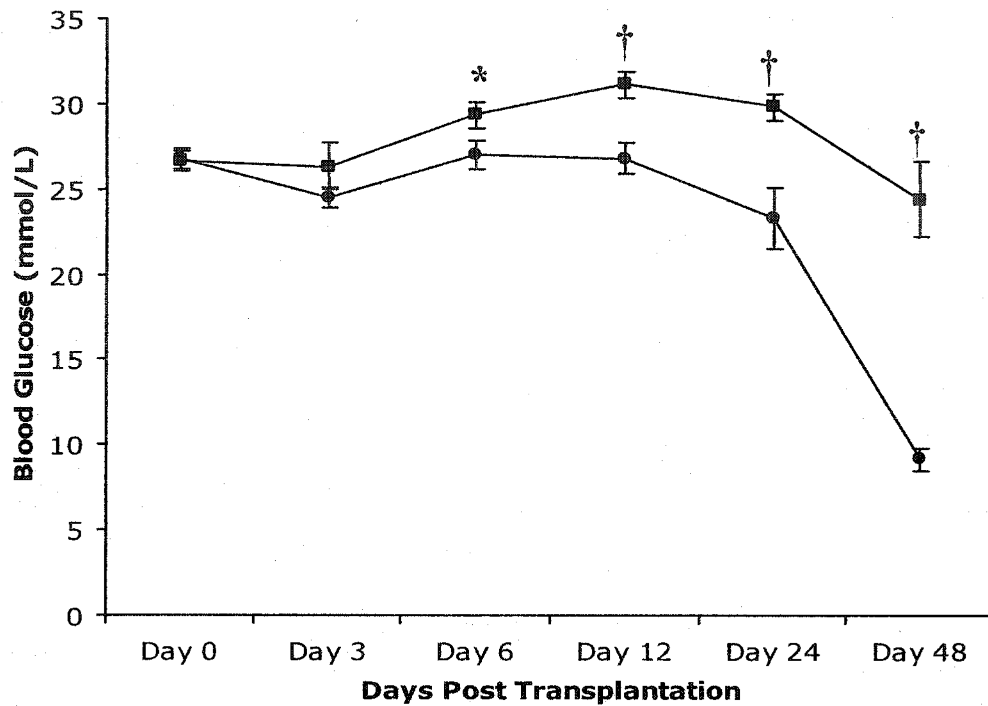

Figure 3-1. NPIs derived from sibling piglets were pooled, and transplanted into immunoincompetent Stz. diabetic mice. Two groups of animals were defined based on the ability of NPIs to correct hyperglycemia within the 48 day observation period (Group 1 – Normalized, (●), n=47 day 0, n=38 day 6, n=29 day 12, n=20 day 24, n=11 day 48) or not able to correct hyperglycemia (Group 2 – Not normalized, (■), n=59 day 0, n=56 day 6, n=48 day 12, n=37 day 24, n=21 day 48). Statistical significance of difference was determined by a Student's unpaired t-test. *$p<0.05$, †$p<0.001$

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Table 3-2. Characteristics of tissue transplanted. Mice receiving transplants from tissue resulting in correction of hyperglycemia within the 48 day observation period were placed into Group 1. Group 2 includes all animals receiving NPI tissue which did not correct hyperglycemia by day 48 in this experiment.

	Per Transplant (ng)		Per Transplant		
	DNA	Insulin	% insulin +ve cells	# of ß-cells (x10^6)	Stz. Lot #
Group 1 - Normalized (n=47)	50420.92 ± 1528.5	2709.81 ± 89.1	15.33 ± 0.3	1.10 ± 0.4	119H1029
Group 2 - Not Normalized (n=59)	33223.83 ± 783.7 *	2567.18 ± 60.2	20.85 ± 0.5 *	0.95 ± 0.2	12K1478

Data are expressed as mean ± SEM from the indicated number of independent experiments.
Statistical significance of difference was determined using a Student's unpaired t-test. *p<0.001 vs. Group 1

3.3.2 Graft insulin content at time points analyzed

Overall, insulin content of grafts recovered from Group 1 animals had increased more than in grafts recovered from Group 2 animals (Table 3-3). At day 6 post-transplantation, grafts from Group 1 had increased 1.20 ± 0.1 fold, whereas Group 2 grafts decreased and contained only 0.24 ± 0.1 fold insulin of pre-transplant values (statistically significant, p<0.005). Insulin content increased in both groups in samples analyzed from day 12, 24, and 48 post-transplant grafts, and differences between the two groups at these time points were not significant. However, grafts from Group 2 took much longer than those from Group 1 to increase in content beyond pre-transplant values (Table 3-3).

Table 3-3. Fold increase in insulin content extracted from grafts removed at the time points indicated post-transplantation from Group 1 and Group 2.

	Fold increase insulin recovered vs. pre-transplant values			
	Days Post Transplantation			
	6	12	24	48
Group 1 - Normalized	1.20 ± 0.1 (3)	0.80 ± 0.3 (3)	2.85 ± 1.3 (3)	12.15 ± 3.9 (4)
Group 2 - Not Normalized	0.24 ± 0.1 (4) *	0.27 ± 0.1 (5)	0.91 ± 0.2 (6)	7.18 ± 1.4 (6)

Data are expressed as mean ± SEM of (n) independent experiments.
Statistical significance of difference was determined by Student's unpaired t-test. *p<0.005 vs. Group 1

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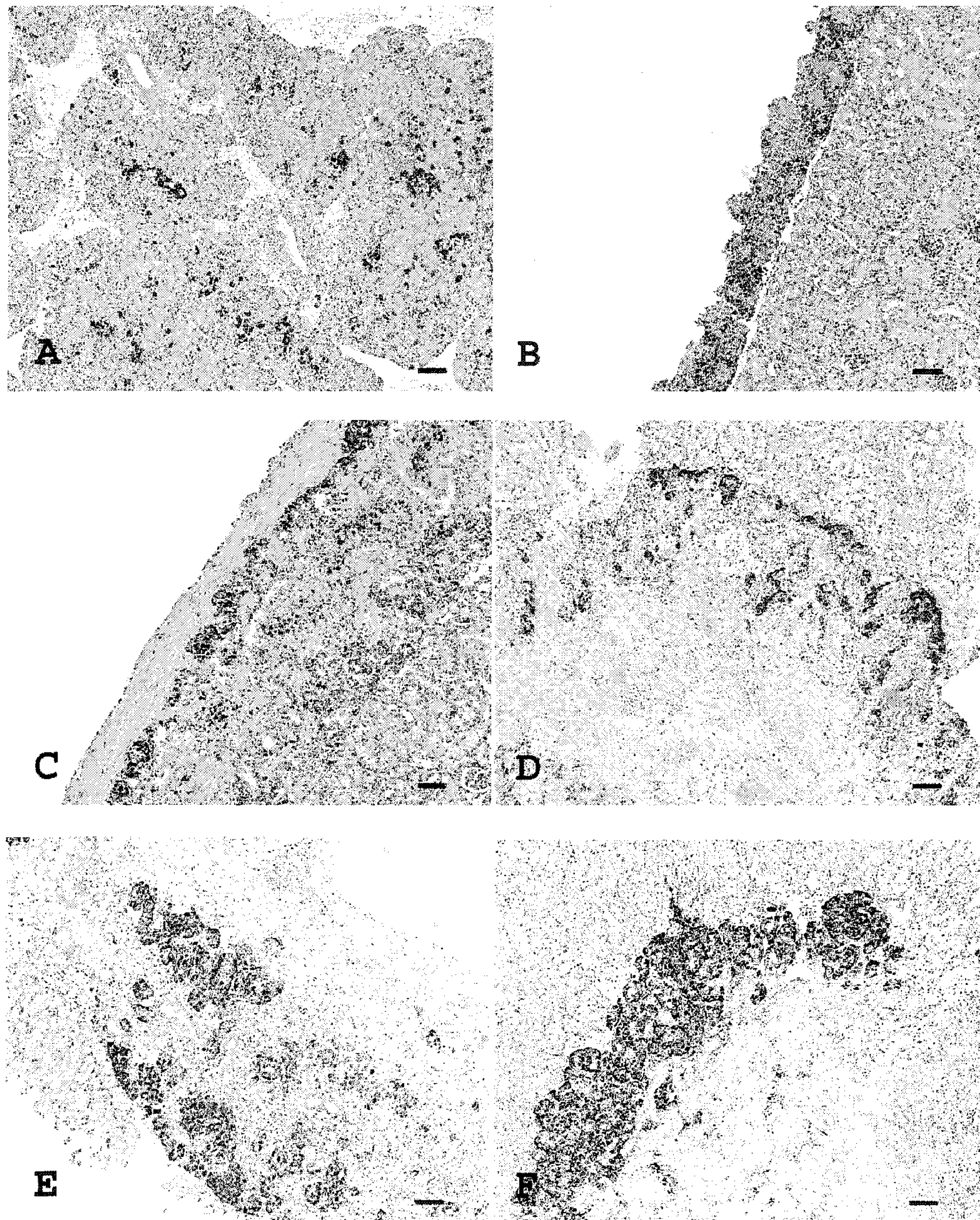

Figure 3-2. Immunohistochemical analysis of insulin-positive tissue during in vivo NPI maturation. (A) Neonatal porcine pancreas. Insulin-producing cells are scattered throughout the pancreas, there are no distinct islets. (B) Day 0 graft. Shortly after transplantation, the isolated NPI transplanted under the kidney capsule appear similar to the neonatal porcine pancreas in regard to lack of islet organization. (C) Day 6 graft and (D) Day 12 graft. Insulin-producing cells begin to cluster together 6 and 12 days post-transplantation. (E) Day 24 graft. By 24 days post-transplant, NPIs have formed distinct clusters. (F) Day 48 graft. The area of the graft staining positively for insulin has clearly increased by day 48, insulin-positive cells have taken over most of the graft. Scale bars represent 50 μm.

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3.3.3 Morphology of recovered grafts and area of insulin-positive tissue

When the NPI grafts from days 0, 6, 12, 24, and 48 were immunohistochemically stained for insulin and analyzed, no qualitative difference was noticed between Group 1 and Group 2. As illustrated in Figure 3-2A, insulin-positive cells are scattered throughout the neonatal porcine pancreas, and not arranged into islets. Grafts retrieved only one hour post-transplant (day 0, Figure 3-2B) have a similar pattern of scattered insulin-positive cells. By day 6 (Figure 3-2C) and day 12 (Figure 3-2D), the insulin staining cells appear grouped together, and by days 24 and 48 post-transplant (Figure 3-2E and F, respectively), the insulin-positive cells are grouped together throughout the graft. Qualitatively, an increase in the area of the graft staining positively for insulin is observed.

This increase was analyzed quantitatively using a technique described in Appendix B of this text to calculate the relative area of the graft stained for insulin. Results from this quantitation are shown in Table 3-4. Samples from Group 1 and

Table 3-4. Results of quantitation of the area of grafts staining positively for insulin, expressed as a percentage of total graft area, at days 0, 6, 12, 24, and 48 post-transplantation. As no significant differences were noted between groups 1 and 2, the values were combined.

	% of tissue area which is insulin-positive				
	Day 0	Day 6	Day 12	Day 24	Day 48
Group 1 - Normalized	5.09±0.7 (2)	5.71±1.0 (2)	5.90±0.8 (3)	8.12±1.6 (3)	15.52±2.7 (3)
Group 2 - Not Normalized	3.60±0.5 (4)	3.51±0.5 (2)	3.29±0.5 (2)	9.59±2.8 (2)	13.55±2.7 (3)
Combined	4.10±0.5 (6)	4.64±0.8 (4)	4.86±0.8 (5)	8.71±1.3 (5)	14.54±2.7 (6)

Data are expressed as mean ± SEM of (n) independent experiments. Statistical significance of differences between groups 1 and 2 were analyzed using a Student's unpaired t-test, no significant differences were found.

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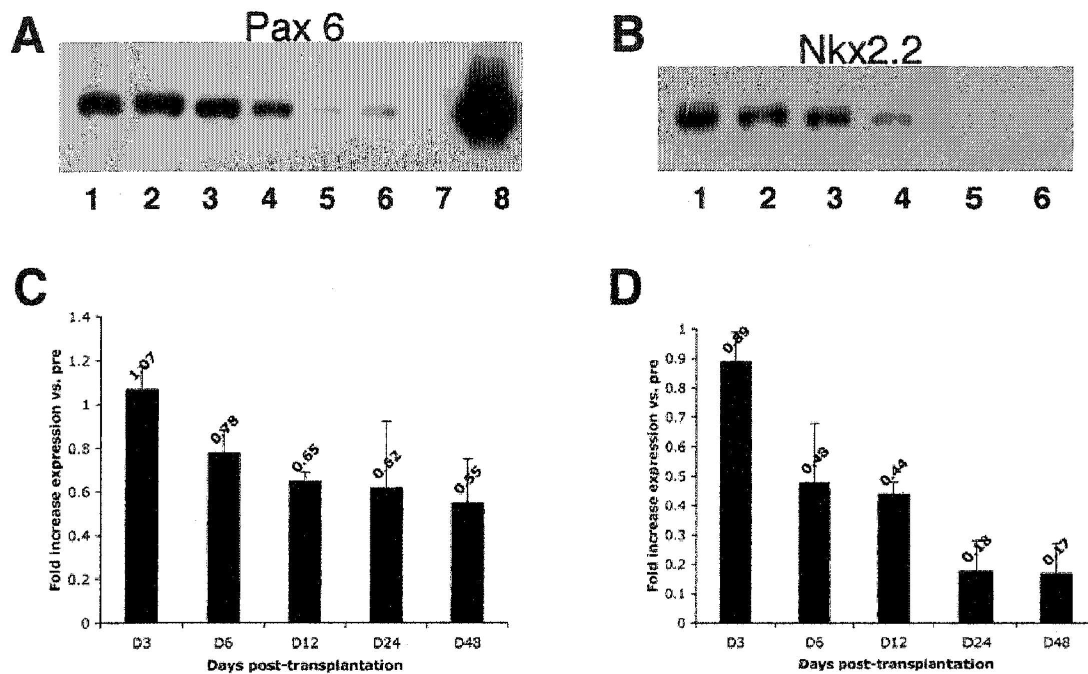

Figure 3-3. Western blot analysis of PAX6 and NKX2.2 expression in in vivo matured NPI grafts. (A) Representative blot probed for PAX6. Lanes were as follows: 1. pre transplant (pre-tx) tissue, 2. Day 3 graft, 3. Day 6 graft, 4. Day 12 graft, 5. Day 24 graft, 6. Day 48 graft, 7. blank, 8. Pax6 transfected NPI control. (B) Representative blot probed for NKX2.2. Lanes were as follows: 1. pre-tx tissue, 2. Day 3 graft, 3. Day 6 graft, 4. Day 12 graft, 5. Day 24 graft, 6. Day 48 graft. (C) Graphical representation of an average of 4 PAX 6 densitometric values from individual experiments, expressed as relative to pre-tx tissue. (D) Graphical representation of an average of 4 NKX2.2 densitometric values from individual experiments, expressed as relative to pre-tx tissue.

Group 2 were analyzed, however no significant differences were found between the two groups at any time point with regards to percentage insulin-positive area, thus results were combined. Similarly to the insulin content of the grafts, percent insulin-positive graft area remained constant from day 0 post-transplant until after day 12 post-transplant

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(4.10 ± 0.5%, 4.64 ± 0.8%, and 4.86 ± 0.8% for days 0, 6, and 12, respectively). By day 24 post –transplant, the area of insulin positive tissue within the grafts had increased to 8.71 ± 1.3%, and by day 48 it was 14.54 ± 2.7% (Table 3-4).

3.3.4 PAX6 and NKX2.2 protein expression throughout in vivo maturation

Western blots analyzed for PAX6 and NKX2.2 combined samples from Group 1 and Group 2, as no difference was noted (data not shown). Figure 3-3A and B illustrate representative Western blots for PAX6 and NKX2.2, respectively. Expression of both transcription factors decreased throughout the time course. Blots were quantitated using densitometry, values from which were normalized to the corresponding coomassie blue stained membrane to correct for any loading inconsistencies. Figure 3-3C graphically represents the values obtained using densitometry to calculated PAX6 expression and Figure 3-3D represents NKX2.2 expression. PAX6 expression is present at all points throughout the time course, and its relative expression only increases 1.07 ± 0.1 fold over pre-transplant values (baseline NPI values) prior to decreasing gradually until at day 48 post-transplant, when PAX6 expression is half of baseline values. NKX2.2 drops in day 3 samples from the baseline value, and by day 24 is barely detectable (Figure 3-3).

3.3.5 RT-PCR analysis of islet genes expressed throughout in vivo maturation

Porcine specific primers were used to detect mRNA expression of endocrine genes throughout the time course of in vivo NPI maturation. RT-PCR is not quantitative, thus results in Table 3-6 indicate the presence of gene expression in all samples analyzed (+), or in some of the samples analyzed (+/-). All of the genes analyzed were expressed during the time course in at least one sample analyzed. Insulin, glucagon, NeuroD,

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somatostatin, and pancreatic polypeptide (PP) mRNA was expressed in all samples analyzed from all time points. Pdx-1 mRNA expression was variable at all time points, and Nkx6.1 mRNA was detected in all pre-transplant samples, and all day 48 samples, but was variable at all other time points.

Table 3-5. RT-PCR analysis of islet gene expression throughout in vivo NPI maturation.

	Insulin	Glucagon	Pdx-1	Nkx6.1	NeuroD	Somato.	PP
Pre-transplant (n=3)	+	+	+/-	+	+	+	+
Day 6 (n=3)	+	+	+/-	+/-	+	+	+
Day 12 (n=3)	+	+	+/-	+/-	+	+	+
Day 24 (n=3)	+	+	+/-	+/-	+	+	+
Day 48 (n=3)	+	+	+/-	+	+	+	+

+ indicates presence of mRNA as detected by RT-PCR.
+/- indicates presence in at least one, but not all, samples.

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3.4 DISCUSSION

Fetal and neonatal porcine islets have great potential for maturation, however this process takes 6 weeks or more post-transplantation (6;7;9;10). It is the hope of many researchers that this tissue will provide an unlimited source of xenotransplantable insulin-producing tissue. Understanding of the processes and signals involved in in vivo maturation of NPIs will provide insight into optimizing tissue and conditions for transplantation. Looking at molecular markers during in vivo NPI development may provide insight as to what signals are involved in maturation, potentially leading to means of hastening the maturation process and eliminating the lag time commonly seen after transplantation of immature tissue.

Animals in this study were killed on days 0, 3, 6, 12, 24, and 48 post-transplantation to observe NPI graft maturation, regardless of whether the graft had corrected hyperglycemia or not. Two distinct groups of animals were found based on blood glucose levels: Group 1, in which the NPIs transplanted had reversed hyperglycemia by day 48, and Group 2, in which the majority of animals killed on day 48 had not reached normoglycemic levels. Animals sacrificed at earlier time points were grouped with respect to the day 48 blood glucose results of mice transplanted with the same NPIs. It is not clear why some NPIs correct hyperglycemia in mice faster than others, however dissecting the differences between the two groups which arose during these experiments may provide some insight. Differences in the ability of NPI isolated and transplanted at different centers to correct hyperglycemia in mice have been documented, however a reason for these differences has not been determined (7). Although it was not the original intent of this study, the animals sacrificed at each time

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point for graft analysis were treated as two separate groups to observe any differences in the in vivo maturation of the NPIs.

The NPIs that were transplanted into Group 1 and Group 2 were characterized (Table 3.2). Interestingly, the insulin content of the NPIs going into the two groups were nearly identical. A higher percentage of dissociated NPI cells stained positively for insulin in Group 2 than in Group 1. More cells went into the Group 1 transplants than the Group 2 transplants as indicated by DNA values, equating to 3500 NPIs per transplant in Group 1 vs. 5500 NPIs per transplant in Group 2. The smaller DNA value in Group 2 samples may indicate death of the tissue post-isolation, in culture prior to transplantation, indicating less viable tissue.

Based on the percentage of insulin-positive cells in the transplanted tissue, more β-cells went into the Group 1 transplants than in the Group 2 transplants. In light of a recent study by Dor et al. emphasizing the importance of β-cell proliferation (19), rather than neogeneis from a precursor cell, this higher number of β-cells may play a role in graft success. Evidence in fetal pancreatic islet-like clusters (ICCs) indicates that a large population of epitheloid cells exists post-transplant of ICCs that decreases during graft maturation. This decrease is accompanied by an increase in the number of β-cells in the graft, but no increase in graft size, thus indicating a role of these epitheliod cells and neogenesis of β-cells (9). It is likely that both neogenesis and proliferation have a role in NPI graft development and functional maturation.

Another difference noted between the two groups of transplanted mice was the lot of streptozotocin (Stz.) used to chemically-induce diabetes. If the activity of lot # 119H1029 was less than that of lot # 12K1478, fewer of the β-cells in the mouse pancreas

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may have been fatally damaged, allowing these cells to participate in correction of hyperglycemia, and explaining the accelerated return to euglycemia in this group. Unfortunately the pancreases of sacrificed mice were not obtained for analysis to confirm this possibility. A further complicating factor is the individual nature of the piglets used to obtain NPIs.

Insulin content of grafts removed 6 days post-transplantation had dropped to significantly lower levels in Group 2, to one quarter of the transplanted content (Table 3-3). In contrast, in Group 1 the graft insulin content had increased slightly. Day 12 samples showed no increase in the Group 2 insulin content, and Group 1 samples had dropped to 80% of the originally transplanted insulin content. At some point between day 12 and day 24 post-transplantation, graft insulin content in both groups increased around 3.5 fold. Between days 24 and 48 post-transplantation, Group 1 animals increase graft insulin content a further 4.3 fold, and Group 2 animals a further 7.9 fold (Table 3-3). As these insulin content values are of the excised graft, residual insulin left in the pancreas will not influence these values. Insulin content alone can explain why the Group 1 animals had normalized by day 48.

It has been reported that there is a period of remodeling post-transplantation, during which transplanted islets undergo substantial death (22). This is observed in syngeneic islet transplants, thus it is not due to an immune response to the foreign tissue, but is likely a response to the surgical procedure itself. Trauma from transplantation of fetal islet-like clusters (ICC) under the kidney capsule of nude mice resulted in an increase of heat shock protein 70 (HSP 70) after transplantation which decreased within 3-9 days post-transplant (23). HSP is produced non-specifically in response to stress

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conditions and is a measure of the stress which islets are exposed to after transplantation (23). It has been shown that revascularization of the grafts, as analyzed by Doppler flowmetry of blood flow to the islet graft and adjacent kidney, is 70% in place only three days post-transplantation, and continues to improve regardless of hyperglycemia (23). Extrapolating from these studies, perhaps the insulin content dropped up to 12 days post-transplant in our NPI grafts due to stress and tissue remodeling, with an increase in insulin content between 24 and 48 days post-transplant, once the graft has revascularized, remodeled, and stress has been relieved.

Figure 3-2 illustrates the scattered nature of insulin-producing cells within the immature graft (Fig 3-2B), and the subsequent arrangement and increase of insulin-producing cells throughout in vivo NPI maturation (Figure 3-2C through F). A technique was developed to quantitate the percent area of the graft which stained positively for insulin (described in detail in Appendix B). No significant differences were noted between Group 1 and Group 2 with regards to percent insulin-positive graft area, and the same pattern of increasing percentages over the time course was observed, so the two groups were combined at this point. Interestingly, as noted in Table 3-2, Group 2 had a higher percentage of β-cells before transplantation (20.85% vs. 15.5% in Group 1), however immediately after transplantation, as observed by quantitating day 0 grafts, Group 2 had a lower total graft area staining positively for insulin (3.60% vs. 5.09% in Group1, Table 3-4). This could be due to the transplant procedure as more cells were transplanted into Group 1, likely more tissue successfully was grafted. In addition, the lower DNA values of Group 2 transplanted tissue may reflect unhealthy tissue in vitro, prior to transplantation.

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Quantitation of insulin-positive area could determine fewer β-cells which happen to be more mature, and are storing more insulin. Other groups have used point morphometry to calculate number of β-cells and β-cell mass of grafts (24). Although we cannot compare our percent insulin-positive graft area numbers directly to increases in β-cell numbers, we can compare the relative increases in these parameters. Weir et al. observe very similar relative increases in β-cell numbers when quantitating insulin-positive area. They report a 1.5 fold increase after the first week in vivo, up to a 2.4 fold increase in β-cells by 56 days post-transplantation (24). The fold increase in insulin-positive area of our grafts is 1.1 fold after 1 week, and 3.55 fold by day 48. It does appear that the observed increase in percentage of graft area staining positively for insulin post in vivo maturation is related to the increase in cell number that Weir et al. observe.

Pancreatic development is controlled by transcription factors turning on and off genes at specific times to determine cell fate (reviewed in (25-27)). Western blotting was used to detect PAX6 and NKX2.2 protein during NPI in vivo maturation to determine when these transcription factors are expressed in NPI development. Expression of both proteins decreased over the time course analyzed (Figure 3-3). PAX6 is associated predominantly with glucagon-producing cells, but has a role in insulin, glucagon, and somatostatin gene transcription as it binds to all three promoters. The insulin-positive area of the graft increases after day 12, thus PAX6 may be expressed at the earlier time points to provide signals leading to endocrine cell differentiation as well as hormone production. NKX2.2 protein expression is highest at day 3 post-transplantation, almost the same as in the pre-transplant samples (0.89 ± 0.1 fold of pre-transplant). NKX2.2

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expression drops at days 6 and 12 post-transplantation, and is nearly undetectable on day 24 and 48. Nkx2.2$^{-/-}$ mice lack mature β-cells (30), thus NKX2.2 protein expression must be required early on for the neogenesis of β-cells, preceeding the increase observed in insulin-positive graft area. By observing transcription factor profiles in in vivo NPI maturation, it may be determined what signals are required at what time points to optimally mature tissue. The use of this model to study other transcription factors, and other time points may provide clues as to how we can accelerate NPI maturation.

RT-PCR was used to determine gene transcription of islet related genes during in vivo NPI maturation, showing that insulin, glucagon, NeuroD, somatostatin, and PP mRNA was present in grafts removed at all the timepoints studied (Table 3-5). In at least one sample analyzed at all time points, Nkx6.1 and Pdx-1 mRNA was present (Table 3-5). Quantitative PCR will reveal expression levels throughout maturation. Knowing time points at which genes are expressed will be valuable as the timing to trigger development will likely be relevant.

This study provides snapshots of what is happening at points in time when the grafts are removed. We know that much of the graft maturation and increase in insulin-producing tissue occurs between day 12 and day 24 post-transplantation. We see a drop in insulin content shortly after transplantation, which may be attributed to stress-associated death of tissue and trauma from the transplantation procedure (23). Remodeling of the transplanted tissue, as well as revascularization of the graft, takes place shortly after transplantation (22;23), after which the NPI tissue begins to mature. Future studies to look at apoptosis in NPI grafts post-transplantation, and throughout the time course must be performed to confirm that apoptosis is occurring. If anti-apoptotic

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factors could be supplied initially to prevent some cell death, or factors supplied to promote angiogenesis, the success of NPI engraftment may be improved. The maturation process does appear to begin immediately after transplantation, as transcription factors involved are expressed at early time points, providing an increase in insulin-producing tissue after 12 days in vivo. This increase in insulin-positive tissue continues until the last time point observed 48 days post-transplantation. It is expected that the amount of insulin-positive tissue will plateau after this time point as has been seen between day 40 and 60 in pig (31), and similarly, but at earlier time points, in rat islet development (32-34).

This study indicates that the number of cells transplanted, as determined by DNA quantitation, but not insulin content of cells transplanted, may have a role in the speed of NPI maturation perhaps by providing some kind of physical protection to the majority of the graft, allowing more tissue to survive the transplantation procedure. Study of the immediate post-transplant period may provide understanding of how engraftment may be improved. This knowledge would be relevant if fewer islets could be required for successful islet transplantation, thus providing some relief to the demand for high numbers of transplantable islets. We believe that the in vivo maturation of NPIs provides a valuable tool for the study of islet development which may be transferred to human islet development. Understanding the signals required for maturation of NPIs will aid in the understanding of how this tissue may be manipulated in order to correct hyperglycemia more quickly post-transplantation.

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CHAPTER 4

Pdx-1 ADENOVIRAL TRANSFECTION OF NEONATAL PORCINE ISLETS DOES NOT INDUCE MATURATION

4.1 INTRODUCTION

Neonatal porcine islets (NPIs) have been identified as a potential source of xenotransplantable insulin-producing tissue due to their ease of isolation and potential for growth and expansion (1). After nine days culture, NPIs have been shown to consist of 55% endocrine and 45% ductal (endocrine precursor (2;3)) cells. NPIs secrete significant amounts of insulin in response to glucose, exhibit growth both in vitro and in vivo, and correct diabetes in nude mice within 2-6 weeks post-transplantation (1). Although this lag time post-transplant is shorter than that seen with fetal porcine tissue (2 months (4)), there is clearly a delay during which time the islets must mature in vivo. In vitro maturation of NPIs prior to transplant will not only expand the pool of transplantable tissue, it will allow for study and understanding of islet cell development.

The homeodomain protein PDX-1 is critical for pancreatic development, as seen in Pdx-1 knockout mice which do not develop a pancreas (5;6). A second role for PDX-1 is as a transcription factor for several β-cell specific genes in terminally differentiated β-cells, such as GLUT 2 (7), glucokinase (8), and insulin (9). Loss of Pdx-1 after initial pancreatic development may direct a common endocrine precursor cell towards a non- β-cell fate, however some δ-cells do express PDX-1 and Pdx-1 does activate the somatostatin gene (10;11).

Ectopic expression of Pdx-1 has been demonstrated in the liver using a recombinant adenovirus, with Pdx-1 controlled by the CMV promoter, by injection into

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the tail vein of diabetic mice (12). Hepatic insulin levels increased, as did serum insulin levels, and the blood glucose of streptozotocin-treated diabetic mice dropped to near normal levels. Transgenic mice expressing ectopic PDX-1 in the liver (13), as well as those receiving a Pdx-1 containing adenovirus directly into the pancreas (14) indicated endocrine pancreatic cell development and β-cell neogenesis, respectively. Induction of intestinal cells or embryonic stem cells to express β-cell specific genes post-Pdx-1 transfection has been reported in vitro (15;16).

We hypothesized that overexpression of PDX-1 would enhance differentiation and proliferation in NPI endocrine precursor cells, thus increasing β-cell content, and resulting in a NPI graft which would correct diabetes faster in mice than untreated NPI. When transfecting clusters of cells, a technique must be chosen which may allow transgene expression in cells other than those on the surface. Viral vectors are an effective means of transducing islets (17-20). A transient infection, as offered by an adenovirus which will not incorporate into the host cell genome, would be ideal to provide signals committing precursor cells towards a β-cell phenotype, without continuous overexpression of the gene. We first ascertained a multiplicity of infection (MOI), which would transfect a large number of NPI cells, thus targeting as many precursor cells as possible, while having no harmful or toxic effect on the NPIs. In order to enhance NPI maturation, an in vitro maturation technique was used (described in Chapter II of this manuscript), as well as the addition of exendin-4. Exendin-4 is a glucagon-like peptide 1 (GLP-1) analogue (21) which has been shown to upregulate Pdx-1 gene expression as well as to stimulate differentiation of pancreatic ductal cells into endocrine cells (22-25). Attempts were made to purify a ductal cell population from

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NPIs which would confirm the role of these cells as precursors to endocrine cells, however these were not met with success (see Appendix A). In order to determine whether an in vivo environment would have an effect on adenoviral Pdx-1 (AdPdx-1) transfected NPI maturation, transplants were performed in naïve and streptozotocin-induced diabetic SCID mice.

4.2 RESEARCH DESIGN AND METHODS

4.2.1 Amplification of recombinant adenoviral construct

A recombinant adenovirus containing mouse Pdx-1 was a kind gift from Dr. Harry Heimberg (Brussels Free University, Brussels, Belgium). Briefly, the coding sequence of mouse Pdx-1 was subcloned into the shuttle vector and constitutively expressed under control of the CMV promoter. pAdTrack-CMV contained the eGFP cDNA behind a second CMV promoter. Replication-deficient recombinant adenovirus expressing Pdx-1 in combination with GFP was generated following the standard protocol as described by He et al. (26). Pdx-1 was sequenced for confirmation following sub-cloning. Amplification was performed in 293 cells (Microbix Biosysems Inc., Toronto, ON) and adenovirus was purified using a cesium chloride gradient. The viral lysate obtained was dialyzed using Slide-A-Lyzer 10K (Pierce, Rockford, IL) in dialysis buffer (10mM Tris pH 7.4, 1mM $MgCl_2$, 10% glycerol). Viral titers were determined by plaque assay on 293 cells and were generally in the range of 10^8 to 10^9 plaque-forming units (PFU)/mL.

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4.2.2 Neonatal porcine islet and serum preparation and NPI transfection

All animal protocols used in this study follow guidelines set out by the Canadian Council for Animal Care (CCAC). The method used to isolate NPIs has previously been described (1). Briefly, donor pancreases were obtained from Landrace-Yorkshire neonatal pigs (1-3 days old, 1.5-2.0 kg body weight) of either sex. Piglets were anesthetized with halothane and subjected to laparotomy and complete exsanguination. Pancreases were removed, cut into small pieces, and digested in 2.5 mg/mL collagenase (Sigma, Oakville, ON). After filtration through a nylon screen (500 μm), the tissue was cultured for 6 days in HAM's F10 medium (Gibco, Burlington, ON) containing 10mM glucose, 50 μm isobutalmethylxanthine (IBMX; ICN Biomedicals, Montreal, PQ), 0.5% bovine serum albumin (BSA) (fraction V, radioimmunoassay grade, Sigma), 2mM L-glutamine, 10 mM nicotinamide (BDH Biochemical, Poole, England), 100 U/mL penicillin, and 100 μg/mL streptomycin. Blood collected from piglets during surgery was aliquotted into Vacutainer® SST® Gel and Clot Activator tubes (Becton Dickinson, Franklin Lakes, NJ), incubated for 20 minutes and centrifuged at 650 *g* for 15 minutes for serum removal. Neonatal porcine serum was heat inactivated at 58°C for 30 minutes and stored at -20°C until use.

NPIs were infected at a variety of multiplicities of infection (MOI), ranging from 0.4 to 22, in order to determine optimal conditions. Initial experiments tested culture times with virus of 24 and 48 hours at 37°C, eventually only a 24 hour culture period was used as protein production was similar to the 48 hour period, and it was felt that a shorter exposure to the virus would be beneficial for the NPIs. After infection, cells were cultured an additional 48 hours to allow for protein expression. Exendin-4 (American

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Peptide Company, Inc., Sunnyvale, CA) was added to media for some experiments at a concentration of 100nM. For dissociation and re-aggregation experiments, NPIs were dissociated into single cells by gentle agitation in calcium-free medium supplemented with 1mM EGTA and 0.5% BSA with 25 μg/mL trypsin (Boehringer Mannheim, Laval, PQ, Canada) and 4 μg/mL DNase (Boehringer Mannheim). Chamber slides (Falcon, Becton Dickinson, Franklin Lakes, NJ) or 6-well plates (Falcon) were coated with either 1 mg/mL Poly-D-Lysine (Sigma-Aldrich, St. Louis, MO), or 0.01% (w/v) Poly-L-Lysine (Sigma) for 30 minutes at 37°C. Dissociated NPIs were plated and/or AdPdx-1 transfected, and cultured for 72 hours in Ham's + 5% autologous serum at 37°C.

4.2.3 NPI Alginate microencapsulation and culture with autologous serum

For alginate microencapsulation, NPIs were washed with HBSS (Sigma) without calcium or magnesium, supplemented with 10 mmol/L HEPES. Islets were resuspended in 0.5mL HBSS and 0.5mL of 1.5% (w/v) highly purified MVG alginate (Pronova Biomedical, Oslo, Norway) dissolved in HBSS. The resulting islet/alginate mixture was vortexed and transferred into a 1mL syringe. Microcapsules (350 – 450 μm in diameter) were formed by passing the alginate/islet suspension through an electrostatic generator followed by collection in a 120mmol/L $CaCl_2$ (10mmol/L HEPES, 0.01% Tween 20) solution. The capsules were washed by gravity sedimentation in HAM's F10 medium. Day 0 samples were taken immediately after the encapsulation procedure. Encapsulated NPI were cultured in HAM's F10 medium supplemented with 10% autologous neonatal porcine serum (prepared as described above) in order to provide ECM and growth factors to the NPI, as described in Chapter 2 of this manuscript. Media was changed every second day for the duration of the culture period. For sampling, alginate capsules were

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dissolved by incubating the encapsulated islets in calcium-free medium for 15 minutes at 37°C.

4.2.4 Insulin and DNA content analysis

All measurements were assessed from duplicate aliquots of samples. Hormone content was measured after extraction in 2 mmol/L acetic acid containing 0.25% BSA. Samples were sonicated in acetic acid, centrifuged (800*g*, 15 minutes), then supernatants were collected and stored at -20°C until assayed for porcine insulin content by radioimmunoassay (Linco Research, Inc., St. Charles, MO). For DNA content, 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. Before being assayed, cell pellets were placed in 1000 μL of lysis buffer (10 mmol/L Tris, 1 mmol/L EDTA, 0.5% Triton X-100, 4°C, pH 7.40), sonicated, diluted 1:40 then a further 1:20, and 100 μL was aliquoted into a 96-well plate with 100 μL Pico Green reagent (5 μL/mL in lysis buffer) (Molecular Probes, Inc., Eugene, OR). Fluorescence was measured at 485nm excitation / 527nm emission of samples in duplicate. Samples were run in parallel with and diluted in proportion to a 9-point (0-1000 ng/mL) standard curve, which was generated using a Molecular Probes standard kit.

A static incubation was used to determine in vitro viability of NPI samples. NPI samples were incubated in low (2.8 mM) and high (20 mM) glucose-containing Ham's for 2 hours in duplicate, and media supernatant samples were assayed for insulin content by radioimmunoassay (Lincon Research, Inc.). Results were expressed as a stimulation index which was calculated as percentage insulin release (insulin released in supernatant

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divided by total insulin as calculated by insulin content of tissue plus the insulin content of the media) at 20 mM glucose divided by percentage release at 2.8mM glucose.

4.2.5 Immunohistochemical and morphological analysis

For determination of populations of cells, samples were dissociated into single cells as described above. After washing cells with phosphate-buffered saline, they were placed on Histobond slides (Marienfeld, Germany) and fixed in Bouin's solution for 12 minutes before storage at 4°C in 70% ethanol. Primary antibodies used were: guinea-pig anti-insulin 1:1000 (Dako Diagnostics, Mississauga, ON, Canada), mouse anti-glucagon 1:5000 (Sigma), mouse anti-CK7 1:200 (Dako), and rabbit anti-PDX-1 253 1:1000 (kind gift from Dr. J.F. Habener, Massachusetts General Hospital, Boston, MA). Microwave antigen retrieval was performed for 6 x 5 seconds in 10mM sodium citrate on high power (Sanyo, 1260W) prior to CK7 and PDX-1 staining. Following quenching with a 20% H_2O_2 /80% methanol solution, dissociated cells were blocked with 20% normal goat serum (Vector Laboratories, Burlingame, CA) for 15 minutes followed by a 30 minute incubation with primary antibody. Slides were washed and incubated with the corresponding biotinylated secondary antibody for 20 minutes (1:200; Vector Laboratories). The avidin-biotin complex/horseradish peroxidase (Vector Laboratories) detection method was used and developed with 3,3-diaminobenzidinetetrahydrochloride (DAB) (BioGenex, San Ramon, CA) to produce a brown colour. Light microscope images were acquired using an Axiocam MRC high resolution colour digital microscopy camera mounted on an Axioskop-2 plus microscope, using AxioVision software (Carl Zeiss Canada Ltd., Calgary, AB).

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For confocal imaging, a previously describe protocol was followed (27). Briefly, islets were fixed in suspension in Zamboni's fixative for 18 hours at 4°C followed by three washes in Sorenson's phosphate buffer. Islets were dropped onto microscopic slides with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame,CA) and coverslipped with #0 thickness glass coverslips for viewing on the inverted confoal laser scanning microscope (with an Argon/Krypton laser) using the 100x lens with oil immersion.

4.2.6 Protein extraction and western blotting to detect PAX6 and NKX2.2 protein

NPI samples were lysed in 100 μL of ice-cold RIPA buffer for 30 minutes at 4°C (150 mM NaCl, 20 mM Tris-Cl, 1 mM EDTA, pH 7.5, 1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 1mM DTT, and 0.1% protease inhibitor cocktail [Sigma-Aldrich Canada Ltd.]). The lysates were spun at 12,000 *g* for 5 minutes at 4°C. The supernatants were removed and assayed for total protein content using the Bradford method (BioRad, Mississauga, ON, Canada). Ten micrograms of cell lysate protein was fractioned on 10% polyacrylamide gels containing SDS in a minigel apparatus (BioRad). After the gel separation, proteins were transferred to Immun-Blot™ PVDF membrane (Bio-Rad Laboratories, Hercules, CA) at 10 mAmps for 16 hours at 4°C in buffer containing 20% (v/v) methanol, 48 mM Tris base, 39 mM glycine, and 0.037% SDS at pH 8.3. Membranes were then incubated in blocking buffer containing 136 mM NaCl, 20 mM Tris, pH 7.6, and 5% skim milk powder for 1 hour at room temperature with gentle shaking. Membranes were washed three times for 10 minutes in PBS-T (0.1%) buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, pH 7.5 and 0.1% Tween-20). Fresh blocking solution was then applied

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containing 1:10,000 dilution of PDX-1 253 rabbit with shaking for 2 hours. Membranes were washed as before, followed by a secondary antibody to rabbit immunoglobulin G coupled to horseradish peroxidase at 1:500 dilution (Amersham Biosciences, Inc., Baie d'Urfé, PQ, Canada) for 1 hour at room temperature, followed by 3 additional 10 minute washes. The blots were developed with an enhanced chemiluminescence Western blotting detection kit (Amersham Biosciences, Inc.).

4.2.7 Transplantation of neonatal porcine islets into SCID mice

All recipients of neonatal porcine islet transplants were 6-8 week old CB17 SCID beige mice (Taconic Farms, Germantown, NY). Diabetes was induced in some recipients using 225mg/kg body weight Streptozotocin (Sigma) injected intraperitonially (i.p.). All diabetic recipients had a minimum blood glucose level of 20 mmol/L prior to transplantation. Blood glucose levels were monitored weekly using a OneTouch Ultra blood glucose monitor (Lifescan, Milpitas, CA). Non encapsulated NPIs were transplanted under the kidney capsule and alginate microencapsulated islets were transplanted intraperitonially (i.p.).

4.2.8 Statistical analysis

Data are expressed as means±SEM of *n* independent observations. Statistical significance of differences for insulin and DNA content, immunohistochemical, and static incubation data was calculated by a one-way ANOVA using Scheffé's post hoc test. A value of $p<0.05$ was considered statistically significant. A Student's unpaired T-test was used to determine statistical significance of difference for blood glucose levels of animals, as well as to compare differences between control and Pdx-1 transfected

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conditions directly (prior to and post-transplantation). $p<0.05$ was considered significant. All tests were performed on Statview (SAS Institute Inc., Cary, NC).

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4.3 RESULTS

4.3.1 AdPdx-1 infects NPIs and does not induce death or maturation

Unfortunately, parallel experiments using an empty adenoviral vector were not performed at the time of this thesis publication, however these experiments are on-going in order to confirm that effects seen were due to actual PDX-1 protein production, and not simply an effect of the adenovirus on the NPIs.

Several multiplicities of infection (MOIs) were used to infect NPIs to determine optimal infection efficiency of the cells, with minimal deleterious effects.NPIs were cultured with virus for either 24 hours or 48 hours, followed by a further 48 hour culture period in order to allow for gene expression and protein translation before samples were taken for analysis. Figure 4-1 illustrates GFP expression in transfected NPI cells at MOIs of 2.2 (Fig. 4-1 A), 4.3 (Fig. 4-1 B), and 22 (Fig. 4-1 C) as imaged using confocal microscopy. As shown in Table 4-1, increasing MOI did correspond to an increase in the percentage of cells which immunostained positively for PDX-1 protein. A representative sample is shown in Figure 4-2, showing cells positively immunostaining for PDX-1 protein (Fig. 4-2 A-E, representing pre, control, MOI 2.2, MOI 4.3, and MOI 22, respectively), and this increasing protein production is reiterated using Western Blotting as shown in Fig. 4-2 F. As seen in Fig. 4-2 F lanes 4 (control) and 5 (pre-culture), NPIs express endogenous PDX-1, however lanes 1, 2, and 3, representing MOIs 22, 4.3, and 2.2, respectively, indicate that, with transfection, PDX-1 is highly expressed in a dose-

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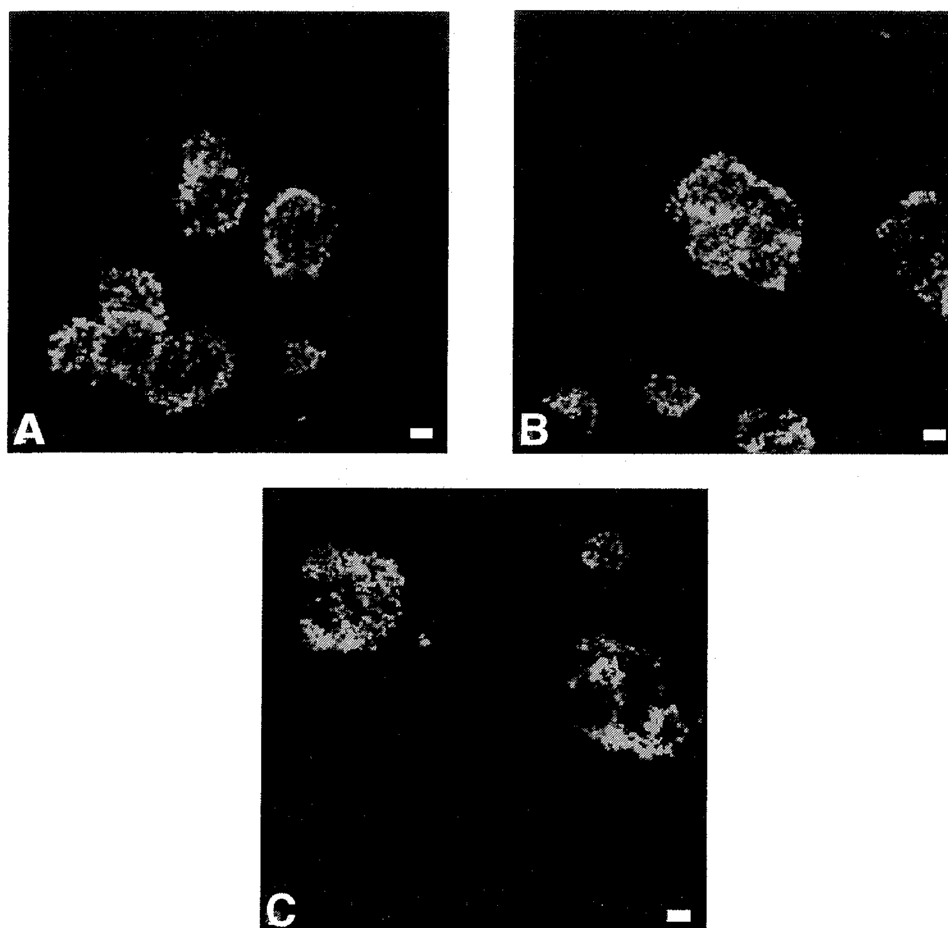

Figure 4-1. Confocal imaging of AdPdx-1 transfected neonatal porcine islets (NPIs). NPIs were exposed to MOIs of 2.2, 4.3, or 22 (A, B, and C, respectively) of AdPdx-1 for 24 hours. The adenoviral construct contained GFP behind a second CMV promoter, allowing for fluorescent visualization of cells transfected with virus. Scale bars represent 50 μm.

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dependent manner. DNA content of samples infected at various MOIs for either 24 or 48 hours revealed no change in the number of cells surviving the culture period when comparing the infected samples to the corresponding controls (Table 4-1). Analysis of insulin content of samples resulted in no change due to overexpression of Pdx-1. At lower MOIs (0.433 and 0.865), with a 24 hour incubation period with the adenovirus, insulin content did appear to go up (to 1.51±0.2 fold and 1.89±0.2 fold over day 0 values, respectively), however these increases were not significant (Table 4-1).

Table 4-1. Effect of different AdPdx-1multiplicities of infection (MOI) and exposure times on NPI in vitro.

	MOI	Increase vs. infection day (day 0): Insulin Content	DNA content	Stimulation Index	% Positive Cells: PDX-1	Insulin	Glucagon	CK7
24 hour Infection								
Control	0	1.39±0.1 (16)	1.43±0.2 (15)	2.64±0.5 (13)	41.64±3.2 (11)	23.71±1.7 (11)	29.27±2.0 (10)	22.65±5.0 (7)
Infected	0.433	1.51±0.2 (5)	1.25±0.2 (5)	3.09±1.3 (5)	49.00±2.7 (4)	17.93±4.7 (3)	26.57±2.3 (3)	12.47±6.3 (3)
	0.865	1.89±0.2 (3)	2.00±0.4 (3)	1.43±0.2 (3)	59.67±4.4 (3)	22.00±3.8 (3)	29.67±2.2 (3)	ND
	2.2	1.49±0.1 (6)	1.63±0.3 (6)	2.10±0.2 (5)	56.07±5.6 (3)	25.87±1.1 (3)	28.10±2.0 (4)	22.33±7.0 (4)
	4.3	1.18±0.1 (6)	1.32±0.4 (6)	2.02±0.2 (5)	45.65±8.7 (4)	25.95±3.0 (4)	27.80±1.1 (4)	17.25±4.6 (4)
	22	1.20±0.2 (6)	1.60±0.3 (6)	1.64±0.1 (5)	64.40±3.4 (4)	23.45±2.4 (4)	27.47±1.9 (3)	20.88±8.3 (4)
48 hour Infection								
Control	0	0.98±0.4 (4)	0.82±0.1 (4)	3.13±0.6 (5)	36.28±9.4 (4)	24.10±1.3 (4)	29.90 (2)	17.71±3.8 (4)
Infected	2.2	1.20±0.2 (4)	0.77±0.1 (4)	2.73±0.3 (5)	47.80±6.9 (4)	22.83±2.9 (4)	30.50 (1)	8.60±1.4 (4)
	4.3	1.35±0.3 (4)	0.74±0.1 (4)	1.89±0.2 (5)	48.67±6.8 (3)	25.33±3.8 (4)	28.20 (2)	7.83±1.5 (4)
	22	0.99±0.2 (4)	0.68±0.1 (4)	1.94±0.3 (5)	52.97±4.1 (3)	19.93±2.4 (4)	25.25 (2)	11.15±1.1 (4)

Data are expressed as mean ± SEM from (n) individual experiments. ND indicates not determined.
Statistical significance of differences was calculated by ANOVA using Scheffe post hoc test, however no significant differences were found.

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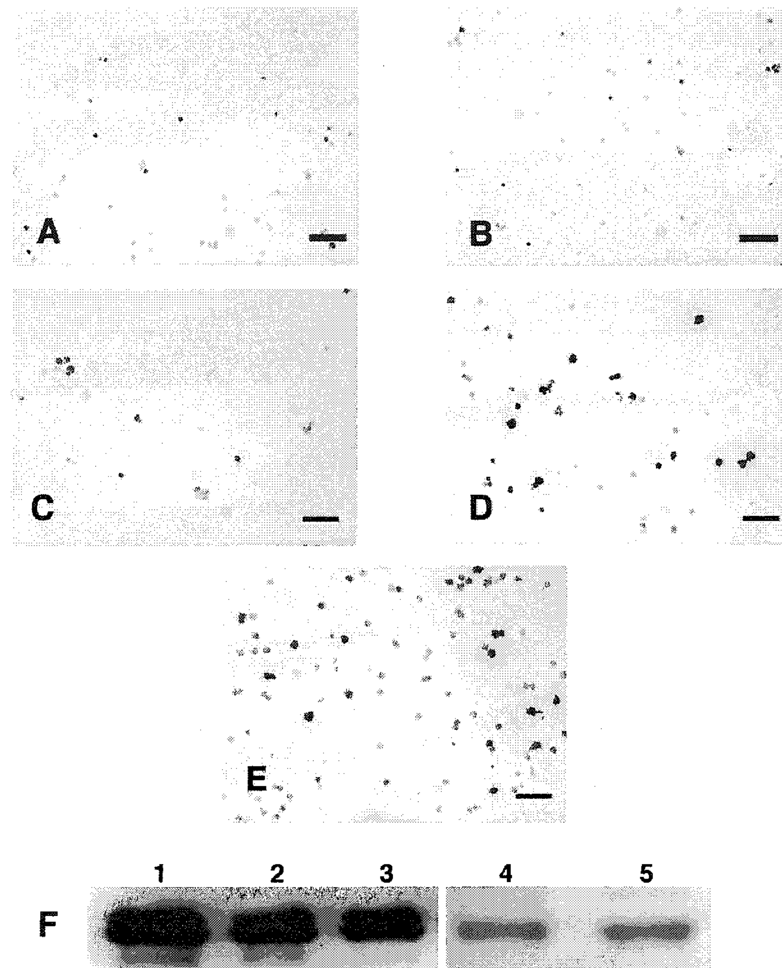

Figure 4-2. AdPdx-1 transfection causes upregulation of PDX-1 protein in neonatal porcine islets as imaged by immunohistochemistry and western blotting. Prior to transduction, NPIs shown were composed of 18.2% PDX-1 positive cells (A). Culture with no adenvirus resulted in 19.2% of cells PDX-1 positive (B). Addition of AdPdx-1 to culture media resulted in a dose-dependent increase in PDX-1 positive cells by immunostaining. C-E) MOIs of 2.2, 4.3, and 22, respectively, with 51.6%, 68.6%, and 73.0% PDX-1 positive cells. Considerable hypertrophy of PDX-1-positive cells was noticed at higher MOIs Scale bars represent 50 μm. F) Western blot analysis showing in lanes:1-5 MOIs 22, 4.3, 2.2, no virus, and pre-culture whole protein samples. Lanes 1-3 are a 1 second exposure, whereas lanes 4 and 5 are from a 2 minute exposure of X-ray film.

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Immunohistochemical analysis of the islet hormones, insulin and glucagon revealed no increase in the proportion of hormone-positive cells in the infected samples as compared to the controls. The number of Cytokeratin 7 (CK7) positive ductal cells appeared to decrease in infected samples compared to non-infected controls, particularly with a longer infection period, however these values were not significant (Table 4-1). NPIs were analyzed for function by static incubation and, although a general decrease in simulation index (S.I.) was observed with transfection, this was not statistically significant (Table 4-1).

4.3.2 Encapsulation and culture of AdPdx-1 transfected NPI with autologous serum

Transfected and control NPIs were encapsulated in alginate microcapsules, and cultured with 10% autologous serum (EC ser$^+$) as previously described (Chapter 2). Samples taken were compared to transfected, non-encapsulated NPIs to determine whether EC ser$^+$ culture had an effect on the transfected NPIs. MOIs of 0.44, 3, and 6 were used following the previous infection efficiency studies which indicated that overexpression of Pdx-1 was achieved at these MOIs with no detrimental effect on NPI health. Figure 4-3 shows GFP expression in encapsulated NPIs at MOIs of 0 (A), 3 (B), and 6 (C), as well as PDX-1 protein expression by western blot (D) in pre (lane 1, endogenous expression), control encapsulated (lane 2, endogenous expression), and transfected encapsulated (lane 3, slight increase over endogenous expression, 1.07 fold as determined by densitometry) samples. Immunohistochemically, an increase in the percentage of cells staining positively for PDX-1 protein was seen, and this was proportional to MOI (Table 4-2).

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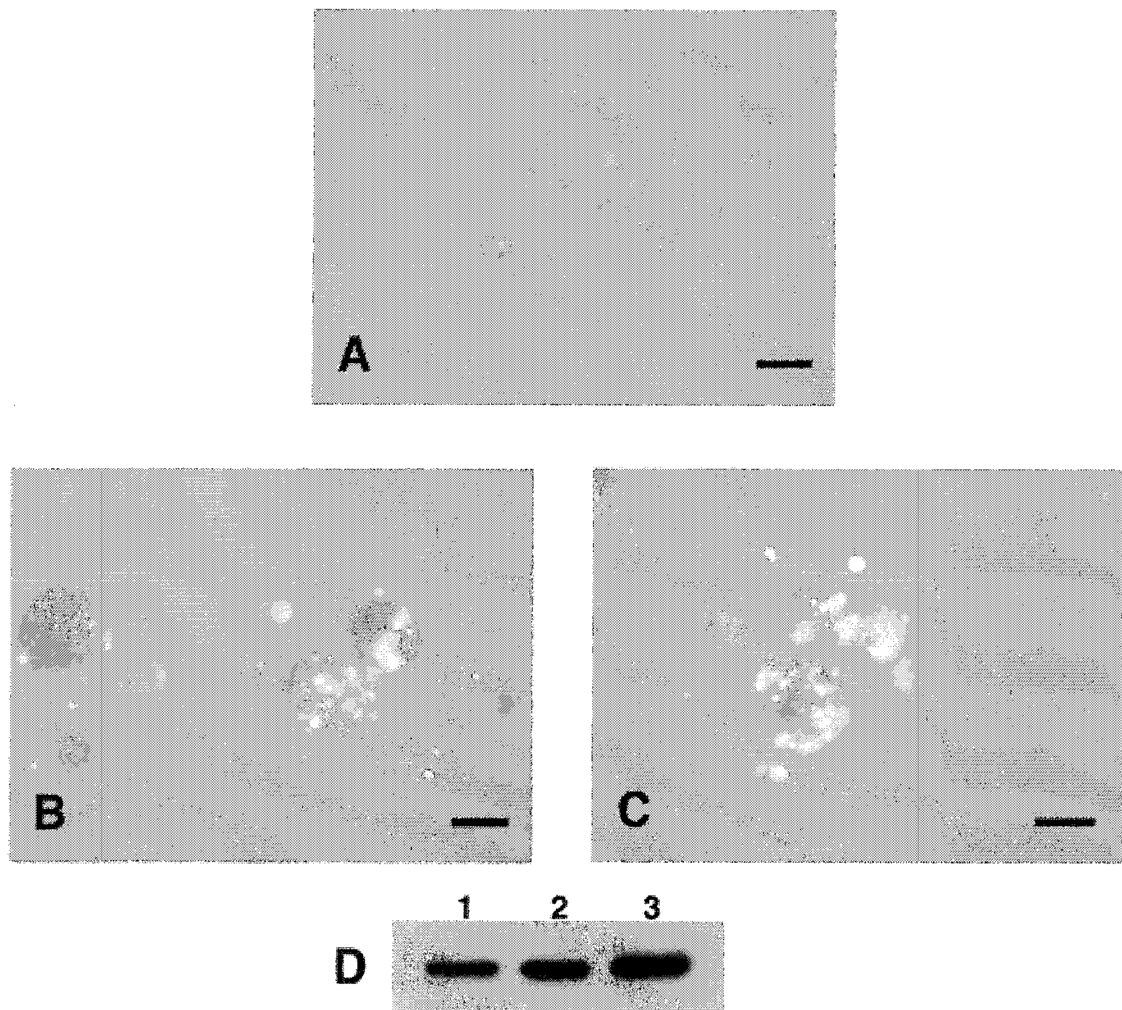

Figure 4-3. AdPdx-1 transfection of NPIs followed by alginate microencapsulation and culture with autologous serum. A-C). Adenoviral transfection of alginate microencapsulated NPIs is visualized by GFP expression post in vitro maturation. A: MOI 0 (control), B: MOI 3, C: MOI 6. Scale bars represent 50 μm. D) Western blot probed for PDX-1 protein indicating in, lane 1, endogenous PDX-1 in NPIs prior to transfection and encapsulation, lane 2, endogenous PDX-1 protein in control in vitro matured NPIs (MOI 0), and lane 3, PDX-1 protein expression in Pdx-1 transfected and in vitro matured NPIs (MOI 6).

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Table 4-2. Effect of encapsulation and culture in autologous serum on AdPdx-1 transfected NPIs. NPIs were cultured with virus for 24 hours, encapsulated, and cultured with autologous serum for a further 6 days.

		Increase vs. day 0			% Positive Cells			
	MOI	Insulin Content	DNA content	Stimulation Index	PDX-1	Insulin	Glucagon	CK7
Encapsulated								
Control	0	3.68±0.6 (18)*	0.39±0.1 (18)	7.26±1.4 (14)	44.09±2.0 (14)	27.08±1.9 (12)	28.94±3.2 (10)	45.61±3.1 (8)
Infected	0.44	3.67±0.5 (5)	0.66±0.4 (5)	7.28±2.3 (5)	46.67±2.0 (3)	29.37±4.2 (3)	25.10±4.4 (3)	43.67±6.7 (3)
	3	2.61±0.5 (8)	0.56±0.2 (8)	5.04±0.9 (6)	47.95±4.5 (6)	31.40±3.1 (6)	30.17±6.8 (3)	39.75±8.8 (4)
	6	1.66±0.6 (4)	0.35±0.1 (4)	5.16±1.1 (4)	47.10±2.5 (4)	26.13±1.9 (4)	31.18±3.6 (4)	49.35 (2)
Non-encapsulated								
Infected	0.44	4.69 (2)	0.47 (2)	3.28 (2)	ND	ND	ND	ND
	3	2.56±0.6 (8)	1.04±0.3 (8)	1.45±0.1 (6)	51.12±2.6 (6)	36.98±2.8 (6)	28.50±4.3 (4)	47.48±5.0 (4)
	6	1.62±0.35 (4)	1.73±1.3 (4)	1.20±0.3 (4)	47.25±0.8 (4)	28.33±2.7 (4)	27.43±2.9 (4)	51.32±4.1 (4)

Data are expressed as mean ± SEM from (n) independent experiments.
Statistical significance of differences was calculated by ANOVA using Sheffe post hoc test. *$p<0.05$ vs. day 0 (pre-transfection and/or EC).

No significant changes in the percentage of cells immunostaining for insulin, glucagon, or CK7 were observed (Table 4-2). Insulin content recovered from samples (both encapsulated and non-encapsulated) transfected with a very low MOI (0.44) was similar to that observed in EC ser$^+$ NPI (MOI 0), MOI 0.44 increased 3.67±0.5 fold whereas control NPIs increased 3.68±0.6 fold (Table 4-2). Other MOIs, regardless of EC ser$^+$ culture of the NPIs, increased very modestly in insulin content from day 0 values, 2.64±0.05 fold and 1.66±0.6 fold for MOIs of 3 and 6, respectively. DNA content dropped in all of the encapsulated samples (Table 4-2) compared to day 0 values, including the control sample. This may be due to incomplete removal of alginate from the samples, which has a detrimental effect on the DNA assay, or due to cell death. An MOI of 0.44 seemed to have the least detrimental effect on DNA content of encapsulated samples, as 0.66±0.4 fold of the original day 0 content was recovered. Only 0.56±0.2 fold and 0.35±0.1 fold of day 0 DNA content were recovered from MOI 3 and 6 encapsulated samples, respectively. The non-encapsulated, transfected samples were consistent with previous results (Table 4-1, 24 hour infections) and remained the same

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(MOI 3, 1.04±0.3 fold) or increased slightly (MOI 6, 1.73±1.3 fold) compared to day 0 DNA content (Table 4-2). The stimulation index (S.I.) was much improved in EC ser^{+} NPI (Table 4-2). MOI 0.44 samples had a S.I. of 7.28±2.3, very similar to non-infected NPIs (7.26±1.4). Increased MOIs resulted in decreased S.I.s (5.04±0.9 and 5.16±1.4, MOI 3 and 6, respectively), however these were much higher than respective S.I.s of non-encapsulated samples (1.45±0.1 and 1.20±0.3, MOI 3 and 6, respectively).

4.3.3 Culture with exendin-4

Although transfection of NPIs was apparent as seen by GFP expression as well as PDX-1 protein, when looking at confocal images cross-sectioning transfected islets, it appears that GFP expression is mainly limited to the peripheral cells of some islets (Figure 4-4). Using a higher MOI was more detrimental then beneficial for NPIs, thus, to attempt to increase transfection efficiency while maintaining a lower MOI, NPIs were gently dissociated into single cells prior to transfection. A recent study indicated that GLP-1, or its long-acting analogue, exendin-4, increased PDX-1 binding to the insulin gene and up-regulated Pdx-1 expression in cell lines (28). Using exendin-4, with and without Pdx-1 transfection, we attempted to mature dissociated NPIs cultured as a monolayer. Dissociated NPIs were plated on both poly L- and D-lysine coated chamber slides and 6-well plates. No differences in the parameters analyzed were observed with the different types of poly lysine (data not shown), thus poly L-lysine was used for the remainder of the experiments. NPIs re-aggregated in chamber slides (Figure 4-5, A-D) after 72 hours culture. Four culture conditions were analyzed: A) Ham's + 5% NBP

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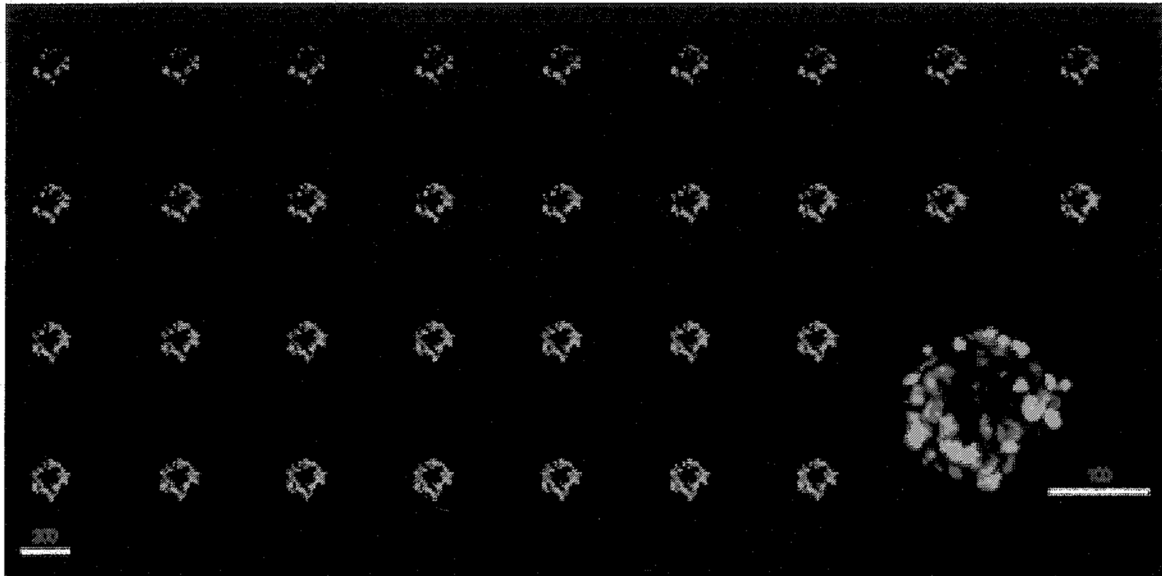

Figure 4-4. Confocal mosaic image of GFP expression in a transfected NPI. By viewing sections throughout the islet, shown here sequentially in a mosaic pattern, it is apparent that mainly cells on the periphery are transfected by the adenovirus. Inlay represents a 3-D reconstruction of the sectioned islet.

serum alone (Figure 4-5A), B) with 100nM exendin-4 (Figure 4-5B), C) with Pdx-1 adenoviral transfection (MOI 3) (Figure 4-5C), and D) with both Pdx-1 adenoviral transfection and 100nM exendin-4 (Figure 4-5D). Using an MOI of 3, preliminary experiments using dissociated NPIs resulted in 44.98% (n=2) of cells becoming GFP positive, (data not shown). No difference was seen in the percentage of cells staining positively for insulin between the four groups (A: 24.4±3%, B: 24.8±2%, C: 29.7±7%, D: 24.8±5%). Using immunohistochemistry, it was difficult to quantitate PDX-1 positive cells in the aggregates. Using Western Blotting, the two transfected conditions (C and D) contained high levels of PDX-1 protein relative to the non-transfected conditions (A and B) (Figure 4-5E, lanes 1-4 represent conditions A-D, respectively). The potential benefit of exendin-4±Pdx-1 transfection was expressed relative to control culture conditions (A).

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DNA content was similar between the 3 experimental groups: B = 1.04±0.1 fold, C = 0.94±0.1 fold, and D = 0.99±0.1 fold as compared to A (Table 4-3). Insulin content recovered from the three experimental groups was lower than the control, dropping to 0.92±0.04 fold, 0.70±0.1 fold, and 0.63±0.1 fold in conditions B, C, and D, respectively, C and D dropping significantly (Table 4-3). Media samples from each culture condition were assayed for insulin release at the end of the experiment. Insulin release dropped in conditions B, C, and D, relative to condition A, to 0.98±0.02 fold, 0.79±0.1 fold, and 0.78±0.1 fold, respectively (Table 4-3).

Table 4-3. Effect of exendin-4 and AdPdx-1 transfection on DNA content and insulin content and release after in vitro culture of NPI monolayers.

	Relative to culture in Ham's + 5% NBP serum (A)		
	DNA content	Insulin Content	Insulin Release
Culture Conditions			
Exendin-4 (B)	1.04±0.1 (8)	0.92±0.04 (8)	0.98±0.02 (8)
Pdx-1 transfect(C)[a]	0.94±0.1 (8)	0.70±0.1 (8) *†	0.79±0.1 (8)
Exendin-4 + Pdx-1 transfect (D)[a]	0.99±0.1 (8)	0.63±0.1 (8) *†	0.78±0.1 (8)

Data are expressed as mean ± SEM of (n) independent experiments.
Statistical significance was determined by ANOVA using Sheffe post hoc test.
*p<0.005 vs. control (A), † p<0.05 vs. exendin-4 (B)
[a] AdPdx-1 transfections were MOI 3

As a beneficial effect was not achieved using the dissociation protocol which required a more aggressive treatment of the tissue, the dissociation protocol was discontinued, and 100nM exendin-4 was added to the previous in EC ser$^+$ culture protocol. Two conditions of encapsulated NPIs were cultured in Ham's supplemented with 10% autologous serum and 100nM Exendin-4; control, and Pdx-1 transfected.

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Table 4-4 illustrates that no change is seen in the insulin content, or in the DNA content recovered from the Pdx-1 transfected NPIs when compared to the control NPIs.

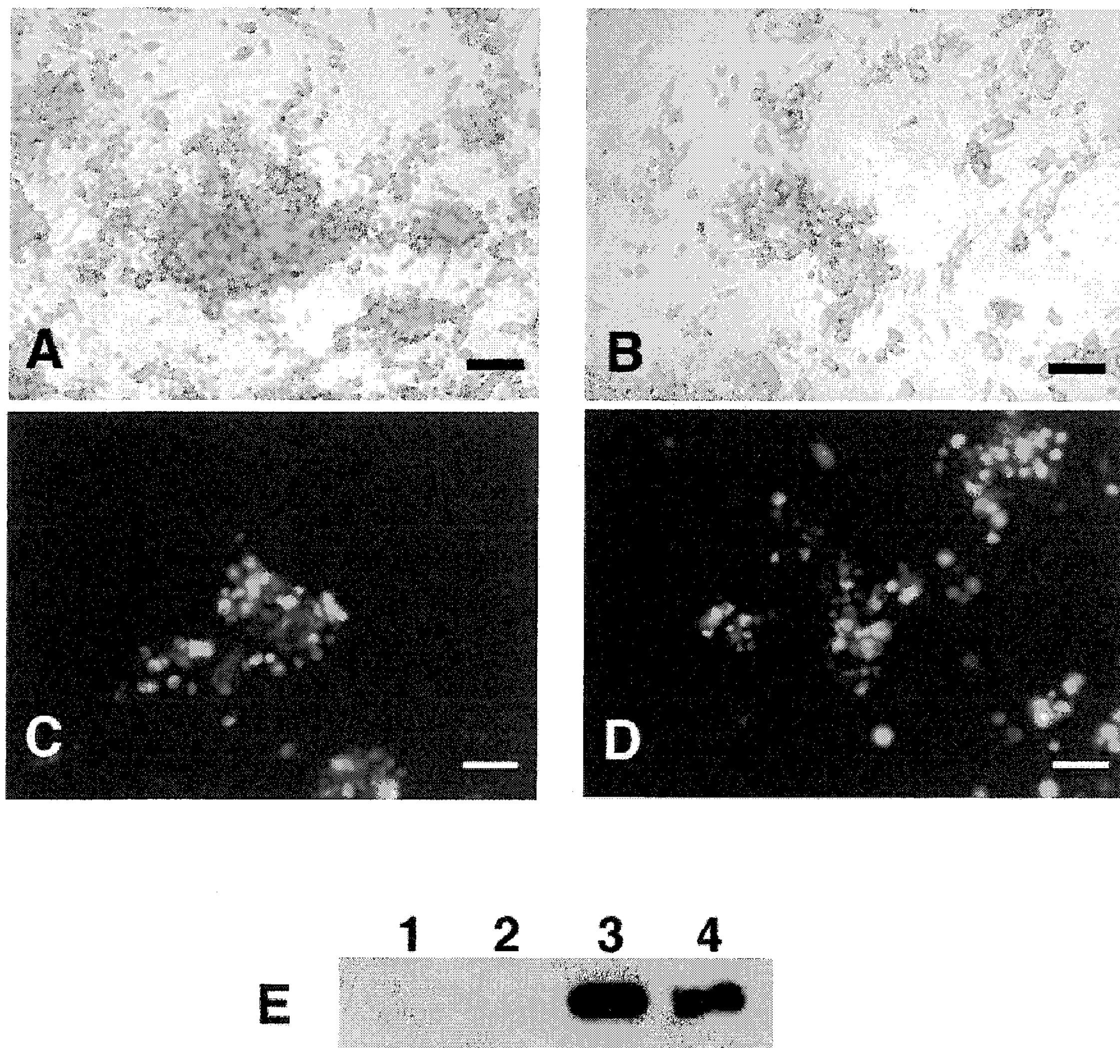

Figure 4-5. Re-aggregated AdPdx-1 transfected NPIs, cultured with and without exendin-4. NPIs were dissociated and allowed to re-aggregate on poly-L lysine coated chamber slides A) in Ham's + 5% autologous serum, B) plus 100nM exendin-4 as control conditions. Pdx-1 transfected dissociated NPIs were cultured in Ham's +5% autologous serum without (C) and with (D) 100nM exendin-4. Scale bars represent 50 μm. A Western blot probed for PDX-1 protein (F) indicates successful production of PDX-1 in the two transfected samples, lanes 1-4 represent culture conditions A-D as described above.

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Table 4-4. Effect of encapsulation and autologous serum on control and AdPdx-1 transfected NPIs cultured with exendin-4.

	Relative content vs. control		% positive cells			
	Insulin	DNA	Insulin	Glucagon	CK7	PDX-1
Control	1 (4)	1 (4)	24.18±1.8 (4)	28.03±3.1 (4)	31.73±9.5 (3)	27.68±2.1 (4)
Pdx-1 Transfected	0.96±0.1 (4)	1.13±0.1 (3)	24.35±2.5 (4)	30.77±1.0 (3)	33.15±7.4 (4)	44.13±1.4 (3)*

Data are expressed as mean ± SEM of (n) independent experiments.
Statistical significance of difference was analyzed using an unpaired Student's T-test. *p<0.005 vs. control

The percentage of cells immunostaining positively for PDX-1 protein was much higher in the Pdx-1 transfected group, 44.13±1.4% compared to 27.68±2.1% of the cells in the control group (Table 4-4). Other immunohistochemical analysis revealed no difference between the two conditions with regards to percentage of cells staining positively for insulin, glucagon, or CK7 (Table 4-4).

4.3.4 In vivo functional analysis of AdPdx-1 transfected NPIs

In vitro, previous work using Pdx-1 transfection alone (15;16) or in combination with GLP-1 or exendin-4 (25) could not be replicated in NPIs. To determine whether factors present in vivo would augment Pdx-1 expression in NPIs, approximately 150 control or AdPdx-1 transfected NPIs were transplanted under the kidney capsule of naïve SCID mice. At days 0, 7, and 24 post-transplant, NPI grafts were removed and analyzed for insulin content. At day 7 post-transplant, control NPI grafts had an increase in insulin content of 4.56±1.67 fold, whereas transfected NPI grafts had only increased 1.18±1.3 fold (Table 4-5). As expected at day 24, the insulin content of control NPI grafts had increased significantly over day 0 and control day 7 values (ANOVA, Sheffé's post-hoc analysis, $p<0.01$) to 17.97±3.6 fold day 0 values, however at day 24, transfected grafts had only increased 9.56±3.7 fold over day 0 (Table 4-5).

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Table 4-5. Effect of encapsulation and autologous serum on control and AdPdx-1 transfected NPIs transplanted into naïve SCID mice.

	MOI	n	Per transplant (day 0)		Fold Increase Insulin Content (vs day 0)	
			DNA / Tx (ng)	Insulin / Tx (ng)	Day 7 post-Tx	Day 24 post-Tx
Control	0	3	1271.42±264.8	385.13±165.5	4.56±1.67	17.97±3.6
Pdx-1Transfected	5	3	1440.45±188.0	416.49±190.7	1.18±0.3	9.56±3.7

Data are expressed as mean ± SEM.
Differences between control and transfected samples at each time point were not statistically significant by an unpaired Student's t-test.

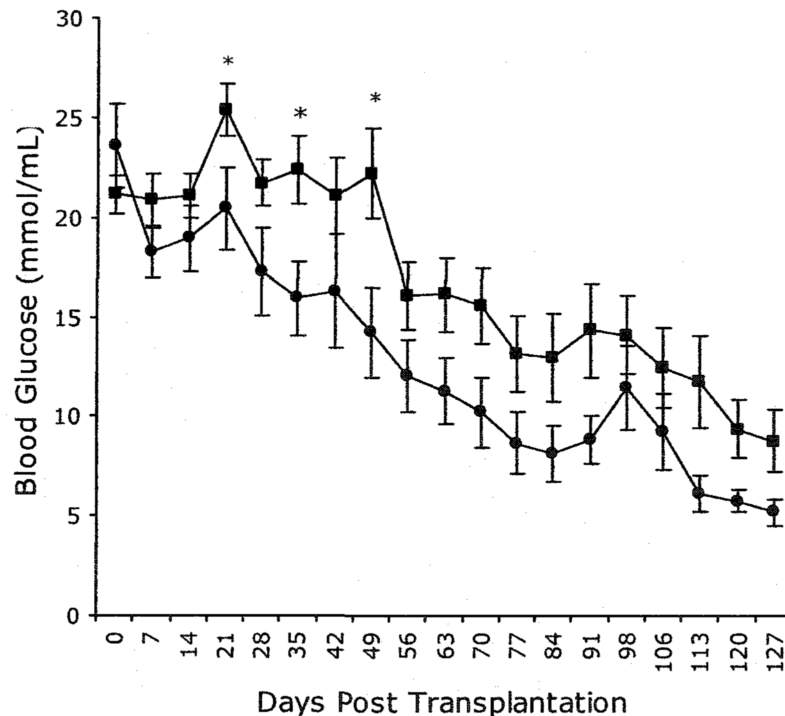

Figure 4-6. Blood glucose levels of diabetic mice transplanted with NPIs. Grafts of 150 NPIs were transplanted under the kidney capsule of diabetic SCID mice. Animals with control NPI grafts (●, n=13) normalized faster than those with AdPdx-1 transfected NPI grafts (■, n=16). Statistical significance of differences between the two groups, as determined by an unpaired Student's t-test, was observed at three time points, *$p<0.05$.

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Further attempts to achieve maturation of NPIs post-AdPdx-1 transfection were performed as transplants in diabetic (streptozotocin-induced) SCID mice, as we wished to explore whether growth factors present due to the damaged pancreas, which might enhance Pdx-1 activity. Approximately 150 control or AdPdx-1 transfected NPIs were transplanted under the kidney capsule, and blood glucose was monitored weekly throughout the experiment. Animals receiving AdPdx-1 transfected grafts had higher blood glucose values throughout (Figure 4-6), and did not normalize until 87.60±10.2 days post-transplant, whereas animals receiving control grafts normalized 61.42±8.1 days post-transplant.

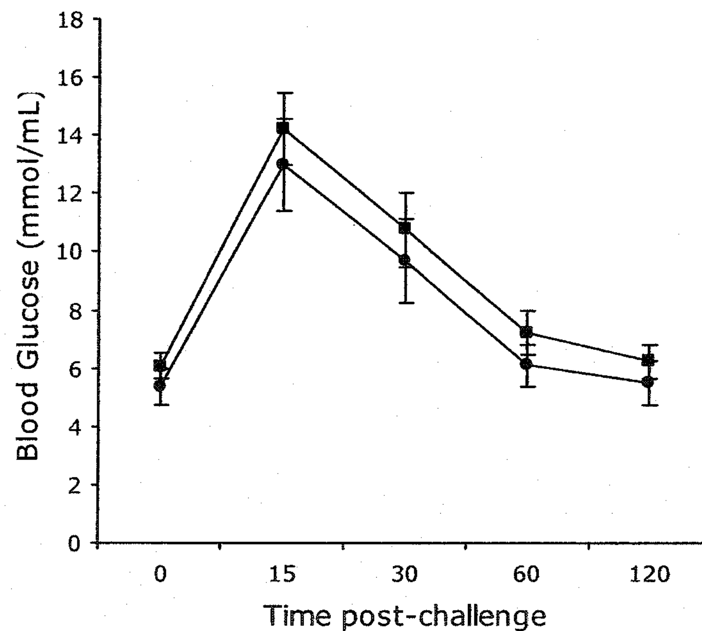

Figure 4-7. Oral glucose tolerance test of recipients of control and AdPdx-1 transfected NPIs. No difference was observed in the blood glucose response of animals to an oral challenge of 3 mg/g body weight of 50% dextrose between animals with control NPI grafts (●, n=11) and those with AdPdx-1 transfected NPI grafts (■, n=15).

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Upon normalization (blood glucose >10mmol/mL), mice were subjected to an oral glucose tolerance test (OGTT) to observe function of the NPI graft. Animals with Pdx-1 transfected grafts had slightly higher blood glucose values at all time points observed than animals with control grafts, however at no time point were these differences statistically significant, and the shape of the curve indicates good function of the graft in both groups of animals (Figure 4-7). Once mice became normoglycemic, they were sacrificed, and grafts removed for analysis of insulin content. Control NPI grafts reached a 40.86±20.5 fold increase in insulin content compared to the amount transplanted initially, and Pdx-1 transfected grafts had an increase of 24.19±13.9 fold (Table 4-6, not statistically significant).

Table 4-6. Transplantation of control and AdPdx-1 transfected NPIs into diabetic SCID mice.

	Per transplant (day 0)		Fold Increase Insulin Content (vs day 0)
	DNA / Tx (ng)	Insulin / Tx (ng)	
Control	15663.48±4630.8 (13)	701.78±91.9 (13)	40.86±20.5 (9)
Pdx-1Transfected	12301.30±3567.8 (16)	943±186.1 (16)	24.19±13.9 (11)

Data are expressed as mean ± SEM of (n) independent experiments.
No significant differences were found between control and transfected groups, Student's t-test p=0.05.

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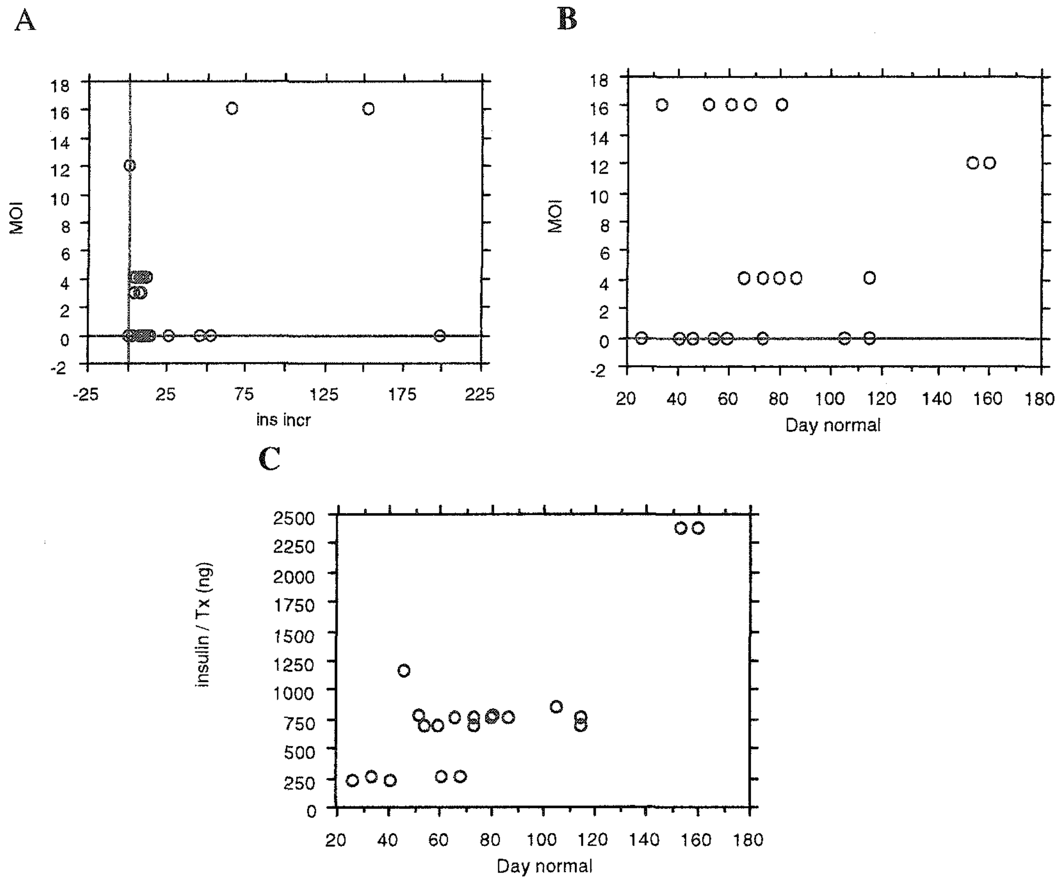

Figure 4-8. Scatter-plot analysis of the effect of MOI and amount of insulin per transplant on the outcome of NPI transplants. **A**. Analysis of the effect of AdPdx-1 MOI on the increase in insulin content of the recovered graft from the day of transplant. **B**. Analysis of the effect of MOI on the number of days required for the diabetic recipient to normalize (blood glucose of 10 mmol/L). **C**. The effect of the amount insulin content transplanted on the number of days required for the diabetic recipient to normalize (blood glucose of 10 mmol/L).

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Various comparisons were done using scatter-plots to decipher what parameters had an effect on the ability of NPIs (control or transfected) to cure diabetes. In Figure 4-8A, the effect of MOI on the increase in graft insulin content throughout the experiment was plotted, indicating that, other than some outliers, any MOI and thus any AdPdx-1 transfection resulted in a smaller increase in insulin content recovered from the grafts. Figure 4-8B plots MOI against the day (post-transplant) the recipient normalized. Animals receiving control NPI (MOI 0) grafts, and those receiving NPIs which had been Pdx-1 transfected at a high MOI of 16 normalized faster than animals receiving NPIs transfected at MOIs of 4 or 12. Figure 4-8C shows the effect of insulin content per transplant on the number of days required for the recipient to normalize. Surprisingly, the lower the amount of insulin per transplant, the faster the animals in these experiments normalized.

A final set of transplants involved encapsulation and culture in autologous serum of NPIs, post AdPdx-1 transfection and prior to transplantation. Both control and AdPdx-1 transfected encapsulated islets increased similarly in insulin content, 2.02±0.5 fold and 2.29±0.4 fold, respectively, after 6 days culture in Ham's + 10% autologous serum (Table 4-7).

Table 4-7. Results of encapsulation and culture with autologous serum on control andAdPdx-1 transfected NPIs prior to transplant into diabetic SCID mice.

	Fold Increase (D6 vs. D0)	Content Per Transplant (ng)		Stimulation Index
	Insulin content	Insulin	DNA	
Control	2.02±0.5 (3)	3931.85±638.8 (3)	5952.37±1637.5 (3)	8.90 (2)
Pdx-1 Transfected	2.29±0.4 (3)	5014.10±1167.6 (3)	4785.29±1485.5 (3)	5.68 (2)

Data are expressed as mean ± SEM of (n) independent experiments.
No significant differences were detected between control and AdPdx-1 transfected samples by unpaired Student's t-test

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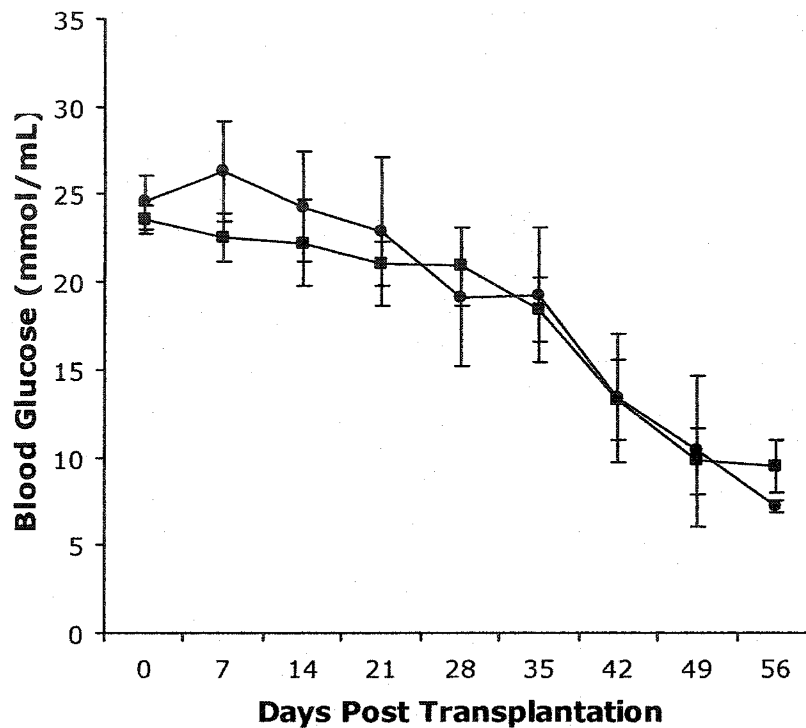

Figure 4-9. Blood glucose levels of diabetic SCID mice transplanted with control or AdPdx-1 transfected microencapsulated NPIs. Recipients of 600 encapsulated control NPIs (●, n=5 day 0-day 49, n=3 day 56) or 600 encapsulated AdPdx-1 transfected NPIs (■, n=8 day 0-day 49, n=5 day 56), transplanted intraperitonially, had no difference in blood glucose levels, but did become normoglycemic after 49 days post-transplantation. Groups were compared using an unpaired Student's t-test, and at no time point were the groups considered significantly different.

Approximately 600 encpsulated NPIs were transplanted intraperitonially (i.p.) into diabetic SCID mice. At the time of transplantation, static incubations were performed on the tissue, indicating that the control NPIs were more functional, with a S.I. of 8.90 as compared to the Pdx-1 transfected NPIs which had an S.I. of 5.68 (Table 4-7). At no time point post-transplantation were the blood glucose levels between the two groups of animals significantly different (Figure 4-9).

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4.4 DISCUSSION

Neonatal porcine islets (NPIs) are a promising source of xenotransplantable insulin-producing tissue, however ex vivo maturation prior to transplantation would be required to provide a more rapid correction of hyperglycemia in a recipient. Due to the high percentage of ductalcells, thought to be precursors, NPIs also represent a model for the study of islet development, and islet precursor manipulation, from which knowledge gained may be directly transferred to human islets due to the physiological similarities between these species for islets.

To determine of the infection efficiency of AdPdx-1, we based our protocol on previous work involving transduction of islets or ductal tissue in suspension with adenoviral vectors (26;29). Intact NPIs were cultured with virus at various MOIs at 37°C. Figure 4-1 illustrates transfection of intact islets as imaged by confocal microscopy detecting GFP, also coded by the adenoviral construct. Table 4-1 shows the effect of varying MOIs of AdPdx-1, cultured with NPIs for either 24 or 48 hours. These culture periods are both longer than those reported in other studies (1 to 2 hours, (26;29;30)), and it appears that longer exposure to virus, especially 48 hours, results in lower DNA content recovery values as compared to MOI 0 control samples, likely reflecting cell damage. The negative effect of the virus is apparent when looking at the stimulation index (S.I.), a measure of islet function in response to glucose stimulation. After any exposure to the virus, the S.I. was lower than the control (MOI 0) values. Perhaps a shorter exposure time to the adenovirus would alleviate the negative effect on the NPIs, thus future experiments should be designed to reflect this. The number of cells positive for the islet hormones, insulin and glucagon, was not affected by transfection

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with AdPdx-1. The CK7-positive ductal cell population appeared to drop in samples exposed to various MOIs of AdPdx-1 for 48 hours, however none of these values were significant, nor did they result in an increase in the percentage of endocrine hormone positive cells, and thus may have died as the DNA content recovery dropped in these samples as well. The number of cells staining positively for PDX-1 did increase in a dose-dependent fashion with increasing MOIs. Protein content, as observed by Western blot (Table 4-1, Figure 4-2), confirmed this, however none of these increases were significant.

As no beneficial effect of AdPdx-1 transfection was noted in initial experiments looking at infection efficiency, transfected NPIs were encapsulated, to provide an extracellular matrix (alginate microcapsule), and cultured in autologous serum to provide growth factors (EC ser$^+$, as described in Chapter 2 of this thesis). Lower MOIs (0.44, 3, and 6) were used from this point on, as was a 24 hour exposure to the virus in culture. GFP was visible in the NPIs where cells had been infected with AdPdx-1 (Figure 4-3). A western blot of NPIs pre-transfection/encapsulation indicated endogenous PDX-1 expression in control NPIs and after EC ser$^+$ culture which increase slightly in AdPdx-1 transfected (1.1x pre samples as determined by densiometry, Figure 4-3 D). Results in Table 4-2 indicate no advantageous effect of AdPdx-1 transfection in combination with Ec ser$^+$ culture. All non-encapsulated samples were controls for the MOIs used, and results were similar to those seen in the infection efficiency experiments (Table 4-1). One apparent advantage of the EC ser$^+$ culture was improved S.I. values compared to the non-encapsulated MOI-matched samples. Insulin content did not increase, however DNA content was substantially lower in the EC ser$^+$ samples, thus insulin/DNA

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recovered from infected samples is higher. This may be due to cell death, however recovery of DNA and insulin from encapsulated samples may result in some loss of tissue as the alginate capsules must be removed, requiring several extra wash steps. Immunohistochemically, no advantage is seen after in vitro maturation when compared to non-encapsulated samples for all proteins observed (insulin, glucagon, CK7, and PDX-1).

One concern in regards to effective transduction of NPIs was whether the virus was able to infect cells in the middle of the islets, or if only the cells on the surface were exposed to virus. Figure 4-4 displays a mosaic image of GFP expression throughout an AdPdx-1 transfected islet as imaged using confocal microscopy. The majority of GFP expressing cells are on the surface. Previously we looked at NPI morphology using confocal microscopy of insulin and CK7 immunostained islets. CK7-positive cells were located mainly within the core of the islet, while ß-cells were on the periphery (data not shown). We hoped to target the CK7-positive ductal cells, which are hypothesized to be precursor cells (2;31;32). The adenovirus not penetrating into the core of the NPIs may be one reason for our lack of increase in insulin-containing cells. To address this issue, we dissociated the NPIs, and plated them on poly L-lysine coated plates or chamber slides for transfection, re-aggregation, and analysis. Reaggregated NPIs, ± AdPdx-1 infection were cultured with 100nM exendin-4 due to recent literature describing the positive effect of glucagon-like peptide 1 (GLP-1) and its analogue exendin-4 on Pdx-1 gene transcription, insulin expression (22-25), and ß-cell neogenesis in a model of pancreatic damage (33). Addition of 100nM exendin-4 resulted in levels of DNA content, insulin content, and insulin release into the media almost identical to the control NPIs (Table 4-3). The addition of AdPdx-1 transfection, either with or without exendin-

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4, resulted in no change to DNA content, however significantly less insulin content and less insulin released into the media than the control NPIs (Table 4-3). Exendin-4 had no beneficial effect, and adenoviral transduction in the dissociated and re-aggregated NPIs seemed to have a negative effect on the amount of insulin. This did not appear to be due to cell death, as DNA levels did not change. Cells appeared to be healthy in all conditions, as they maintained a rounded morphology and adhered to the plates.

Figure 4-5 illustrates the four culture conditions as well as a Western blot confirming the production of PDX-1 protein in the two AdPdx-1 transfected conditions. As dissociating NPIs did not result in positive results, one final in vitro experiment was performed involving intact NPIs. Control and AdPdx-1 transfected NPIs were encapsulated and matured (as described previously) in the presence of 100nM exendin-4. Table 4-4 displays the results comparing AdPdx-1 transfected NPIs to control (non-transfected). The only difference observed was the percentage of the cells immunostaining positively for PDX-1, which was significantly elevated in the transfected group, however this had no effect on the other parameters analyzed.

With dissociation, our infection efficiency may have been much higher than with the intact islets, which may have had a negative effect on insulin gene transcription. This phenomena has been noted previously (34) where too much or too little PDX-1 both have the effect of decreasing insulin expression. Stanojevic et al. attribute the down regulation of insulin gene transcription in the presence of overexpression of PDX-1 to sequestering of the p300 co-activator (35). At normal levels, p300 interacts with PDX-1 in the nucleus to activate genes. In excess, PDX-1 may bind to p300 in the cytoplasm, thus not allowing translocation and squelching the effect of gene transcription. Similarly,

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interacting partner proteins are required for PDX-1 function (36-38), which may not be abundant, and the overexpressed PDX-1 would be unable to activate gene transcription on its own.

A third issue involved in gene transcription is chromatin structure. If the chromatin surrounding PDX-1 downstream targets is repressed, gene transcription may not occur (36). In addition, Chakrabarti et al. noted that PDX-1 associates with insulin and Pdx-1 promoters in ß-cells four-times more strongly than in non-ß-cells, which may indicate overexpression of PDX-1 not having a dramatic effect on immature ductal cells (38). Presumably, in vivo these partner proteins and co-activators may be present, and chromatin structure may be regulated, thus the next step was to attempt to mature AdPdx-1 transfected NPI in vivo, in SCID mice.

In naïve SCID mice, 200 control or AdPdx-1 transfected NPI were transplanted under the kidney capsule. Grafts were removed and insulin was extracted and measured using a radioimmunoassay (RIA) at two time points, 7 and 24 days post-transplantation. Insulin content of the control NPI grafts had increased more than that of the transfected grafts, thus AdPdx-1 transfection did not enhance maturation (Table 4-5). It was thought that perhaps the signals provided in a hyperglycemic environment may encourage turning on of the insulin gene, and thus require PDX-1 binding. The next group of transplants involved 250 NPIs, either control or AdPdx-1 transfected, transplanted under the kidney capsule of streptozotocin-induced diabetic SCID mice. The blood glucose of the mice was monitored weekly to track graft function. As seen in Figure 4-6, mice that received control NPI grafts normalized faster then mice receiving AdPdx-1 transfected grafts. Once animals had become normoglycemic, an oral glucose tolerance test (OGTT)

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indicated that the function of transfected grafts was very similar to that of control grafts. However, when grafts were removed at the end of the experiment and analyzed for insulin content by RIA, control grafts had increased nearly two-fold more than transfected grafts when compared to pre-transplant (day 0) values (Table 4-6). Scatter plots (Figure 4-8) were analyzed to attempt to determine if there were any differences within parameters which could explain the lack of desired effect with AdPdx-1 transfection. From these, it may be concluded that either non-transfected (MOI 0) or samples transfected with a high MOI of 16 (n=2) resulted in a greater increase in insulin content (of the retrieved graft at the end of the experiment, compared to the amount of insulin in the initial transplant) (Figure 4-8 A). However, these high MOI values may simply be outliers, indicating that any virus causes less maturation of NPIs in vivo. When MOI was plotted against how long it took mice to normalize, again non-transfected (MOI 0) and NPIs transfected with an MOI of 16 resulted in faster normalization of diabetic animals (Figure 4-8 B). Insulin per transplant was plotted against the length of time required for animals to normalize post-transplant, and interestingly the less insulin the initial graft contained, the faster the mice achieved normoglycemia. These results do not agree with our in vitro results, where it appeared that lower MOIs resulted in less harm than higher MOIs. A logical step for future experiments would be to attempt some NPI transfections at an MOI of 20, and transplant a small amount of tissue (and therefore low amount of insulin per transplant) to observe if these trends are indicating requirements for more successful transplants.

Due to our previous success usind EC ser^{+} culture of NPIs in vitro to shorten the lag time seen post-NPI transplantation required to cure hyperglycemia in mice (as

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described in Chapter 2 of this thesis), we cultured AdPdx-1 NPIs in EC ser^+ conditions prior to transplantation. Six hundred encapsulated islets, either control or AdPdx-1 transfected (MOI 6) were transplanted intraperitonially (ip) into diabetic SCID mice after EC ser^+ culture. As shown in Table 4-7, our results culturing the NPIs EC ser^+ prior to transplantation were very similar to those from in vitro experiments not using the EC ser^+ culture protocol (Table 4-2). The function of the EC ser^+ NPIs was slightly better, as indicated by S.I. when compared to non-encapsulated NPIs. AdPdx-1 transfected NPIs cultured EC ser^+ had a slightly lower S.I.than the non-transfected EC ser^+ NPIs. The blood glucose of the transplanted mice was monitored weekly. The two types of transplants behaved very similarly, with control NPIs having corrected hyperglycemia only 2 days faster than the transfected NPIs (Figure 4-9).

In our in vivo experiments, overexpression of Pdx-1 alone did not provide necessary signals to trigger precursor cells present to mature faster. The NPIs do mature, even with AdPdx-1 transfection, as evidenced by the increase in insulin content of grafts removed (Tables 4-5, and 4-6). In addition to the lack of partner proteins, or sequestering of p300 as mentioned earlier, a much simpler explanation of why AdPdx-1 transfection does not enhance NPI maturation would be that mouse PDX-1 does not bind to the porcine insulin promoter. This is hard to believe, simply because other studies have indicated that mammalian PDX-1 protein shares 100% homology in the homoeodomain portion of the protein with the Xenopus XIHbox protein as well as significant (50%) homology throughout the remainder of the protein (39;40). Thus, one would expect that as we are using a mammalian (mouse) Pdx-1 sequence, that binding to the porcine insulin

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promoter would occur, however, the possibility cannot be ruled out until it is proven otherwise by performing an electrophoretic mobility shift assay.

In our hands, Pdx-1 adenoviral transfection of NPIs did not increase the maturation of NPIs as hoped. No transdifferentiation of ductal cells to ß-cells was noted either by increase of insulin content of samples, or by immunohistochemical analysis of populations of cells. On-going experiments to determine the effect of an empty adenoviral vector on NPIs will confirm whether the up-regulation in PDX-1 protein expression seen is due to AdPdx-1 transfection, or simply stimulation of NPIs due to presence of the virus. Previous success using Pdx-1 adenoviral transfection of various cell types, in vitro and in vivo, with resulting increased insulin content or ß-cell numbers, encourages further research.

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CHAPTER 5

PORCINE ENDOGENOUS RETROVIRAL NUCLEIC ACID IN PERIPHERAL TISSUES IS ASSOCIATED WITH MIGRATION OF PORCINE CELLS POST ISLET TRANSPLANT[1]

5.1 INTRODUCTION

Islet transplantation has proven to be an effective treatment for brittle type 1 diabetes (1-3), however in order to make islet transplantation a therapy for more patients, several obstacles must be overcome. In addition to eliminating the need for chronic immunosuppression, thus making this treatment available to juveniles, an alternative tissue supply must be found as the number of cadaveric pancreases available does not meet the demand for transplantable tissue. Neonatal porcine islets (NPI) present an attractive alternative source of islet tissue (4-6), since they are simple to isolate and provide large numbers of islet cells with the capacity to grow and differentiate *in vivo* once transplanted (4).

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In addition to T-cell mediated rejection (7) and hyperacute rejection (8;9), the presence of porcine endogenous retrovirus (PERV) presents a major concern when considering porcine-to-human xenotransplantation. Although more than 160 humans have been exposed to porcine tissue either as a transplant (10-12) or via an extra-corporeal liver assist device composed of porcine hepatocytes (13), no PERV has been detected in any recipients. In these patients however, only peripheral blood and saliva samples were assayed for PERV, thus neglecting to consider that the virus may be sequestered in peripheral tissues. There is fear of potential recombination of PERV with human endogenous retrovirus (HERV), however Suling et al. recently indicated that such recombination events between these two viruses does not occur (14).

PERV is classified structurally as a C-type retrovirus belonging to the gammaretroviridae family. Closely related viruses include mouse and feline leukemia virus which are known to cause leukemia in an infected host (12). A retrovirus contains genomic RNA, which once reverse transcribed into double-stranded DNA, may incorporate into the host genome. Once a retrovirus enters the germline, it is transmitted vertically, thus, if PERV incorporates into a human germline, it will be passed to subsequent generations, allowing potential rescue of the virus by later recombination events with other endogenous retroviruses. PERV DNA is present in every porcine cell analyzed (15), however the mode and possibility of PERV transmission remain unclear. Humans working closely with porcine tissue (farmers, butchers) have shown no signs of zoonosis, and neither have patients who have been transplanted with porcine tissue (10-13).

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In vitro studies have demonstrated that human cell lines (16;17), and primary human cells (18-20) may be infected with PERV when co-cultured with PERV producing cell lines. Ritzhaupt et al. showed PERV infection of both non-human primate primary cells and cell lines, however no reverse transcriptase activity was detectable at any time, indicating that no viral replication was occurring (21). There has been no convincing in vivo evidence reported of productive PERV infection (10;22-25). Experiments showing evidence of PERV viral gene transcription also indicate porcine specific gene transcription in the same peripheral tissue analyzed, therefore microchimerism cannot be ruled out (26). Heneine et al. retrospectively examined serum samples of 10 patients who received fetal porcine islet transplants, and no PERV was detected in any of these samples (11). In contrast, Van der Laan et al. detected PERV expression in peripheral tissues of immunoincompetent NOD/SCID mice transplanted with adult porcine islets (26). However, in all tissues in which PERV expression was identified, evidence of porcine tissue was also present which would suggest that migration and then cell-to-cell contact of porcine islet cells with peripheral tissues may account for PERV detection. In addition, since this study was conducted in immuno-deficient mice (26), it is conceivable that PERV is found in peripheral tissues due to the absence of an active immune system that would normally destroy the migrated cells, and thus PERV.

One approach to prevent contact of porcine islets with host tissue and thereby potentially lower the risk of viral transmission, is to use immunoisolation devices (27). A report by Elliott et al. (28) demonstrated no in vitro release of viral particles or nucleic acid from cultured alginate encapsulated fetal porcine islets. When transplanted in nude or NOD mice as well as two patients with type 1 diabetes, there was no detection of

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PERV nucleic acid. This is surprising, since alginate microcapsules often do not completely cover the islets and, thus, allow cell exposure (29;30). We hypothesize that PERV is detected in peripheral tissues following transplantation of porcine islets in SCID mice because there is not an intact immune system to eliminate any cells which migrated from the graft. It is also hypothesized that alginate encapsulation of porcine islets will allow detection in peripheral tissues due to incomplete covering of all islet tissue as well as the degradation of capsules post-transplantation (29).

The aim of this study was to examine the incidence of PERV in peripheral tissues of mice transplanted with NPI. We hypothesize that cell migration and/or cell contact allow PERV detection in tissues. We first looked at the role of the immune system by reconstituting NPI transplanted SCID mice and examining for the incidence of PERV in peripheral tissues. Secondly, we examined two immunoisolation approaches to prevent migration of cells from the transplant, namely alginate microencapsulation, and macroencapsulation using a Theracyte™ device.

5.2 RESEARCH DESIGN AND METHODS

5.2.1 Preparation of neonatal porcine islets

All animal protocols used in this study follow guidelines set out by the Canadian Council for Animal Care (CCAC). The method used to isolate NPI has previously been described (4). Briefly, donor pancreases were obtained from Landrace-Yorkshire neonatal pigs (1-3 days old, 1.5-2.0 kg body weight) of either sex. Piglets were anesthetized with halothane and subjected to laparotomy and complete exsanguination.

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Pancreases were removed, cut into small pieces, and digested in 2.5 mg/mL collagenase (Sigma, Oakville, ON). After filtration through a nylon screen (500 μm), the tissue was cultured for 6 days in HAM's F10 medium (Gibco, Burlington, ON) containing 10mM glucose, 50 μm isobutalmethylxanthine (IBMX; ICN Biomedicals, Montreal, PQ), 0.5% bovine serum albumin (fraction V, radioimmunoassay grade, Sigma), 2mM L-glutamine, 10 mM nicotinamide (BDH Biochemical, Poole, England), 100 U/mL pennicillin, and 100 μg/mL streptomycin.

5.2.2 Encapsulation and transplantation of NPIs into diabetic SCID mice

Diabetes was induced using 225mg/kg body weight Streptozotocin (Sigma) injected intraperitonially (i.p.) in 6-8 week old CB17 SCID beige mice (Taconic Farms, Germantown, NY). All diabetic recipients had a minimum blood glucose level of 20 mmol/L prior to transplantation. Blood glucose levels were monitored weekly using a OneTouch Ultra blood glucose monitor (Lifescan, Milpitas, CA). Under the left kidney capsule of eighteen SCID mice, 3,000 non-encapsulated (non-EC) NPI (28.3±4.0 μg DNA, 4.2±0.8 μg insulin per graft) were transplanted. In a second group of fifteen SCID mice, 1000 alginate microencapsulated (EC) NPI (6.2±0.3 μg DNA, 6.6±1.0 μg insulin) were transplanted i.p.. For alginate microencapsulation, NPI were washed with HBSS (Sigma) without calcium or magnesium, supplemented with 10 mmol/L HEPES. Islets were resuspended in 0.5mL HBSS and 0.5mL of 1.5% (w/v) highly purified MVG alginate (Pronova Biomedical, Oslo, Norway) dissolved in HBSS. The resulting islet/alginate mixture was vortexed and transferred into a 1mL syringe. Microcapsules (350 – 450 μm in diameter) were formed by passing the alginate/islet suspension through an electrostatic generator followed by collection in a 120mmol/L $CaCl_2$ (10mmol/L

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HEPES, 0.01% Tween 20) solution. The capsules were washed by gravity sedimentation in supplemented HAM's F10 medium and cultured overnight prior to transplantation. In a third group, NPI were contained in a TheraCyte™ macroencapsulation device and the device was implanted in the abdominal fat pad of normoglycemic SCID mice. Previous attempts to cure diabetes were technically difficult, due to little, if any, fat pad remaining in the recipient diabetic mice. Prior to transplantation, TheraCyte™ devices (4.5 μL TheraCyte™System, TheraCyte, Inc., Irvine, CA) were soaked overnight at 37°C in HAM's F10. NPI (5000 islets per transplant) were loaded into the TheraCyte™ devices using a 25 μL Hamilton Syringe (Hamilton Company, Reno, NV) and cultured in HAM's F10 at 37°C until time of transplantation (30-60 minutes). A midline incision was made to reveal the abdominal fat pad, which was pulled out and spread to form a layer. The loaded device was placed on top of the thin fat layer, and the fat was folded to enclose the device and sealed using DERMABOND (Ethicon, Cincinnati, OH) and the incision was closed.

5.2.3 Reconstitution of SCID mice

Once diabetic mice had returned to a normoglycemic state (blood glucose < 10mmol/L, 56.8±1.7 days post transplantation), 10 of 18 SCID mice from the non-EC group were reconstituted with $10x10^6$ Balb/c splenocytes. To isolate splenocytes, spleens were removed from Halothane anesthetized Balb/c mice (University of Alberta colony) and suspended in cold HBSS. In a petri dish containing sterile saline, spleens were minced to $3mm^2$ fragments, homogenized using the frosted edges of two sterile glass slides, then filtered through a 63 μm mesh filter and the filtrate was washed twice in sterile saline. Red blood cell lysis buffer (0.15M NH_4Cl, 1.0mM $KHCO_3$, 0.1mM

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Na_2EDTA, pH 7.2) was added for 4 minutes and the splenocytes were washed three times with saline, and injected i.p..

5.2.4 DNA/RNA isolation and histological analysis

All animals were anaesthetized with halothane, prior to euthanization by cervical dislocation. Recipients of non-EC NPI that were not reconstituted (n=8), were sacrificed and tissues harvested for DNA and RNA isolation between 74 and 87 days post-transplantation. Mice that achieved euglycemia by implantation with alginate EC NPI were sacrificed and tissues harvested at 74 (n=6), 87 (n=7), and 150 (n=2) days post-transplantation. Reconstituted mice were sacrificed and tissues collected once they rejected their grafts and reverted to hyperglycemia (12.7±1.0 days post reconstitution). Those mice receiving NPI placed in TheraCyte™ devices (n=5) were sacrificed at 76 days post-transplantation.

All tissues were removed under aseptic conditions utilizing RNase/DNase free reagents and instruments to prevent possible RNA/DNA cross-contamination. Duplicate tissue samples from each brain, heart, liver, spleen, and non-engrafted contralateral kidney were collected from 38 SCID mice (non-EC n=18, EC n=15, TheraCyte™ n=5). Samples for DNA analysis were immediately frozen and stored at –80°C until extraction, while samples for RNA analysis were crushed with sterile RNase/DNase free pestles, resuspended in 1 mL of Trizol (Invitrogen, Carlsbad, CA) and stored at –80°C until extraction.

For DNA extraction, samples were thawed on ice, resuspended in 350 µL lysis buffer (50 mM Tris, 100 mM EDTA, 400 mM NaCl, 0.5% SDS), and treated with 0.2 mg/mL proteinase K (Sigma) overnight at 55°C. Following proteinase K inactivation,

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Table 5-1. Primers used for PCR amplification of porcine endogenous retrovirus pol, porcine COII, and mouse GAPDH DNA and cDNA [from Deng et al. (31)].

Primer		Sequence									Amplified Product (bp)
Perv-pol											
1stPCR	Forward:	GCA	TTC	AGT	GCT	GCT	ACA	AC			
	Reverse:	ATT	GGA	CAG	GAA	CTA	GGA	TG			
2ndPCR	Forward:	GCT	ACA	ACA	ATT	AGG	AAA	ACT	AAA	AG	326
	Reverse:	AAC	CAG	CAG	TGT	ATA	TCT	TGA	TCA	G	
COII											
1stPCR	Forward:	CGT	TAC	CCT	TTC	CAA	CTA	GGC	TTC		
	Reverse:	TTC	GAA	CTA	CTT	TAA	TGG	GAC	AAG		
2ndPCR	Forward:	CAC	ACA	CTA	GCA	CAA	TGG	ATG	CC		315
	Reverse:	GAG	GAT	ACT	AAT	ATT	CGG	ATT	GTT	AT	
GAPDH	Forward:	AAT	CCC	ATC	ACC	ATC	TTC	CAT	TC		335
	Reverse:	GGC	AGT	GAT	GGC	ATG	GAC	TCA	AG		

chloroform extraction, and ethanol precipitation, DNA pellets were washed in 70% ethanol, air-dried, and dissolved in 100 µL DNase/RNase free water (Sigma). Total RNA was extracted from any organs in which PERV polymerase DNA was detected by PCR. RNA was isolated using Trizol (Invitrogen) according to the manufacturer's protocol. To remove any genomic DNA, 1µg of RNA was treated with 1.0 U of Amplification Grade DNase I (Invitrogen). The RNA was then reverse transcribed in 20 µL at 42°C for 60 minutes using Superscript RNase H-Reverse Transcriptase (Invitrogen) and oligo dT12-18.

PERV sequence and or messenger RNA (mRNA) was detected using primers to PERV polymerase gene (*pol*) (31) (Table 5-1). The *pol* gene is shared between PERV

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class A and B, whereas PERV *env* is highly variable (31), and PERV *gag* shares some sequence homology to mouse endogenous retrovirus (MERV) (31). Although our primers do not detect PERV class C, it has been shown that PERV-C is unable to infect human cells (32;33). Primers to the porcine mitochondrial gene encoding cytochrome oxidase subunit II (COII) were used to distinguish PERV incidence from microchimerism (Table 5-1). Primers to GAPDH housekeeping gene were used to verify DNA/RNA integrity (Table 5-1). Both the *pol* and COII PCRs were two stage (nested pcr) while GAPDH was a single stage reaction. The starting template in the 1st amplification of the nested and single stage PCR reactions was either 500 ng of DNA or 1 μL of RT reaction in a 50 μL reaction volume (1X PCR buffer, 2mM $MgCl_2$, 300 nM of each primer, 200 μm dNTP, and 1.5 U Taq DNA Polymerase (Invitrogen)). In the nested/two stage PCR reaction, 2% of the first reaction served as the template in the second round of amplification. PCR was performed using the Gene Amp PCR System 9700 (Applied Biosysems, Foster City, CA) using the following conditions: 25 cycles (1st PCR) or 35 cycles (2nd PCR) at 94°C denaturing for 30 seconds, 56°C annealing for 30 seconds, and 72°C extension for 30 seconds.

All PCR products were electrophoresed through an ethidium bromide stained agarose gel (1.5%) and photographed. PERV and COII PCR and RT-PCR products of the expected size were ligated into the pCR4-TOPO vector (TOPO TA Cloning Kit for Sequencing, Invitrogen) and sequenced (University of Alberta DNA Core Lab). Unknown sequences were analyzed using BLAST (NCBI) and compared with known GenBank sequences. PERV *pol* sequencing results revealed that the primers recognized both PERV class A and B.

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At the time of sacrifice, NPI graft bearing kidneys (from both non- and reconstituted mice) and TheraCyte™ devices were removed for histological analysis. Tissues were immersed in Z-fix (Anatech Ltd., Battle Creek, MI), processed, and embedded in paraffin. After deparaffinization and rehydration, tissue sections (3µm) were stained using guinea-pig anti-insulin antibody (1:1000; Dako, Mississauga, ON). Sections were then incubated with biotinylated goat anti-guinea-pig IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA). The avidin-biotin complex/horseradish peroxidase (Vector Laboratories) method of detection was used and developed with 3,3-diaminobenzidinetetrahydrochloride (DAB) (BioGenex, San Ramon, CA) to produce a brown colour. Sections were counterstained using hemotoxylin (Zymed Laboratories Inc., San Francisco, CA) and eosin Y solution (Sigma).

5.2.5 Statictical Analysis

Data are expressed as means±SEM of *n* independent observations. A student's unpaired T-test was used to determine statistical significance of difference for blood glucose levels of EC versus non-EC transplants. $P<0.05$ or $P<0.005$ was considered significant. The Fisher's Exact Test was used to determine statistical significance of difference for levels of PERV *pol* and porcine COII DNA from tissues analyzed. $P<0.05$ was considered significant.

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5.3 RESULTS

5.3.1 NPI correct diabetes and are rejected following splenocyte reconstitution

Diabetic SCID mice, transplanted with 3000 non-EC NPI (n=18), required 5-11 weeks (43.8±4.0 days) to achieve euglycemia (Figure 5-1A). Recipients of 1000 EC NPI (n=15), corrected hyperglycemia more rapidly than the non-EC NPI recipients, (37.7±4.2 days) at which time the mice achieved a blood glucose level of 10 mmol/L (Figure 5-1A). Significant differences in glycemia between the non-EC and EC group were noted 7, 14, 21, and 56 days post-transplantation. Between 74 and 87 days post-transplantation, mice were sacrificed. In a third group, SCID mice, with non-EC grafts, were reconstituted with syngeneic splenocytes (56.8±1.7 days post-transplantation, n=10). These mice rapidly rejected the NPI grafts 12.7±1.0 days post-reconstitution, and returned to a diabetic state (blood glucose >20 mmol/L; Figure 5-1B). Immunohistochemical analysis of the grafts recovered from the reconstituted SCID mice revealed few surviving ß-cells and extensive lymphocyte infiltration (Figure 5-2A). In contrast, grafts from non-reconstituted SCID mice exhibit well granulated insulin-positive cells. (Figure 5-2B). In a fourth group, NPI implanted in TheraCyte™devices were transplanted under the fat pad of naïve SCID mice (n=5, 5000 islets). For this study, TheraCyte™device transplants were not performed in diabetic animals because of difficulty in achieving normoglycemia, possibly due to lack of a sufficient fat pad. Animals in this group were sacrificed 76 days post-transplant. Immunohistochemical staining revealed large insulin positive cells populating the majority of the graft (Figure 5-2C).

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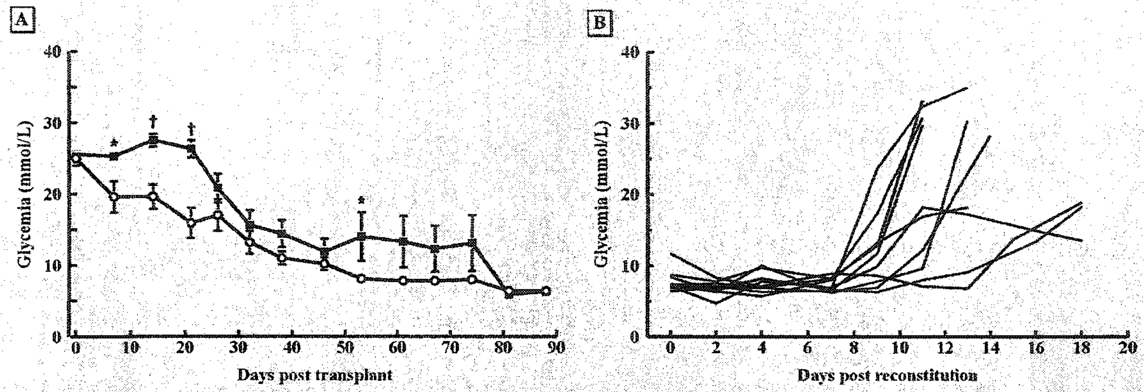

Figure 5-1. (A) Blood glucose levels in SCID mice transplanted intraperitonially with alginate microencapsulated NPI (white circles), or with non-encapsulated NPI under the kidney capsule (black squares). In the encapsulated group, n=18 ≤ day 46 post-transplant, n=8 day 47-74 post-transplant, and n=2 > day 74 post-transplant. In the non-encapsulated group, n=15 ≤ day 74 post-transplant, and n=9 > day 74 post-transplant. Statistical significance of differences was calculated by the Student's unpaired T-test. *$P<0.05$, $P<0.005$. (B) Blood glucose levels in SCID mice previously transplanted with non-encapsulated NPI under the kidney capsule and subsequently reconstitued with Balb/c splenocytes (n=10).

5.3.2 Presence of PERV pol and porcine COII DNA in peripheral tissues

DNA collected from peripheral tissues (spleen, liver, kidney, brain, heart) was analyzed by PCR for the presence of PERV *pol* gene as well as for porcine-specific mitochondrial cytochrome oxidase II (COII) as previously described (31). Negative controls included non-transplanted mouse kidney to ensure that our PERV primers were not cross-reacting with mouse DNA, while freshly isolated NPI were used as positive controls. Results from analysis of individual tissue types are outlined in Tables 5-3 and 5-4.

Peripheral tissues were analyzed from four groups of mice: non-EC, EC, non-EC then reconstituted, and TheraCyte™ (Table 5-2). The following four criteria were used to

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analyze PCR and RT-PCR results: PERV *pol*/COII double positive, PERV *pol* only, COII only, and negative. Of the tissues retrieved from non-EC NPI grafts, 32.5% (13/40) were double positive for PERV *pol*/ COII DNA. One liver was found to be PERV *pol* positive alone (COII negative) (Figure 5-3A, Table 5-3), while 37.5% (15/40) were positive for COII DNA only. The remaining tissues analyzed were found to be negative for both PERV *pol* and COII DNA (11/40, 27.5%). For any tissues positive for PERV *pol* DNA (alone or in the presence of COII), RNA was extracted and analyzed for viral gene expression using RT-PCR. In the non-EC group, only one out of twenty-one tissues analyzed for PERV *pol* mRNA was positive (Table 5-2), and this spleen was also positive for COII mRNA (Table 5-2, Figure 5-3B). Eight out of the twenty-one tissues (38.1%) were positive for residual porcine tissue, but not PERV *pol* mRNA as indicated by the presence of COII.

In mice receiving alginate EC NPI, similar results were obtained (Table 5-2). Of tissues analyzed 37.3% (28/75) were double positive for PERV *pol*/ COII DNA. As in the non-EC group, one liver (out of 75 tissues analyzed) was found to be PERV *pol* positive and COII negative (Figure 5-3A), and 32.0% (24/75) of tissues were positive for COII DNA only. The remaining samples, 24.3% (22/75) of tissues, were negative for both PERV *pol* and COII DNA. When tissues positive for PERV *pol* DNA were screened using RT-PCR, results were similar to the non-EC group; one spleen of 27 tissues (3.7%) was positive for PERV *pol* gene expression, but this spleen was also positive for COII mRNA (Figure 5-3B). Seventeen of 27 tissues analyzed by RT-PCR were positive for COII gene expression alone (Table 5-2) indicating presence of residual porcine tissue, and no (0/27) tissues were positive for PERV *pol* alone.

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When SCID mice were reconstituted with Balb/c splenocytes (n=10), they returned to a hyperglycemic state (Figure 5-1B). They exhibited significantly lower levels ($p<0.01$) of PERV *pol*/COII double positive DNA in peripheral tissues than either the non-EC or the EC group (8% compared to 32.5% and 37.3% respectively, n=50 tissues analyzed) (Table 5-2). Levels of COII positive DNA alone were also significantly reduced in these tissues as compared to the non-reconstituted animals ($p<0.01$) (8% vs. 37.5% and 32.0% for non-EC and EC, respectively). This reconstituted group had a significantly higher percentage of tissues negative for both PERV *pol* and COII DNA ($p<0.0001$) (84% vs. 27.5% of non-EC and 24.3% of EC). When RT-PCR was used, mRNA was not detected for PERV *pol* whereas COII mRNA alone was found in 30.8% (4/13) of tissues. This is much lower than the EC group (63.0%), but similar to the non-EC condition (38.1%).

Table 5-2. PCR and RT-PCR results for porcine endogenous retrovirus and COII from peripheral tissues collected from SCID mice transplanted with neonatal porcine islets.

	DNA								RNA							
	PERV/COII^{+VE}		PERV^{+VE} ONLY		COII^{+VE} ONLY		NEGATIVE		PERV/COII^{+VE}		PERV^{+VE} ONLY		COII^{+VE} ONLY		NEGATIVE	
Non-encapsulated	13/40	32.50%	1/40	2.50%	15/40	37.50%	11/40	27.50%	1/21	4.80%	0/21	0%	8/21	38.10%	12/21	57.10%
Alginate Microencapsulated	28/75	37.30%	1/75	1.30%	24/75	32.00%	22/75	29.30%	1/27	3.70%	0/27	0%	17/27	63.00%	9/27§	33.30%
Reconstituted	$4/50^{\dagger\ddagger}$	8%	0/50	0%	$4/50^{\dagger\ddagger}$	8%	$42/50^{*\ddagger}$	84%	0/13	0%	0/13	0%	4/13	30.80%	9/13	69.20%
Theracyte™ Macroencapsulated	$0/28^{*\dagger}$	0%	0/28	0%	$0/28^{*\ddagger}$	0%	$28/28^{\S*\ddagger}$	100%	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

N/A indicates that no tissues were analyzed for PERV or COII message in this group, as no DNA was detected by PCR.
* $p< 0.001$ *vs. non-EC*, † $p< 0.01$ *vs. non-EC*, ‡ $p < 0.005$ *vs. EC*, § $p < 0.05$ *vs. reconstituted.*

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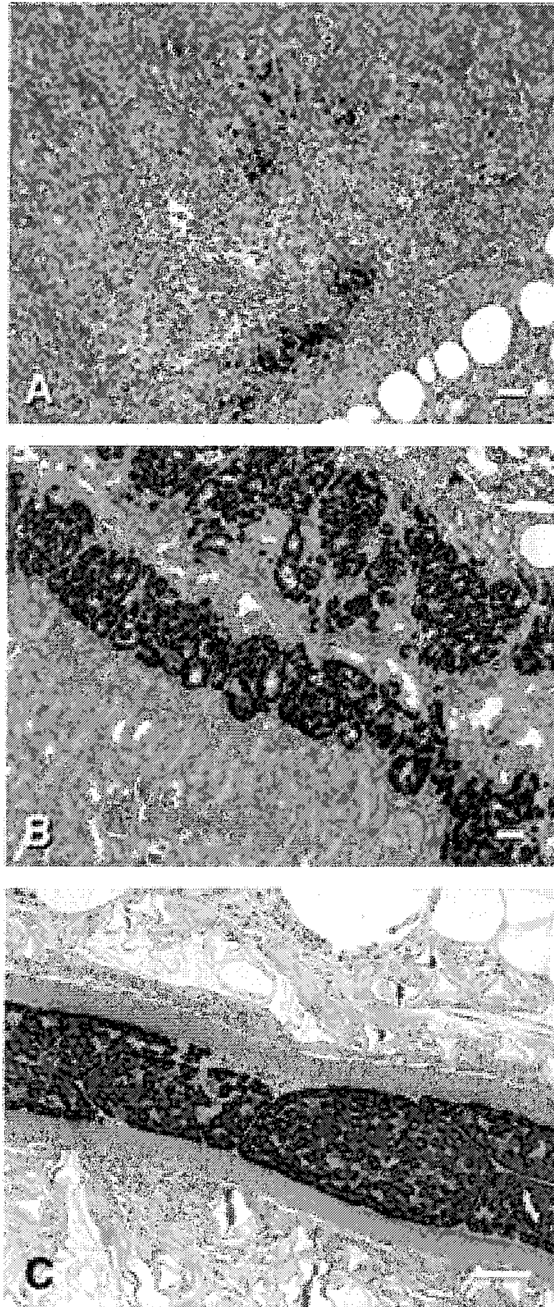

Figure 5-2. Histological analysis of NPI grafts stained for insulin, detected using DAB chromogen (brown), and counterstained using Harris's hematoxylin and eosin Y. (A) A graft from a SCID mouse reconstituted with Balb/c splenocytes and removed after hyperglycemia returned. Very few insulin-positive cells remain in the heavily lymphocytic infiltrated graft. (B) NPI graft recovered from a non-reconstituted SCID mouse 76 days post-transplantation. (C) Survival of NPI in a TheraCyte™ device removed from the abdominal fat pad of a SCID mouse 76 days post-transplantation. Sections reveal many well granulated insulin positive cells. Scale bars represent 50 μm.

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Table 5-3. PCR analysis of peripheral tissues retrieved from SCID mice transplanted with neonatal porcine islets.

		Tissues Analyzed by PCR											
		Spleen		Kidney		Liver		Heart		Brain		Total Tissues Analyzed	
Non-encapsulated	*PERV*	**5/8**	62.5%	**1/8**	12.5%	**5/8**	62.5%	**1/8**	12.5%	**2/8**	25%	**14/40**	35%
	COII	**5/8**	62.5%	**5/8**	62.5%	**7/8**	87.5%	**4/8**	50%	**6/8**	75%	**27/40**	67.5%
Alginate Microencapsulated	*PERV*	**8/15**	53.3%	**3/15**	20%	**11/15**	73.3%	**7/15**	46.7%	**0/15**	0%	**29/60**	48.3%
	COII	**8/15**	53.3%	**13/15**	86.7%	**11/15**	73.3%	**12/15**	80%	**6/15**	40%	**40/60**	66.7%
Theracyte™ Macroencapsulated	*PERV*	**0/5**	0%	**0/5**	0%	**0/4**	0%	**0/4**	0%	**0/5**	0%	**0/23**	0%
	COII	**0/5**	0%	**0/5**	0%	**0/4**	0%	**0/4**	0%	**0/5**	0%	**0/23**	0%
Reconstituted	*PERV*	**1/10**	10%	**0/10**	0%	**2/10**	20%	**1/10**	10%	**0/10**	0%	**4/50**	8%
	COII	**2/10**	20%	**0/10**	0%	**2/10**	30%	**2/10**	20%	**1/10**	10%	**8/50**	16%

Results are expressed as a ratio of positive tissues over total tissues analyzed, and as a percentage.
PERV, porcine endogenous retrovirus.

Table 5-4. RT-PCR analysis of tissues retrieved from mice which were positive for porcine endogenous retrovirus *pol* DNA in one or more tissues as analyzed by PCR.

		Tissues Analyzed by RT-PCR											
		Spleen		Kidney		Liver		Heart		Brain		Total Tissues Analyzed	
Non-encapsulated	*PERV*	**1/5**	20%	**0/3**	0%	**0/6**	0%	**0/3**	0%	**0/4**	0%	**1/21**	4.8%
	COII	**4/5**	80%	**1/3**	33.3%	**1/6**	16.7%	**1/3**	33.3%	**1/4**	25%	**8/21**	38.1%
Encapsulated	*PERV*	**1/8**	12.5%	**0/3**	0%	**0/10**	0%	**0/6**	0%	**N/A**		**1/27**	3.7%
	COII	**5/8**	62.5%	**1/3**	33.3%	**7/10**	70%	**4/6**	66.7%	**N/A**		**17/27**	63%
Reconstituted	*PERV*	**0/2**	0%	**0/3**	0%	**0/3**	0%	**0/2**	0%	**0/3**	0%	**0/13**	0%
	COII	**2/2**	100%	**1/3**	33.3%	**1/3**	33.3%	**0/2**	0%	**0/3**	0%	**4/13**	30.8%

Results are expressed as a ratio of positive tissues over total tissues analyzed, and as a percentage.
PERV, porcine endogenous retrovirus.

In mice implanted with TheraCyte™ macroencapsulated NPI, no porcine COII was detected in any of the tissues (n=28) (Table 5-2). Similarly, no PERV *pol* DNA was detected in peripheral tissues. Subsequently, since no PERV *pol* or COII DNA was detected in these tissues, no RNA was extracted for RT-PCR analysis.

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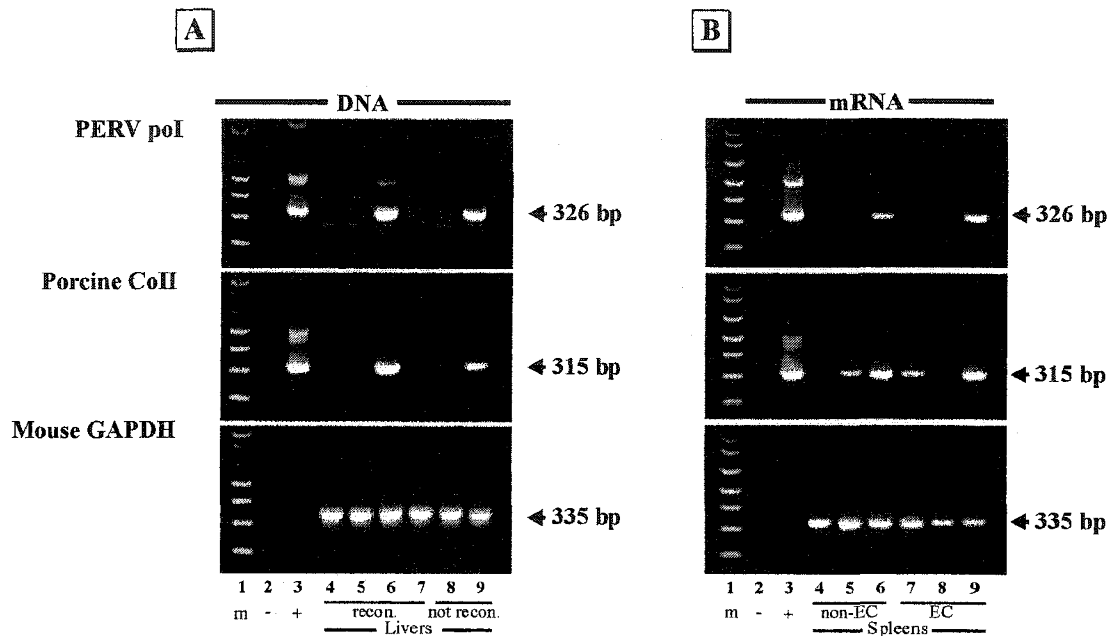

Figure 5-3. Representative PCR (A) and RT-PCR (B) detecting PERV pol (top), porcine COII (middle), and mouse GAPDH (housekeeping gene to ensure nucleic acid integrity, bottom panel). Lane 1 is a 100kbase ladder, lane 2 is a negative control, lane 3 is NPI (positive control). (A) Livers harvested from SCID mice (n=6) transplanted with NPI under the kidney capsule. Lanes 4, 5, 6, and 7 are liver samples from SCID mice reconstituted with Balb/c splenocytes, lanes 8 and 9 are samples from SCID mice which were not reconstituted. One reconstituted mouse (lane 6) and one non-reconstituted mouse (lane 9) were positive for PERV pol DNA, both of these samples were also positive for porcine COII DNA. (B) Spleens harvested from SCID mice transplanted with non-EC (n=3) or EC (n=3) NPI. Lanes 4, 5, and 6 are spleen samples from non-EC islet transplanted SCID mice, lanes 7, 8, and 9 are samples from EC islet transplanted SCID mice. One EC (lane 6) and one non-EC (lane 9) spleen sample are positive for PERV pol gene transcription, but these spleens are also positive for porcine COII gene transcription.

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5.4 DISCUSSION

In previous studies, mice transplanted with adult or fetal porcine tissue, have been shown to harbor PERV DNA, not only at the site of transplant, but also in peripheral tissues such as the liver and spleen (26;31;34). In our study, NPI transplanted into SCID mice achieved similar results. We, and others (26;34), have also observed the presence of PERV *pol* gene expression in these peripheral tissues. However, in our recipient animals which did indicate transcription of PERV *pol* RNA, this always coincided with the presence of porcine COII RNA (Figure 5-3B). Thus, it has been difficult to distinguish microchimerism of tissue from actual PERV infection of host tissue. In our study, PERV *pol* RNA was only detected in two samples: one spleen from the non-EC group, and one spleen from the EC group. It is interesting to note that the site of engraftment in both groups is close in proximity to the spleen; either the kidney in the case of the non-EC group, or the intraperitonial space in the EC group. It is common to observe adhesion of the spleen to the kidney in those animals receiving islets under the kidney capsule, and when alginate encapsulated islets are injected into the intraperitonial space, microcapsules do adhere to the spleen and liver.

It was shown by Elliott et al. (28) that alginate microencapsulation of porcine islets prevented leakage of PERV nucleic acid into media when cultured for 2 months. Transplantation of these islets into NOD or nude mice and subsequent analysis of peripheral tissues (liver, spleen, and heart) 3 months post-transplant revealed no evidence of PERV or porcine nucleic acid sequestered. In our hands, animals transplanted with alginate EC islets had very similar levels of both PERV and porcine COII DNA to the non-EC group (Table 5-2). This contradiction may be a result of the animal models used

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or simply the preparations of the microcapsules, as Elliott et al. used polylysine alginate capsules (28) as opposed to our alginate capsules. If any non-encapsulated porcine tissue is present, upon culture with the alginate microcapsules this tissue would adhere to the outside of the capsule, thereby allowing cell-to-cell contact between the graft tissue and the host, as would any non-complete capsules. Occasional broken alginate microcapsules have been observed when capsules are examined histologically post-transplantation (data not shown, (29)). Smaller capsule formation is associated with some islets actually protruding from the capsules (data not shown, (30)), creating the potential for porcine tissue to become exposed to the host or to migrate into the host's peripheral tissues. Because our data is not in agreement with the previous study (28), this exemplifies the importance of carefully selecting an effective immunoisolation device that does avoid all cell-to-cell contact or cell migration and may then prevent transmission.

Use of an alternative encapsulation device, the TheraCyte™ macroencapsulation device, results in total avoidance of porcine cell and PERV nucleic acid migration and may prevent the possibility of PERV transmission, as exemplified by our analysis of peripheral tissues from SCID mice transplanted with NPI in these devices. As Table 5-2 indicates, no trace of PERV *pol* or COII DNA was detected in all tissues examined. This is especially interesting as the manufacturer of the TheraCyte™ device indicated that virus (specifically PERV) would be able to move in and out of the device (personal communication: Tom Loudovaris, Oct. 24, 2002 via email). We believe that these results indicate that PERV sequestered in peripheral tissues is likely due to migration of porcine cells from the graft site as evidenced by the presence of COII, as opposed to movement of virus from the graft.

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In order to better understand the role the immune system plays in preventing PERV infection, we allowed NPI to engraft in diabetic SCID mice, and, once the animals were normoglycemic, we reconstituted the animals (n=10) with Balb/c splenocytes. This model was used because direct transplantation of NPI into immunocompetent mice results in rapid destruction of the graft. Levels of PERV *pol* /COII double positive DNA were significantly lower in tissues recovered from reconstituted animals when compared to tissues removed from non-reconstituted SCID mice which had received either non-EC or EC islet grafts ($p<0.005$, Table 5-2). Similarly, the number of tissues positive for COII DNA only was significantly lower in the reconstituted group when compared to either the non-EC or the EC group ($p<0.005$, Table 5-2). This indicates that the immune system not only clears the virus present, but also the porcine tissue. When examining recovered grafts from reconstituted mice histologically, lymphocyte infiltration had taken over the graft and little insulin-positive tissue remained (Figure 5-2A). No PERV *pol* mRNA was transcribed in any of the tissues analyzed from reconstituted animals (Table 5-2), and levels of COII transcription were reduced from those seen in non-reconstituted animals. Although we cannot determine from these results that the same pathways of killing are involved in the lower levels of PERV *pol* DNA and mRNA as well as in the lower total presence of porcine tissue, it does appear that the disappearance of xenografted tissue corresponds to the disappearance of virus. From these findings we may extrapolate and propose that PERV was not detected in the fetal porcine islet transplanted patients (11) due to rejection of the grafted tissue as anti-porcine antibodies were detected in the patients despite the fact they underwent maintenance immunosupression.

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It is important to note that, in our results, we see a higher incidence of tissues containing COII DNA than peripheral tissues positive for PERV *pol* DNA. This was also noticed, and addressed, by Deng et al.(31). They hypothesized that this is due to higher copy number of mitochondrial DNA than genomic DNA due to multiple organelles within each cell, as the primers used were tested for sensitivity and were found to be identical pertaining to copy number detectable per 1 μg DNA present (31). This explanation is acceptable, however we also propose that the presence of only COII DNA in some tissues may be due to the presence of necrotic cells, which may contain mitochondria but have lost genomic DNA, or DNA has been destroyed during apoptosis. Another possibility is that, as a result of taking only a small portion of each organ in order to extract total DNA and RNA, we have only a representation of what is in a portion of peripheral tissue. Extracted samples are in a highly diluted sample of mouse nucleic acid, also allowing for a discrepancy in the DNA detection. Thus, detection of COII in some PERV *pol* DNA negative tissues emphasizes the sensitivity of the PCR method employed.

Although very few receptors for PERV have been identified, two human proteins identified by Ericsson et al. to be active PERV A (viral subtype specific) receptors have baboon and porcine homologs which are also active as receptors, however the murine homolog was not active (35). Choosing a model which will provide clinically relevant data is essential, and although the probability of the mouse lacking a PERV A receptor has also been indicated by Deng et al. (31), mouse cell lines have been infected with PERV in vitro, indicating some susceptibility to the virus (26), perhaps to other viral subtypes.

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Previous work has shown that PERV is not transmitted to recipients of porcine tissue transplants, particularly in the presence of an immunoisolation device (28;36). When analyzed, saliva and peripheral blood mononuclear cells (shown to be infected by PERV in vitro) (20), from humans who have been exposed to porcine tissue show no sign of PERV DNA or RNA as analyzed by PCR (10-12). In addition, no humans exposed to porcine tissue, via transplant or liver-assist device, have exhibited any adverse affects which could be attributed to exposure to porcine tissue, and none have developed antibodies to any analyzed pig viruses (13). However, the analysis done to follow up any human recipients of xenotransplants has yet to look at peripheral tissues for potential sequestering of virus.

In order to prevent rejection of xenografts, recipients likely will undergo an immunosuppressive regime. Although this may prevent rejection of porcine tissue, it may also effectively remove the recipient's ability to clear PERV infections. Much more information must be gathered to determine the risk involved before we can entirely ignore PERV. The possibility of in vivo PERV transmission to a model other than the immunocompromised SCID mouse must be determined. In order to demonstrate this, a model reflective of clinical xenotransplantation must be chosen. Finally, it must be determined whether PERV is pathogenic in humans. Overall, the outlook regarding cellular (particularly versus whole organ) xenotransplantation is extremely encouraging, however we must be aware of the potential risks and make every attempt to avoid them.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

6.1 GENERAL DISCUSSION

In 1922, with the discovery of insulin, a diagnosis of diabetes went from being a death sentence to a lifestyle change. People afflicted with type 1 diabetes must constantly plan and fine tune their daily exercise, meals, and insulin injections to best regulate their blood glucose. In 1993, the Diabetes Control and Complications group released the results of a study indicating that intensive insulin therapy, to avoid fluctuations in blood glucose, reduced the chances of secondary complications of diabetes (1). Unfortunately, intensive insulin therapy increases the risk of hypoglycemic episodes which can lead to coma and death if not treated quickly. To achieve near physiological control of blood glucose, transplantation of insulin-producing tissue is the most promising treatment for type 1 diabetes. Recent success in islet transplantation has brought this minimally invasive treatment to the forefront of attention (2-4). This procedure, however, requires the recipient to trade daily use of exogenous insulin for chronic immunosuppression. Strategies to avoid immunosuppression, such as tolerance, immunoisolation, or co-transplantation with sertoli cells (5-7) must prove successful before a young type 1 diabetic could justify islet transplantation as a superior treatment.

In the United States, approximately 3000 cadaveric donor pancreases are donated every year. Each year, 30,000 new cases of type 1 diabetes are developed in the U.S.A., so even if one pancreas will be able to supply an adequate islet mass for one transplant, and all immunological considerations aside, the donor tissue supply will clearly not meet

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the demand (8;9). Several alternative sources are being considered for islet-transplantation, including embryonic stem cells (10;11), potential adult pancreatic precursor (ductal) cells (12;13), mature β-cell expansion (14), β-cells transdifferentiated from other terminally differentiated cell types (15-18), and xenogeneic sources (19-24).

Neonatal porcine islets (NPIs) are a practical source of insulin-producing tissue and are the focus of discussion in this manuscript. Korbutt et al. described the ease and reproducibility of NPI isolation, as well as the potential for NPI maturation and expansion (7). Studies further characterizing NPIs have shown immunological properties, such as the age dependent expression of Galα(1,3)Gal (25) and the reaction of the human immune system to NPIs (26-28). Other groups have provided information on factors inducing in vitro NPI maturation (28) and in vivo NPI maturation (29;30).

In this study, we developed a method of in vitro NPI functional maturation that involved alginate microencapsulation and culture with autologous serum (Chapter 2 of this manuscript). Increases in insulin content of NPIs, as well as in the number of endocrine cells in EC ser^+ samples were observed, which resulted in a much faster restoration of euglycemia in diabetic mice post-transplantation than had been seen previously in NPI transplantation. It is believed that the alginate microcapsule provides a three dimensional network which seems beneficial for islets, improving the microenvironment of islets. Autologous serum likely contains growth factors, which may be specific to the neonatal age of the islets as serum was derived from the same piglets as the islets. Knowledge of pancreatic development is improving rapidly, and new signals and growth factors involved in development are still being identified. With new information, improvement of culture conditions of NPIs may be improved further to

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mimic the pancreatic microenvironment and further stimulate NPI maturation. The in vitro functional maturation of NPIs provides a model with which islet development can be studied. Due to the availability of NPIs, and the similarities between porcine and human development, this model will provide information on islet development which may be applied to human studies, including work with expansion of human islet precursor populations, and embryonic stem cell studies.

The ultimate goal being utilization of NPIs for transplantation into humans to treat type 1 diabetes, observation of in vivo development of NPIs is essential (Chapter 3). Using a time course to obtain "snap shots" of in vivo NPI development, we observed an initial drop in insulin content lasting approximately 12 days post-transplantation, after which dramatic increases in insulin content as well as area of the graft staining positively for insulin were observed. Our results can be explained by previous work on a remodeling of transplanted tissue (31) as well as studies examining trauma inflicted on tissue during transplantation (32). An additional observation was the impact of number of cells, and not insulin content, on the time required to correct diabetes post-transplantation. Transplants receiving more NPIs, as determined by DNA content of transplanted tissue, seemed to fare better. It is thought that physical numbers may provide some protection, allowing some NPIs to take the brunt of the trauma, allowing a larger tissue mass to survive the transplant process. However, the lower DNA content of NPIs resulting in a less rapid cure of diabetes may also reflect less viability, and overall less healthy tissue being transplanted. Taken together, if apoptosis of NPIs and trauma to the tissue can be avoided immediately post-transplantation, the lag time required for this immature tissue to correct diabetes may be further shortened. Further work in this area

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should include examination of the remodeling period. TUNEL staining may confirm the influence of apoptosis on loss of tissue post-transplantation. Immune cell infiltration may also play a role in loss of tissue. Preliminary examination of cell proliferation in NPI grafts, taken throughout the in vivo maturation time course, revealed BrdU positive cells surrounding the graft, but not within the graft at six days post-transplantation. Very few proliferating cells were observed at later time points, and these were within the grafts. These proliferating cells must be identified to determine whether they play a role in tissue remodeling or immune attack. Our in vivo model will prove valuable in these types of studies. Information about cell death immediately post-transplant may be transferable to human islet transplantation. Two or three islet isolations from cadaveric pancreases are required to transplant enough islets to correct hyperglycemia in one recipient (2;3), indicating that many more islets are being transplanted than are physiologically required to remove the need for exogenous insulin. By understanding insult to transplanted islets, measures may be developed to prevent loss of islets immediately upon transplantation, and fewer islets may be required per islet transplant recipient. Transcription factor profiling utilizing this time course provides valuable information about islet development. We observed PAX6 and NKX2.2 protein expression remaining near pre-transplant values immediately post- transplantation and dropping by day 12 post-transplantation, indicating the importance of timing of transcription factor expression in development. Development of primers and probes specific for porcine genes will allow us to further examine gene expression throughout in vivo NPI maturation using quantitative RT-PCR. As these techniques have just become available to us, we plan to evaluate endocrine gene expression levels throughout the time course.

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An adenoviral construct containing mouse Pdx-1 behind the CMV promoter (AdPdx-1) was used to transfect NPIs in the hope that transient overexpression of PDX-1 might initiate differentiation of precursor cells toward a β-cell fate (in Chapter 4 of this manuscript). Although overexpression of PDX-1 was seen in our samples (by Western blot and immunohistochemistry), there was no significant increase in insulin content of samples or number of cells staining positively for insulin. The transfected islets were transplanted to determine whether additional signals provided in vivo would aid in PDX-1 mediated maturation of NPIs, as an AdPdx-1 construct had been injected into the liver of mice and induced ectopic insulin expression in a previous study (33). No beneficial effect on NPI maturation was observed. As mouse Pdx-1 was expressed by the adenoviral construct, one concern raised was the ability of a mouse-derived PDX-1 protein to bind to the porcine insulin promoter. Pdx-1 is the mammalian homolog of Xenopus XlHbox 8, (34). The two proteins are closely related, and antibodies raised against one seem to recognize the other (35). Conservation of Pdx-1 between chick, human, and mouse has been observed (36), which would lead one to believe that the mouse and porcine Pdx-1 would both recognize and bind to similar regions on the porcine insulin promoter. However the only way to truly know whether species incompatibility would explain the inability of the mouse Pdx-1 induce maturation in NPIs, an electrophoresis mobility shift assay must be performed. Too much and too little PDX-1 both have the effect of decreasing insulin gene expression (37), and p300 co-activator, required for PDX-1 binding, may also sequester excess PDX-1 in cytoplasm, preventing translocation of the transcription factor to the nucleus and, thus, subsequent gene transcription (38). Horb et al. designed a construct which provided VP16

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transcriptional activation domain in addition to Pdx-1 which replaces partner binding proteins required for transcription of PDX-1 regulated genes (39). Collaboration with Dr. Horb has been initiated. We propose to create an adenoviral construct containing Pdx-1-VP16 behind the CMV promoter which will be used to transduce NPIs, in the hope that the VP16 will substitute for necessary partner binding proteins which may have been lacking in our model, and that Pdx-1 expression in NPI precursor cells will increase the β-cell population. Prior to these experiments, the ability of exogenous Pdx-1 binding to the porcine promoter must be confirmed.

In characterizing NPIs as a transplantable tissue source, we also evaluated potential for PERV transmission. To date, more than 160 humans have been exposed to porcine tissue, in the form of fetal porcine islet transplants (40-42), or by use of an extra-corporeal liver assist device (43), with no indication of PERV transmission. Despite this, in vitro experimental infection of human cell lines (44;45) and primary cells (46-48) with PERV, and the fear of an epidemic, have caused researchers in most countries to impose a moratorium on xenotransplantation. Recent reports of unpublished studies of neonatal porcine islet transplantation into adolescents with type 1 diabetes in Mexico indicate that xenotransplantation is a current reality (49), thus making research on PERV and other potential porcine pathogens much more relevant. An intact immune system, provided to mice with established NPI grafts in our study (Chapter 5 of this manuscript), indicated an ability to clear porcine cells which had migrated to peripheral tissues. This is relevant as many studies claiming in vivo transmission of PERV do so because of detection of PERV in peripheral tissues. PERV detection is usually coincident with markers of porcine cells which cloud the issue of transmission versus microchimerism or just existence of

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migrated cells in this tissue. The transplanted children in Mexico are not undergoing any immunosuppressive therapy which, in light of our results, could lower the risk of PERV infection. We observed that site of transplant and type of encapsulation device used in NPI transplantation will have a role in potential PERV transmission.

6.2 CONCLUSIONS

Success in islet transplantation has increased the demand for transplantable insulin-producing tissue. To address the tissue shortage, we have focused on neonatal porcine islets, primarily as a source of xenotransplantable tissue, but also as a readily available and reproducible model to study islet development. In this manuscript, we attempted to mature NPIs, through in vitro functional maturation, in vivo maturation, and by overexpression of Pdx-1, a transcription factor known to be necessary for both pancreatic development, and β-cell function. In vitro and in vivo maturation of NPIs resulted in expansion of insulin-producing tissue, and both models provide insight into NPI development which may be transferred to human sources of tissue for islet development. Overexpression of Pdx-1 did not induce maturation of NPIs. NPI are a valuable tissue source for studying the effect of gene overexpression on islet development, provided that controls are used to confirm species compatibility should gene sequences from species other than porcine be used. One challenge associated with xenotransplantation is how to avoid transmission of disease with transplantation of tissue. Although microchimerism of tissue confounds PERV research, it would appear from our studies that avoidance of migration of porcine tissue using some encapsulation devices, or by the presence of an intact immune system, may prevent PERV nucleic acid detection

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in host tissue. NPIs will provide a realistic means of overcoming the tissue shortage problem, either as a xenotransplantable islet source, or as a model by which further knowledge of islet development will be gained, allowing the development of appropriated tissue sources.

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APPENDIX A

ISOLATION OF A NEONATAL PORCINE DUCTAL CELL POPULATION

A.1 OBJECTIVE

To obtain an enriched ductal cell population from isolated neonatal porcine islets (NPI) in order to study the precursor potential of this tissue source.

A.2 RATIONALE

It is well documented that NPI contain a mixed population of cells composed of 45% endocrine cells and 55% endothelial, described as non-granulated, cells (1). A population of cytokeratin 7 (CK7) immunoreactive ductal cells makes up from 25-45% of the NPIs. Within this population, it is believed, exist precursor cells which will become mature endocrine cells through neogenesis. By isolating these cells, we proposed to study neogenesis as well as to attempt to accelerate the maturation of these precursors through in vitro maturation (see Chapter 2), or by transfection with Pdx-1 (Chapter 4).

Pipleers et.al., using Fluorescence Activated Cell Sorting (FACS), isolated pure β-cell populations from islets by exploiting the granularity of these cells, thus the amount of light scattered, and levels of β-cell autofluorescence (2;3). We believed that by using the same parameters, we could separate out the more mature, granulated endocrine cells from the non-granulated CK7 precursor cells using the same technique.

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A.3 EXPERIMENTAL PROTOCOL

NPIs, isolated as outlined in Chapter 2, were dissociated into single cells by manual disruption with a 10mL glass pipette in calcium-free medium supplemented with 1mM EGTA and 0.5% BSA at 32°C for 8 minutes, followed by a further 5 minutes manipulation after the addition of 25 μg/mL trypsin (Boehringer Mannheim, Laval, PQ, Canada) and 4 μg/mL DNase (Boehringer Mannheim). The dissociated NPIs were then filtered through 70 μm mesh and subjected to FACS analysis. An EPICS® Altra (Beckmen Coulter, Hialeah, FL) cell sorter was used to separate islet cells based on forward and side-scatter of light (due to granularity of cells), as well as autofluorescence. Sorted populations were re-run to ensure clear populations were separated (Figure A-1, C and D).

Sorted populations of cells were collected and samples were fixed onto microscope slides (12 minutes in Bouin's fixative) for immunohistochemical analysis. The remainder of the cells were cultured in Ham's +10% autologous serum in order to attempt to reaggregate the islet cells. Most samples became contaminated after a short culture period (likely due to the multiple manipulations), however when possible, cells were collected after approximately 9 days culture, and transplanted under the kidney capsule of SCID mice (Taconic Farms, Germantown, NY) for in vivo maturation (n=3), however these grafts were not analyzed.

Immunohistochemical analysis of the sorted populations was preformed to detect insulin, glucagon, and CK7. All dilutions and methods have previously been described (Chapter 2, RESEARCH DESIGN AND METHODS).

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Table A-1. Immunohistochemical characterization of FACS sorted NPI populations.

Population	% of cells positive		
	insulin	glucagon	CK7
less granulated	26.81 ± 2.1 (18)	3.68 ± 0.5 (18)	26.60 ± 4.0 (18)
more granulated	3.43 ± 0.4 (18)	52.47 ± 4.4 (18)	11.48 ± 2.5 (16)

Data are mean ± SEM of (n) independent experiments.

A.4 RESULTS

Two distinct populations were sorted using FACS, herein called "less granulated" (corresponding to population A indicated in Figure A-1) and "more granulated" (corresponding to cells in both populations C and D as indicated in Figure A-1). When these two populations were characterized immunohistochemically, results were not as expected. It was felt that the less granulated population would be comprised mainly of non-endocrine CK7-positive cells, however this population was largely insulin-positive (26.81 ± 2.1%) along with a relatively high number of CK7-positive cells (26.60 ± 4.0%) (Table A-1). The granulated population, which was expected to contain the mature, granulated endocrine cells, was mainly glucagon-positive cells (52.47 ± 4.4%) with a very low number of granulated insulin-positive cells (3.43 ± 0.4%) as well as some CK7-positive cells (11.48 ± 2.5%) (Table A-1).

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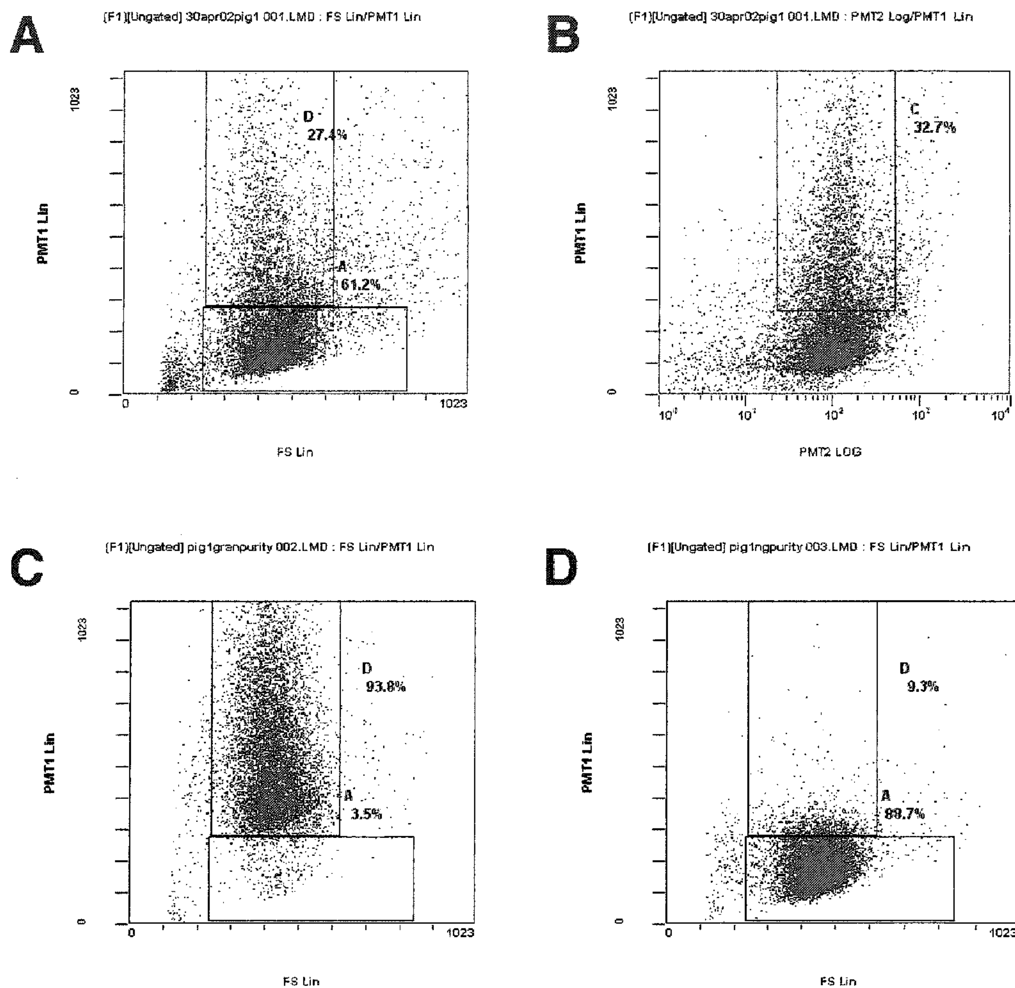

Figure A-1. FACS analysis of dissociated NPIs reveals two distinct populations of cells based on separation due to granularity and auto-fluorescence. A and B) A representative cells sort. Less granular cells were gated as "A", 61.2% in this sort. More mature cells were gated as "D" based on higher granularity and "C" due to auto-fluoresce, "C+D" were sorted out as one more granular population. C) A purity check of the cells sorted into the more granular population revealed a 93.8% pure population. D) A purity check of the cells sorted into the less granular population revealed a 88.7% pure population.

A.5 CONCLUSIONS

Using FACS, it was not possible to sort out a population of primarily ductal cells from NPIs. The less mature (less granulated) population of cells was highly "contaminated" with cells which were immunoreactive for insulin. Although this

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confounded our attempt to separate a population with which to study β-cell neogenesis, this study clearly indicated the immature nature of the NPIs. In pancreatic development, it is well established that glucagon-positive cells are seen at least one day earlier (in the embryonic pancreas) than insulin-positive cells (4). We may conclude from sorting cells based primarily on their granularity, that our NPI model follows this trend. Although insulin-positive cells are clearly seen in NPIs, these are less granulated than the glucagon-positive cells, indicating their immaturity. CK7 positive cells were seen in both the less- and more-granulated populations of cells. A higher number of CK7 cells were observed in the less-granulated population, as would be expected. Cells, which were immunoreactive for CK7 in the more granulated population, may represent transitional cells which are transdifferentiating from a ductal phenotype to an endocrine phenotype, however this remains to be studied.

Although this experiment did not achieve the desired effect; a ductal-enriched population with which we could study endocrine cell neogenesis, it did provide further insight into NPI characterization, and is thus included appended to this thesis.

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APPENDIX B

INSULIN POSITIVE TISSUE AREA QUANTITATION FROM NEONATAL PORCINE ISLET GRAFTS

B.1 OBJECTIVE

To develop a method to determine the area that insulin-positive cells occupy in grafts retrieved post kidney capsule transplantation in mice.

B.2 RATIONALE

Methods of determining ß-cell mass or the number of ß-cells in the pancreas of experimental animals have been previously described (1-5). Due to the, qualitatively obvious, increasing amount of grafted neonatal porcine islet (NPI) tissue staining positively for insulin over time post transplantation, we were interested in quantitating this increase. Trivedi et. al. described a method of determining ß-cell mass from retrieved grafts using non-overlapping fields of view from serial sections (6). Incorporating these ideas, as well as those provided by Dr. Diane Finegood during a one-day session on image analysis and morphometry (Vancouver, B.C., July 28, 2000), we developed a method of quantitating the percentage of graft tissue which immunostained positively for insulin.

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B.3 EXPERIMENTAL PROTOCOL

Tissues were immersed in Z-fix (Anatech Ltd., Battle Creek, MI), processed, and embedded in paraffin. Grafts were not trimmed prior to, or after embedding. Initially, the entire graft was sectioned (4μm), and all of the sections were immunostained for insulin. After deparaffinization and rehydration, tissue sections were incubated with 20% normal goat serum, followed by guinea-pig anti-insulin antibody (1:1000; Dako, Mississauga, ON). Biotinylated goat anti-guinea-pig IgG secondary antibody (1:200; Vector Laboratories, Burlingame, CA) was added for 20 minutes, followed by the avidin-biotin complex/horseradish peroxidase (Vector Laboratories) method of detection, and development with 3,3-diaminobenzidinetetrahydrochloride (DAB) (BioGenex, San Ramon, CA) to produce a brown colour. Sections were counterstained using hemotoxylin (Zymed Laboratories Inc., San Francisco, CA).

Photos were acquired of non-overlapping fields, covering all sections of the graft, using a Zeiss Axiocam camera (Carl Zeiss Canada Ltd., Calgary, AB) mounted on a Zeiss Axioskop 2-Plus microscope (Carl Zeiss Canada Ltd.), and using Zeiss Axiovision software (Carl Zeiss Canada Ltd.). Images were imported into Universal Imaging MetaMorph® Software (Universal Imaging Corporation™, Downingtown, PA). The region containing the graft was defined manually, and the image was split into red, green, and blue images. The red image was discarded, and the defined region was transferred to the blue and green images. First, the green image was thresholded to define the total tissue area. Integrated Morphometry Analysis (IMA) was used to determine area (measured in pixels), and the data was recorded. Next, the blue image was thresholded to define the brown, insulin-positive tissue area was calculated using IMA and the data was

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recorded. Once this procedure was completed for all fields of all sections covering the entire graft, the following equation was used to determine insulin-positive area:

Equation B-1.

Ins +ve area = IMA value from blue image / IMA value from green image x 100%

and an average was determine of all fields.

Subsequent analysis of grafts quantitated only 20% of the graft. For every five sections cut (placed on slides labeled A-E, representing five series of the graft for future staining with up to five antigens of interest), 15 sections were discarded, followed by five sections placed on the A-E series slides, etc. until the entire graft was sectioned. Staining and image analysis proceeded as described above.

B.4 RESULTS

Quantitation of the entire graft, using non-overlapping fields of view, determined that 5.23% of the graft contained insulin-positive tissue. Numbers derived from the 527 fields examined from 96 tissue sections were plotted on a spreadsheet, and used to determine the number of fields of view, as well as the number of sections, which must be analyzed in order to determine an accurate representation of the percentage of graft tissue which is insulin-positive. Observation of 10% of the fields yielded a value similar to observation of all fields, however this would still require sectioning of the entire graft, requiring more histological slides as well as immunohistochemical reagents. Because observation of one section out of every five sections resulted in almost an identical value

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to the entire graft (5.25% vs. 5.23%, Table B-1), and would require staining of only 20% of the tissue sections derived from the graft, we chose to use this value.

Table B-1. Values from quantitation of insulin-positive tissue in one graft. Quantitating one out of every five sections provides an accurate representation of the entire graft.

	all fields of all sections	1 section per 5 sections	1 field per 10 fields	1 field per section	1 field per 3sections
% area insulin-positive	5.23% (527)	5.25% (159)	5.41% (53)	4.68% (96)	6.37% (21)

Data are expressed as area brown stain / total tissue area x 100% of (n) fields.

B.5 CONCLUSIONS

Qualitatively, it is apparent that the area of neonatal porcine tissue staining positively for insulin increases over time when transplanted under the kidney capsule of immunoincompetent mice. We hoped to develop a means of quantitating this increase in insulin-positive tissue.

Techniques were based on previously established morphometry techniques (3;4;6), as well as on advice from Dr. Diane Finegood (personal communication, Vancouver, B.C., July 28, 1000). Using random sampling of the entire graft (20% of the graft), a representation of insulin-positive tissue throughout all regions of the graft is determined. Pixels were used as a measure of the amount of tissue thresholded as positive for insulin. Although pixels are dependent on the brightness of the image, we still feel this is a valid measurement as it is relative area, because insulin-positive area is reported as a percentage of total tissue at the same light intensity.

Relative to the percentage of dissociated NPI cells staining positively for insulin prior to transplantation (15 – 20%, Chapter 3, Table 3-1), values of insulin-positive tissue in grafts retrieved on day 0 (sacrificed approximately 1 hour post-transplant) seemed low (3.5% - 5%, Chapter 3, Table 3-3). This illustrates that we are not calculating the number

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of ß-cells in the retrieved grafts, but the percentage on the graft containing insulin. Analysis of the percentage of insulin-positive tissue does, however, provide insight into the increase of this tissue over time post-transplantation.

Determination of the area of insulin-positive tissue provides insight into maturation of NPIs in vivo (Chapter 3). We have developed a method by which we can determine and compare the increase in insulin-positive tissue in NPI grafts.

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