Augmentation of Clinical Grade Neonatal Porcine Islets with a Novel Maturation Media

by

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Abstract

Current clinical islet cell transplantation relies solely on human cadavers as a source of graft tissue. Much research has been focused on neonatal porcine islets as an alternative due to their ability to be mass-produced, showing strikingly similar physiology and biological activity to human insulin, and extensive research in various type I diabetic animal models. Our lab has demonstrated the feasibility of transplanting neonatal porcine islets in diabetic induced mice with successful reversal of diabetes. However, islet composition and time-to-normalization have remained suboptimal, which has been thought to be attributed to a low number of β-cells within reach islet transplanted. We formulated a maturation protocol for islets in long-term culture in hopes of maintaining a purely β-cell phenotype that will exclusively produce insulin and reverse diabetes sooner than islets grown in standard media.

Pancreata from 1 to 3-day old neonatal pigs were procured and cultured in standard HAMS-F10 media for 4 days. Subsequently, each pancreas was split and further cultured in DMEM-F12 maturation media containing a variety of growth added in a stepwise fashion, or cultured in control HAMS-F10 media. After 20 days, a portion of islets were transplanted into immunodeficient diabetic mice under the kidney capsule. Remaining islets underwent static incubation to assess insulin secretory capacity, DNA quantification to assess β-cell composition, RT-PCR, total cellular insulin content per pancreas, and immunohistology. Insulin content from static incubation and β-cell composition was expressed amount produced per pancreas.

Higher levels of insulin expression, insulin positive cells, and β-cells were observed in matured islets compared to control islets. Increased insulin, β-cell progenitors, and PCNA positive cells were observed in mature islets compared to control islets. Less TUNEL staining and amylase were observed among mature islets compared to control. Mice transplanted with matured islets had significantly lower blood glucose values at weeks 18 and 20 compared to control islets.

It is clear that long-term culture of islets in a maturation media have proven to be successful *in vitro*. The increase insulin production did translate into faster achievement of normalization after transplantation. However, more transplantation experiments are needed in other animal models to suggest that a purely β-cell phenotype is beneficial for a type I diabetic recipient.

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Table of Contents

Abstract ii
List of Key Abbreviations viii
Chapter 1: General Introduction 1
1.1 The Pancreas 1
1.1.1 Anatomy and Physiology 1
1.1.2 The Islet 2
1.2 Type I Diabetes 4
1.2.1 Description of Disease 4
1.2.2 Signs and Symptoms 5
1.2.3 Diagnosis 7
1.2.4 Etiology 9
1.2.5 Other forms of Diabetes 10
1.3 Treatment for Diabetes 10
1.3.1 Insulin Therapy 10
1.3.2 Other Treatment Modalities 11
1.3.2.1 Immunotherapy 11
1.3.2.2 Pancreas Replacement 12
1.3.2.3 Islet Cell Transplantation 13
1.3.2.4 Neonatal Pig Islets (NPIs) 14
1.3.2.4.1 Functional improvement 17
1.3.2.4.2 Maturation 18
1.3.2.4.3 Growth Factors 18
1.3.2.4.4 Maturation Protocols 20
1.4 Objective of Thesis 22
1.5 References 23
Chapter 2: Augmentation of Clinical Grade Neonatal Porcine Islets with Novel Maturation Media 28
2.1 Introduction 28
2.2 Materials and Methods 30
2.2.1 Preparation of Porcine Islets 30
2.2.1.1 20-day Maturation Protocol 30
2.2.1.2 11-day Maturation Protocol 31

2.2.2 Characterization of Islet Preparations 31
2.2.3 RT-PCR 32
2.2.4 Transplantation and Metabolic Follow-Up 33
2.2.5 Characterization of Harvested Islet Grafts 34
2.2.6 Statistical Analysis 34
2.3 Results 35
2.3.1 Cellular Composition of Neonatal Porcine Islets 35
2.3.2 Preparation of Islets 39
2.3.3 Insulin Secretory Capacity 42
2.3.4 Expression Levels of Islet-Specific Genes 43
2.3.5 Transplantation of Control and Matured Islets Into Diabetic Mice 46
2.3.6 Composition of Islet Grafts 47
2.4 Discussion 49
2.5 References 55
Chapter 3: General Discussion 58
3.1 Discussion 58
3.2 Conclusion 62
3.3 References 63
Full Bibliography 64

List of Figures

Figure 1 Normal physiology of glucose metabolism 2
Figure 2 Pathogenesis of Diabetes 7
Figure 3 Suggested Etiology of T1D 9
Figure 4 At 20x magnification - photograph shows neonatal pig islets, an often spherical group of hormone-producing cells. Insulin is labeled here in green, and the nuclei in blue. (Seeberger et.al 2014) 15
Figure 5 Immunohistochemistry of islets grown in control media and maturation media in 20-day protocol. A) immunostaining for insulin and glucagon, insulin and PCNA, and insulin and TUNEL B) Immunostaining for PDX-1 and CK7, and Amylase. Size bar = 10 to 50 μm 38
Figure 6 Immunohistochemistry of islets grown in control media and maturation media in 11-day protocol. A) immunostaining for insulin and glucagon, insulin and PCNA, and insulin and TUNEL B) Immunostaining for PDX-1 and CK7, and Amylase. Size bar = 10 to 50 μm 39
Figure 7 Expression levels of islet-specific genes in islets cultured in control media and maturation media for 20 days. NPIs were used as calibrators and expression level was set to 0 upon plotting log10 values. Expression levels of endogenous control (GAPDH) is also set to 0. N = 10 control, 10 mature. 45
Figure 8 Expression levels of islet-specific genes in islets cultured in control media and maturation media for 11 days in Nic+Ex4. NPIs were used as calibrators and expression level was set to 0 upon plotting log10 values. Expression levels of endogenous control (GAPDH) is also set to 0. N = 10 control, 10 mature. 46
Figure 9 Transplantation of NPIs cultured in control media and maturation media for 20 days and 11 days into diabetic mice. (A) Blood glucose levels over 20 weeks time (B) Percentage normalized curves for transplanted mice that normalized (blood glucose of 15 mmol/L considered normalized) (C) Oral glucose tolerance test (OGTT) (D) Area under curve (AUC) of subsequent OGTT result. Solid line = control, Dotted line = mature, micro-dotted line = 11 days Nic + Ex4. *$p<0.05$ (20-day control vs. mature) 47
Figure 10 (A) H&E + Insulin staining of NPI graft under kidney capsule in control vs. mature transplanted mice. Last recorded blood glucose values: control – 10.3 mmol/L, mature – 9.6 mmol/L. Size bar = 10 and 50 μm. (B) Hormone content after transplantation of NPIs. 49

List of Tables

Table 1 Cellular Composition of Neonatal Porcine Islets during Tissue Culture control and maturation media. 37
Table 2 Preparation of Neonatal Porcine Islets 41
Table 3 Effect of maturation media on Insulin Secretory Capacity of Neonatal Porcine Islets after 20 days or 11 of culture. 43

List of Key Abbreviations

NIC - Nicotinamide

Ex-4 – Exendin - 4

TGF-β1 – Transforming growth factor β1

OSM – Oncostatin-M

Control islets– Immature islets grown in HAMS F-10 media

Experimental islets – Matured islets grown in DMEM-F12 with supplementation

Chapter 1: General Introduction

1.1 The Pancreas

1.1.1 Anatomy and Physiology

The human pancreas is a glandular organ that possesses both digestive and endocrine functions. The main blood supply arises from the celiac trunk where the splenic and pancreatic-duodenal arteries supply the neck, body, tail and head of the pancreas respectively (Drake, Vogl, Mitchell, & Drake, 2005). Venous drainage takes place in the splenic vein, which further drains into the portal venous system carrying insulin, glucogon and somatostatin. Insulin travels through the portal venous system to reach the target organ and allows glucose to enter the cell via specified GLUT receptors present on various tissues (Dimitriadis, Mitrou, Lambadiari, Maratou, & Raptis, 2011). This in turn leads to increased glycogen synthesis by muscle, glycolysis by all other cells, and increased triglyceride synthesis by adipose tissue (Dimitriadis et al., 2011).

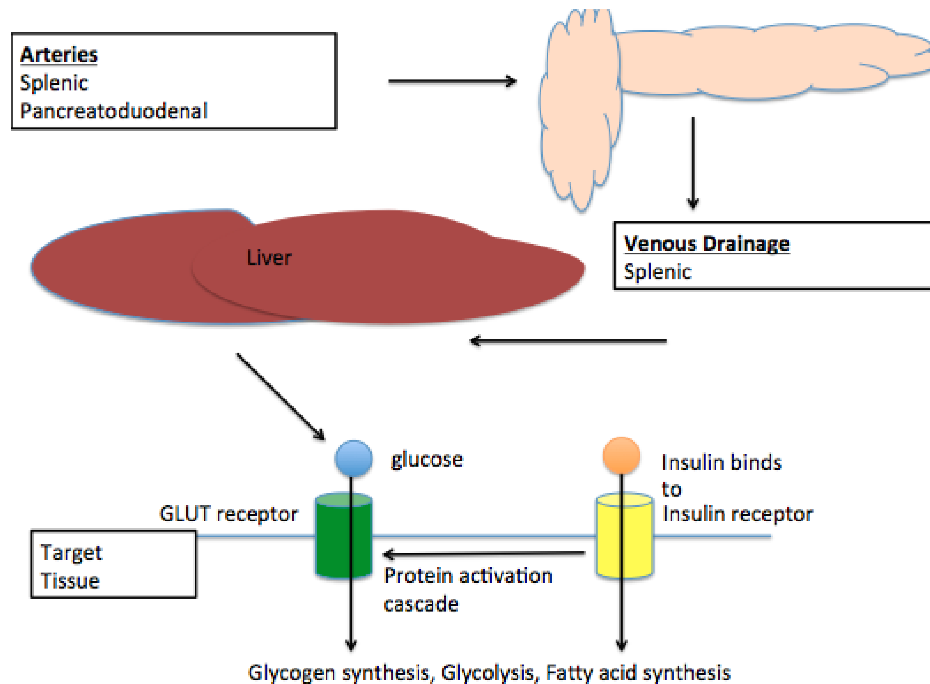

Figure 1 Normal physiology of glucose metabolism

1.1.2 The Islet

The average human pancreas consists of approximately 300,000 islets (Barshes, Wyllie, & Goss, 2005) that secrete insulin, glucagon, somatostatin, or pancreatic polypeptide. They are encompassed by heterogeneous tissue consisting of acinar, ductal, vascular, and nerve cells. Human islets are structured such that β-cells, α-cells, and δ-cells are dispersed throughout the organ. It should be noted that dissociation of islets into single cells severely impacts glucose stimulated insulin secretion (Linn et al., 2006) and implies that cell-to-cell communication and an organized structure is imperative for proper β-cell function.

Pancreatic islets are well a vascularized cluster of cells comprising only 1-2% of the pancreatic mass (Brissova & Powers, 2008). Yet, they receive 15-20% of blood flow to

the pancreas (Brissova & Powers, 2008). There is a greater density of blood vessels within the islets compared to pancreatic exocrine tissue, with features that provide greater partial pressure of oxygen. Islet capillaries and blood vessels are lined with fenestrated endothelial cells, and form during embryonic development through endothelial-endocrine signaling. This process is involved in pancreatic differentiation and influences morphology.

1.2 Type I Diabetes

1.2.1 Description of Disease

Diabetes is a chronic illness described as inadequate or defective insulin production by the pancreas. Type I diabetes (T1D) results from the autoimmune destruction of insulin-producing beta cells in the pancreas. Subsequently, the lack of insulin leads to increased non-metabolized glucose in the blood and urine resulting in symptoms such as polyuria, polydipsia, polyphagia, and weight loss. Patients of T1D classically present as children or adults who are generally thin with no other comorbidities. The chronicity of the disease has created a catastrophic impact on the health and economy. An estimated 382 million people have diabetes worldwide(Guariguata, 2013). In 2012 and 2013, diabetes resulted in 1.5 to 5.1 million deaths per year worldwide (Patterson et al., 2014), making it the 8th leading cause of death. Economic costs globally were estimated at $548 billion (Guariguata et al., 2014) in 2013 and in the United States $245 billion (American Diabetes, 2013) in 2012 due to complications and chronic care. Overall, diabetes has received much attention from leading scientists all over the world in efforts to develop a cure.

1.2.2 Signs and Symptoms

In the presence depleted insulin and high blood glucose, the most common acute and life threating complications among T1D patients is diabetic ketoacidosis (DKA). The classic presentation of DKA includes nausea, vomiting, dehydration, deep gasping breathing (Kausmal breathing), confusion, and coma. In severe DKA – according to the American Diabetes Association - patients are acidotic with a blood pH below 7 with a bicarbonate level below 10 mmol/L and are usually comatose Mild to moderate DKA presents with a blood pH ranging from 7.00 to 7.30 and bicarbonate levels ranging from 10-18 mmol/L. Patients are generally alert or mildly drowsy. Long term, these patients develop high circulating blood sugar levels which lead to glycosylated hemoglobin and oxidative damage to the microvascular system. This leads to end organ damage to eyes, kidneys and nerves – diabetic retinopathy, nephropathy, and neuropathy respectively. Mild to moderate DKA presents with a blood pH ranging from 7.00 to 7.30 and bicarbonate levels ranging from 10-18 mmol/L. Patients are generally alert or mildly drowsy. Long term, these patients develop high circulating blood sugar levels which lead to glycosylated hemoglobin and oxidative damage to the microvascular system. This leads to end organ damage to eyes, kidneys and nerves – diabetic retinopathy, nephropathy, and neuropathy respectively. (Mannucci, Bardini, Ognibene, & Rotella, 1999).

Diabetic retinopathy is progressive blindness due to the accumulation of glucose and fructose in small blood vessels of the retina. At first, patients are initially asymptomatic. However, blood vessels begin to proliferate and surround the vitreous humor inside the eye. These new vessels bleed, cloud vision, and ultimately destroy the

retina, which leads to blindness. This, diabetic retinopathy is the leading cause of blindness in individual's age 65 or older, and account for approximately 10,000 of the newly diagnosed cases of blindness each year. Treatment involves photocoagulation to prevent vision loss.

In diabetic nephropathy, patients initially develop asymptomatic microalbuminuria due to glycosylation damage of glomerular filtration system. This progresses to proteinuria and eventually renal failure. Screening is recommended 5 years after diagnosis for type 1 diabetics and at time of diagnosis for type 2 diabetics. Treatment involves better control of blood sugars and angiotensin-converting enzyme inhibitors to increase blood flow to kidneys.

Diabetic neuropathy involves distal symmetric sensorimotor neuropathy, which most commonly presents as tingling, burning and numbness of extremities. Patients with chronic untreated neuropathy are subject to non-traumatic amputations. Other forms of diabetic neuropathy include gastroparesis, vomiting, diarrhea, erectile and bladder dysfunction. Annual sensory exams of lower extremities are part of treatment.

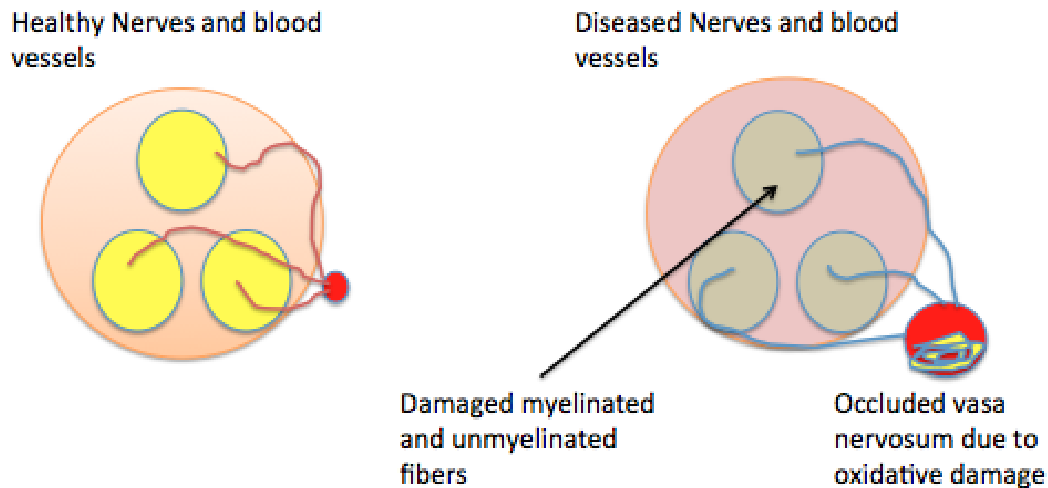

Figure 2 Pathogenesis of Diabetes

Coronary artery disease (CAD) is major complication and leading cause of death among diabetics. Patients typically present asymptomatically and overtime may develop heart failure and sudden death. CAD in diabetics usually manifests itself as a multi-vessel disease, and current guidelines recommend coronary artery bypass grafts as first line treatment before percutaneous intervention and stenting (Farkouh et al., 2012). Other complications include stroke with a 2.5 times higher incidence than non-diabetics. Peripheral vascular disease presents as pain, foot ulcers and gangrene as a result of claudication of major arteries. Screening with ABI is necessary and should be performed on high-risk patients.

1.2.3 Diagnosis

Normally, fasting blood glucose is considered <6.1 mmol/L (<110 mg/dL) after a two-hour oral glucose challenge test (Lu et al., 2006). The World Health Organization (WHO) defines both type 1 and type 2 DM as having a single raised glucose reading with

symptoms, otherwise raised values on two occasions without symptoms. A fasting plasma glucose ≥ 7.0 mmol/l (126 mg/dl) or with a glucose tolerance test, two hours after the oral dose a plasma glucose ≥ 11.1 mmol/l (200 mg/dl) is considered diabetes (Lu et al., 2006). A random blood sugar of greater than 11.1 mmol/l (200 mg/dL) in association with typical symptoms or an HbA_{1c} of greater than 6.5% is another method of diagnosing diabetes.

1.2.4 Etiology

The cause of T1D is unknown. Studies suggest that variants in the IDDM1 gene located on MHC class II region of chromosome have been linked to increased risk for the disease (Redondo, Fain, & Eisenbarth, 2001; Todd, 1990, 1997). Viruses, including Coxackie and Rubella, have been known to cause beta cell destruction through molecular mimicry (Fairweather & Rose, 2002). Drugs such as streptozotocin have been used in the past for treatment of pancreatic cancer but was later discovered to be toxic to normal pancreatic tissue(Brentjens & Saltz, 2001). Overall, no single cause exists and the etiology is likely multifactorial for both types.

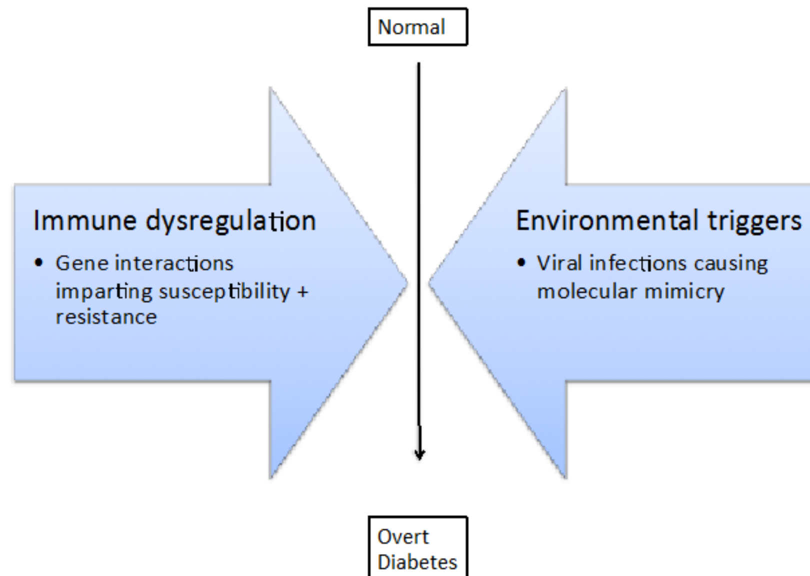

Figure 3 Suggested Etiology of T1D

1.2.5 Other forms of Diabetes

Type II diabetes is characterized as a metabolic disorder involving insulin resistance and insufficient insulin secretion from β-cells. Approximately 90% of diabetes mellitus cases are type II, with obesity and lack of exercise being route causes. Thus, exercising regularly, eating properly, and adjunctive pharmacotherapy are recommended. Gestational diabetes is characterized as high blood glucose levels during pregnancy without previously diagnosed diabetes. Infants born to mothers with untreated gestational diabetes are at increased risk of large for gestational age and hypoglycemia. Thus, pregnant patients are often screened for GDM with glucose non-glucose challenging tests, glucose challenging tests, and oral glucose tolerance tests.

1.3 Treatment for Diabetes

1.3.1 Insulin Therapy

Before the discovery of insulin, T1D was treated with a starvation diet allowing only carbohydrates to be consumed until glucose appeared in the urine. In 1922, the University of Toronto made the initial discovery of insulin and it became widely available in 1923(Banting, Best, Collip, Campbell, & Fletcher, 1922). Currently, it remains the mainstay of treatment for T1D. Each formulation has a specific onset, peak effect and duration of action. Clinicians most often prescribe a combination of formulations (short acting with long acting) to achieve normoglycemia, as well as regular eye and extremities exams to prevent chronic complications such as retinopathy and neuropathy. However, managing diabetes requires a combination of pharmacological therapy, lifestyle modification, and complication prevention. The idea is to maintain

homeostasis, avoiding hypoglycemic or hyperglycemia episodes. Strict glycemic control ultimately reduces the risk of long-term complications. In spite of a treatment rigorous regimen, diabetes is still an epidemic and has taken an economic toll on health care systems worldwide. Therefore, strong efforts have been made by researchers and clinicians to venture into other therapeutic avenues to reduce morbidity, mortality, and ultimately develop a cure for patients with diabetes.

1.3.2 Other Treatment Modalities

Insulin therapy is not entirely an ideal management for T1D. Even with a specified duration of action and onset, there are issues that hinder its full optimization. Subcutaneous injection cannot restore the natural physiologic distribution of the two-fold portal to systemic insulinemia due to poor tissue absorption. Thus, exogenous insulin cannot perfectly mimic normal pancreatic function, which results in poor glycemic control. Additionally, adolescent diabetics tend be non-compliant with multiple daily injections and consequently are subject to high HbA_{1c} levels. They are also at increased risk of hypoglycemic episodes by 3-fold (McCall, 2012), which can occur without warning. Percutaneous insulin pumps have their advantage of providing stable blood glucose levels, reducing the risk of hypoglycemia, and enhancing quality of life. However, they are relatively expensive, with a single unit costing up to $7000 and $1200 yearly in supplies (Hanaire et al., 2008). Additionally, patients are still required to have frequent blood glucose checks with careful adjustment of both background rates—particularly at night—and insulin dose adjustment at each meal.

1.3.2.1 Immunotherapy

Immunotherapy such as cyclosporine, azathiopurine plus prednisone, CTLA4Ig, rituximab, and FC receptor-nonbinding anti-CD3 monoclonal antibody have been shown in a variety of trials to reduce the fall of C-peptide responses that occur in the first two years after onset of disease (Herold et al., 2005; Herold et al., 2002; Orban et al., 2011; Pescovitz et al., 2009). Unfortunately, efficacy diminishes overtime and some individuals respond better compared to others. Novel long acting insulin immunotherapy such as Teplizumab has been shown to preserve insulin production and reduce the use of exogenous insulin in some new-onset type I diabetics (Herold et al., 2013). However, patients were susceptible to opportunistic infection including tuberculosis, cytomegalovirus, and herpes zoster.

1.3.2.2 Pancreas Replacement

In 1966, William Kelly and Richard Lillehei sought to transplant the first whole pancreas in efforts for patients to remain insulin independent (Kelly, Lillehei, Merkel, Idezuki, & Goetz, 1967). There was a relatively high success rate, with a 60% graft survival rate for patients receiving simultaneous pancreas/kidney transplants and 50% for pancreas only transplants at ten years post transplant (Gruessner, Sutherland, & Gruessner, 2012). The advantage of whole organ transplant was immediate vascularization of the tissue (Kandaswamy & Sutherland, 2006) since vascular structures remain intact from the donor. However, whole pancreas transplantation involved major surgery and was associated with higher morbidity. In addition, after massive doses of steroids and complications of sepsis and inflammation, grafts had failed. To reduce the intensity of an immune response, surgeons examined the possibility of segmental transplantation whereby only portions of pancreas rich in beta islets were transplanted. It had proven to

be a success in the 1970's after a recipient remained insulin independent for nearly eighteen years (Dubernard, Traeger, Neyra, Blanc-Brunat, & Ruitton, 1978). Again, complications arose due to the presence of immune-triggering exocrine tissue.

1.3.2.3 Islet Cell Transplantation

The concept of islet transplantation was brought forth to overcome surgical issues associated with whole organ transplant. At the start of the 21st century, a group in Edmonton drew much attention to the scientific community after successfully transplanting human cadaveric islet cells and achieved insulin independence in seven out of seven patients (Shapiro et al., 2000). The protocol utilized collagenase digestion of pancreases in seven patients on a steroid-free immunosuppressive regimen. The pancreas was enzymatically dissociated to yield islet-rich endocrine tissue, which comprises 1-2% of the pancreas. Tissue was separated into top and bottom layers via gradient purification, resulting in the topmost layers containing significantly more islets than bottom layers. Transplanted islets were then grafted by infusion into the portal venous system and suggested to seed in the liver. While this method had proven to be successful, patients resorted back to their exogenous insulin regimens due to graft failure. The initial expectation was not actualized because in a five-year follow up study, only 10% of patients receiving islet transplantation were able to sustain insulin independence. Reasons for loss of graft function were thought to be due to a minimal islet mass used, inflammation, ischemia, and toxic effects excreted by immunosuppressive drugs. These included emerging novel immunosuppressents such as Tacrolimus, Daclizumab, and Sirolimus, which cause mouth ulcers, diarrhea, acne, edema, weight loss, hypertension, hyperlipidemia, and decreased renal function (Rother & Harlan, 2004). It has been

suggested that up to 60% of islets undergo apoptosis, half of which occur within the first 3 days following transplant (Barshes et al., 2005). Ischemia also plays significant role in graft failure, as islets are avascular for several days when first transplanted. Even when revascularization is complete, they do not have the same oxygen tension as normal pancreatic tissue. Thus, research in vascularization is key component to islet survival and important for successful clinical translation.

1.3.2.4 Neonatal Pig Islets (NPIs)

There were many obstacles facing proper clinical translation of human islet cell transplantation after the Edmonton protocol. Aside from the overall chronic shortage of cadavers, there are an insufficient number of islets isolated from donor pancreases during the enzymatic digestion phase. Even with an optimal dose of enzyme and mechanical agitation, it does not translate to an improved clinical outcome. Each patient also requires approximately 800,000 islets to achieve normoglycemia (Gruessner et al., 2012), which translates into at least two donors per patient. Secondly, procuring pancreata from optimal cadaveric donors is rare as they need to be obtained after brain death (Rother & Harlan, 2004) but while the heart is still beating. As a result, if the heart stops beating, warm ischemia ensues and results in a non-viable pancreas. One study demonstrated a better outcome of using islets isolated from pancreata from live donors, for example, due to trauma, than cadaveric sources (Jung et al., 2007). Thirdly, the capacity of human islets to function is relatively poor. This has been demonstrated *in vitro* prior to transplantation where islets undergo glucose stimulation insulin secretion (GSIS) and insulin DNA content tests showing suboptimal insulin production due increased oxygen

consumption (Sweet et al., 2005; Sweet et al., 2008). Overall, there is a strong need for more efficient donor pancreases and alternative sources.

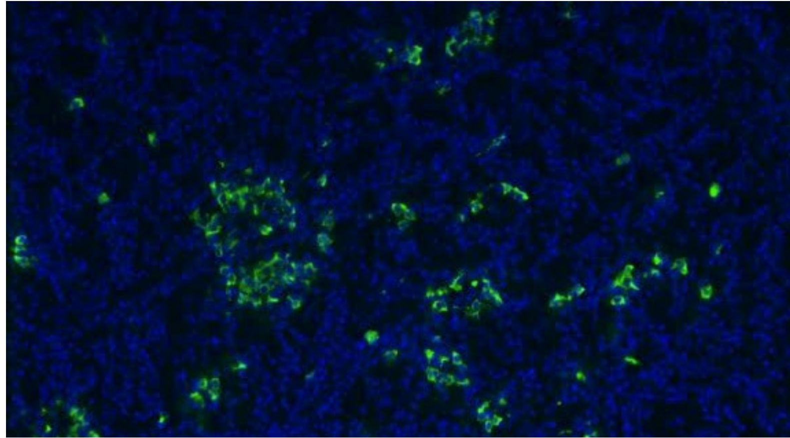

Figure 4 At 20x magnification - photograph shows neonatal pig islets, an often spherical group of hormone-producing cells. Insulin is labeled here in green, and the nuclei in blue. (Seeberger et.al 2014)

Neonatal porcine islets (NPIs) have shown great promise towards being a feasible source of graft tissue(Korbutt et al., 1996). NPIs are more often chosen than adult due to their reliability, resistance to hypoxia, and capability to mature and differentiate in the recipient's body. One of the main benefits is that approximately 27-35% of an NPI consists of both β-cells and non- β-cells and 40-57% duct precursor cells(Korbutt et al., 1996; Yoon et al., 1999), which ensure that a graft can potentially grow *in vivo*. NPIs have consistent tissue quality and unlimited availability due to a short breeding cycle and litter size. This would allow manufacturing companies to scale up and mass produce piglets specifically designed for type I diabetes treatment. Another advantage is a single amino acid difference between human insulin and porcine insulin, making it responsive to glucose in a similar physiological manner as human islets. Finally, there have been several mouse, pig, non-human primate (Cardona et al., 2006; Cardona et al., 2007; van

der Windt et al., 2009), and human studies in the literature that showed NPIs could effectively reverse diabetes. For example, a longitudinal study in Mexico was conducted in 23 patients with T1D who received porcine islets between 2000 and 2004 (R. Valdes-Gonzalez et al., 2010). Results showed glycosylated hemoglobin reduced significantly after the first transplantation. Overall, these advantages and demonstrations have shown considerable promise towards the use of NPIs to treat T1D.

Several issues must be resolved in order for successful translation of clinical grade NPIs. These include graft rejection caused by autoimmune destruction, discerning optimal sites of transplantation, revascularization, and enhancing islet function are most important. As previously mentioned, Valdez-Gonzalez showed NPIs lowered HbA1C levels after transplantation. However, 14 of the 21 patients presented mild chronic complications including graft rejection. Porcine xenotransplants contain a variety of antigens that may trigger a large immune response. Two immunologic epitopes - alpha 1,3 galactose (Gal) and N-glucolylneuramic acid (Neu5Gc) - in NPIs have been found to elicit an immune response causing possible hyper acute rejection in human recipients (Lutz et al., 2013). In addition, efforts to develop barriers with microencapsulation or mesenchymal stem cells (Yoshimatsu et al., 2015; Zhou et al., 2015; Zhu, Yu, He, Lyu, & Wang, 2015) have been researched and proven to be successful. With advent of transgenic pigs - Gal and Neu5Gc knockouts - there has been significant gain with experience from non-human primates (Komoda et al., 2005; Thompson et al., 2011). However, there are many other antigens that can trigger the same immune response and render the graft useless. Site of transplantation is also of concern during the transplantation process. In a detailed review, Shapiro et.al discussed the advantages and

disadvantages of various surgical sites with regards to cellular, endocrinological, immunological, and surgical features (Merani, Toso, Emamaullee, & Shapiro, 2008). While there are several sites of transplantation, our lab has mostly experimented with kidney sub-capsular site as this makes it possible to revert back to hyperglycemic state via survival nephrectomies. Optimal vascularization is also essential for oxygen and nutrients to reach the graft. Liver, kidney sub-capsular, pancreas, and scaffold do offer the best sites for revascularization. However, mesenchymal stem cells promote angiogenesis when co-cultured with islets as evidenced by CD43 positivity. Therefore, the above issues are being heavily investigated in order for future use in the clinical setting.

1.3.2.4.1 Functional improvement

Ensuring islet functionality is also pressing issue worth investigating. NPIs have shown to reverse diabetes in several animal models including mice, pigs, and non-human primates. However, time to blood glucose normalization is prolonged which is thought be attributed to inadequate β-cell mass and decreased functional maturity. According to recent literature, mice transplanted with NPIs can normalize between 9 and 16 weeks (Lopez-Avalos, Tatarkiewicz, Sharma, Bonner-Weir, & Weir, 2001) when cultured in enhanced culture media, while others reported as short as 42 days (Kin & Korbutt, 2007). Our transplanted diabetic mice can take up to 25 weeks to normalize. Hence, there is strong motivation to functionally improve current islet grafts for full optimization in human trials.

1.3.2.4.2 Maturation

There have been attempts to successfully isolate precursor cells from islets that give rise to insulin containing cells (X. Xu et al., 2008). Ductal ligation can activate Ngn3 positive β-cell precursors in adult ductal epithelium, which raises the possibility of β-cell regeneration. However, Kushner et.al discovered that through thymidine analog labeling in mice, β-cell precursors rarely replicate during normal β-cell growth (Teta, Rankin, Long, Stein, & Kushner, 2007). It was argued that β-cells are the products of uniform self-renewal, slowed by a replication refractory period that prevents β-cells from immediately re-dividing. Thus, controversy revolves around presence or absence of β-cell precursors and whether or not β-cells can be regenerated. However, NPIs can be matured in an environment promoting growth and differentiation into a desired phenotype. Regulatory factors such as PDX-1 and Nkx 6.1 control cell type lineages within the pancreas and are detected at various stages of embryonic development. PDX-1 is critical in maintaining β-cell mass, while Nkx 6.1 has a role in β-cell precursor proliferation. More details of pancreatic developmental genes will be discussed in subsequent chapters.

1.3.2.4.3 Growth Factors

Several growth factors have been implicated in islet development across various species. For example, prolactin has been shown to stimulate insulin release and β-cell proliferation in rat islets (Boschero, Crepaldi, Carneiro, Delattre, & Atwater, 1993; Brelje, Parsons, & Sorenson, 1994). Insulin growth-like factor 1 and 2 have been shown

to increase DNA content and β-cell proliferation (Hogg, Han, Clemmons, & Hill, 1993; Oberg-Welsh, Sandler, Andersson, & Welsh, 1997), and VEGF has been show to stimulate duct cell proliferation and insulin production in vitro (Oberg-Welsh, Sandler, Andersson, & Welsh, 1997).

Nicotinamide (NIC) is a water-soluble vitamin that is part of vitamin B_3, and specifically inhibits DNA repair enzyme poly-ADP-ribose polymerase and prevents of β-cell NAD depletion. It has been shown to increase in insulin content in porcine islets in vitro and perfusion of graft-bearing kidneys in mice 18 weeks after transplantation showed five times higher insulin release than control grafts (Korsgren, Andersson, & Sandler, 1993; G. Xu, Stoffers, Habener, & Bonner-Weir, 1999). Interestingly, the European Nicotinamide Diabetes Intervention Trial (ENDIT) assessed whether high dose nicotinamide (1.2g/m2) can prevent or delay clinical onset of diabetes in children with a first-degree family history of type 1 diabetes (Gale, Bingley, Emmett, Collier, & European Nicotinamide Diabetes Intervention Trial, 2004). The study demonstrated no difference in the development of diabetes between treatment groups.

Exendin 4 (Ex-4) is a GLP-1 agonist marketed as Exenatide that promotes insulin secretion. It has been shown to differentiate β-cells from ductal precursor cells in rats (G. Xu, Stoffers, Habener, & Bonner-Weir, 1999) and significantly reduce the apoptotic rate of cultured porcine β-cells subjected to hypoxia (Padmasekar et al., 2013). It was found in a short-term study that 63% of exendin-4-treated mice achieved graft function compared with 21% of untreated mice (Sharma et al., 2006). In the long-term study, 88% of treated mice had functioning grafts compared with 22% of controls.

Dexamethasone (Dex) has been shown to suppress expansion and transdifferentiation of transplanted NPIs into β-cells (Ko et al., 2004). However, it has been found to enhance insulin release in rat islets (Rafacho, Cestari, Taboga, Boschero, & Bosqueiro, 2009) and increase insulin mRNA levels in NPIs when combined with NIC (Korsgren et al., 1993).

TGF-β1 is a key activator of pancreatic MMP-2, which is necessary for islet morphogenesis (Miralles, Battelino, Czernichow, & Scharfmann, 1998). Oncostatin M (OSM) is structurally similar to leukemia inhibiting factor (LIF), and is expressed in pancreatic ducts. This cytokine plays a key role in controlling duct cell proliferation and is involved in repair processes following pancreatic injury (De Breuck, Baeyens, & Bouwens, 2006). Thrombin accelerates aggregation in an early stage of human IPSC differentiation in vitro, indicating it may be involved in islet development in vivo (Wei, Geras-Raaka, Marcus-Samuels, Oron, & Gershengorn, 2006). Recently, it has documented to increase insulin secretion in thrombin treated mouse islets (Hanzelmann et al., 2015).

1.3.2.4.4 Maturation Protocols

Protocols that functionally mature NPIs in long-term culture using various methods have been described in the literature using NPIs (Korsgren et al., 1993; Lopez-Avalos et al., 2001) and human infants islets(Fox et al., 2013). For example, Bonner-Weir subjected NPIs to various growth factors such as nicotinamide, exendin-4, fibroblast growth factor, insulin-like growth factor-1, and VEGF individually or in combination for 21 days. The

result was increased insulin and β-cell composition, higher expression of insulin, GLUT-2, Nkx6.1, and somatostatin according to RT-PCR. However, transplantation results showed that normoglycemia was achieved at the same rate in the control group – strictly nicotinamide - and experimental group receiving islets cultured in nicotinamide, serum, IGF-1, and butyrate. It was proposed that a longer culture period was needed to reach maturity among the islets. In a similar study, human infant islets were subjected to a 24-28 day protocol comprised of various growth factors added in a stepwise fashion to DMEM-F12 media (Fox et al., 2013). Islets were tested for their secretory capacity, gene expression, and β-cell composition. Consequently, there were an increased proportion of insulin positive cells in the maturation protocol compared to standard short-term culture. In addition, patch clamp electrophysiology showed increased exocytotic function in 10-week old donor islets subjected to the maturation protocol compared to short-term culture. Overall, it is clear islets can benefit from long-term culture.

There are other methods promoting functional maturation of islets. For example, co-cultured Sertoli cells (SC) can accelerate β-cell maturation and differentiation by providing trophic support for the islets (Mancuso et al., 2010). *In vitro* insulin release in response to glucose, as well as mRNA insulin expression, was significantly higher in SC+ NPIs compared to islets alone. Incretins such as GLP-1 and cholecystokinin (CKK) have also shown to enhance maturation of NPIs. For example, Tuch et.al showed that after 4 days of culture with supplemented GLP-1 and CKK, β-cells proliferated 20% more versus 8 % from control. This further proves that functional maturation of islets is amendable through various experimental approaches.

1.4 Objective of Thesis

The main objective of this thesis is to functionally mature islets through augmentation in a maturation media. We are hoping that by accelerating the maturation process of NPIs, they can be fully functional and translatable to the clinic. As previously stated, Manning-Fox developed a 24-28 day maturation protocol for human infant islets that comprised of various growth factors added in a stepwise fashion. Using a modified protocol in our experiment, it was hypothesized that this maturation media will optimize function and morphology of current islets. In other words, islets will contain greater percentage of β-cells and subsequently produce more insulin. This will result in reversing diabetes sooner and will ensure greater efficacy than islets grown in traditional media.

1.5 References

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Chapter 2: Augmentation of Clinical Grade Neonatal Porcine Islets with Novel Maturation Media

2.1 Introduction

Clinical islet cell transplantation is continually evolving from an experimental strategy to a standard therapy for type I diabetic patient. With over 750 islet transplants performed worldwide, 44% of recipients have remained insulin independent for up to 3 years (Barton et al., 2012). These transplants have relied solely on human cadavers as a source of graft tissue. However, due to shortage of cadavers and human islet instability, it has remained disadvantageous in the transplantation setting. In an attempt to overcome the supply problem, research has focused on porcine islets – both neonatal and adult – as a promising alternative: Their short breeding cycle and litter sizes allow them to be mass produced; they show strikingly similar physiology and biological activity to human insulin, and have been experimented in various type I diabetic animal models including non-human primates (Elliott et al., 2005; Isaac et al., 2005) and humans (Elliott et al., 2007; R. A. Valdes-Gonzalez et al., 2005). Neonatal porcine islets (NPIs) have attracted much attention in the surgical research community. They are distinguished from adult porcine islets as they consist of 27-35% endocrine cells and 40-57% duct precursor cells, which give them the capacity to differentiate and proliferate in vivo. The average normalization time transplanted diabetic mice of 25 weeks post transplant, which is suboptimal for clinical transplantation. Relatively immature islets with an inadequate β-cell mass has been attributed to such delay in normalization times.

Many studies have demonstrated the benefits of various growth factors to mature NPIs in vitro. For example, the combination of IGF-1, nicotinamide, fetal calf serum and sodium butyrate has been shown to increase insulin content and hormone expression in islets cultured for 14 days (Lopez-Avalos et al., 2001). Cholecystokinin's incretin effect has been shown to enhance insulin secretion in NPIs in combination with Exedin-4 (Hardikar et al., 2002). Nicotinamide and Exedin-4 alone have also shown to increase β-cell mass and insulin secretion respectively in culture among porcine islets (Korsgren et al., 1993) and rat islets (Yoon et al., 1999) in vitro.

Fox et.al previously demonstrated a 24-28 day maturation protocol on human infant islets to understand mechanisms pertaining to islet growth and differentiation (Fox et al., 2013). Matured islets demonstrated increased exocytotic function, hormone expression and proportion of insulin positive cells. The current study adapted a similar protocol in efforts to optimize NPI use in clinical transplantation.

2.2 Materials and Methods

2.2.1 Preparation of Porcine Islets

Donor pancreata from Duroc-cross neonatal piglets at 1 to 3 days old were processed in detail according protocols described by Korbutt (Korbutt et al., 1996). Briefly, pancreata were cut into 1- to 3-mm tissue fragments and exposed to 2.5 mg/mL collagenase (type XI; Sigma, St. Louis MO). Fragments were filtered through a 500-µm nylon screen and washed in Hank's Basic Salt Solution (Gibco, Grand Island NY). NPIs were cultured in non-tissue culture-treated Petri dishes containing Ham's F10 tissue culture media (Gibco, Grand Island NY) and were supplemented with ROCK inhibitor (Stemcell technologies, Vancouver BC) and protease inhibitor (Sigma, St. Louis MO).

2.2.1.1 20-day Maturation Protocol

After 5 days culture in HAMS-F10, plates were split and half were subjected to maturation media in the following steps: NPIs were aggregated with Dulbecco's Modified Eagle Medium Nutrient Mixture F-12 (DMEM-F12; Invitrogen) was supplemented with the following: 1% pig serum, 1X Insulin Transferrin Selenium + PreMix (Gibco). In step 1, DMEM-F12 was supplemented with 20 ng/mL of OSM (R&D systems) and 1µM Dex (Sigma-Aldrich) for 3 days. In step 2, media was supplemented with 10 mM Nic (Sigma-Aldrich) for 3 days. In step 3, media was supplemented with 10 mM Nic and 10 nM Ex-4 (Sigma) for 3 days. In step 4, 10 ng/mL of TGF-ß1 (EMD Millipore) was included with Nic and Ex-4 for 3 days. In Step 5, 1% ITS+Premix was included with Nic, Ex-4, TGF-ß1 and 1U Thrombin (EMD Millipore)

for 3-5 days. Media changes were performed after each step. The other half remained in Ham's F10 media and was designated a control.

2.2.1.2 11-day Maturation Protocol

In efforts to reduce cell loss, we abbreviated the above protocol in the following manner: After 3 days culture, each plate was split and half and subjected to DMEM-F12 with only Nic and Ex-4 for 11 days. The other half remained in HAMS-F10 media. Media changes were performed every 3 days.

2.2.2 Characterization of Islet Preparations

After isolation and 20 days or 11 days in control or maturation media, immunostaining was performed on paraffin sections of islets that had first been fixed with 1% formalin (Fisher Scientific) and embedded in 2% low melting point agarose (Sigma-Aldrich). Paraffin sections were processed and immunostained as previously described by Pipeleers (Pipeleers et al., 1991). Briefly, sections were blocked with 20% NGS for 1 h. Primary antibodies were diluted in 5% NGS at the following concentrations: 1/1000 anti-insulin (company), 1/5000 anti-glucagon (Sigma), 1/300 anti-PCNA, 1/300 anti-proliferating cell nuclear antigen (Dako), 1/2000 anti-amylase (LifeSpan Biosciences), 3/100 anti-CK7 (Dako), and 1/1500 anti-PDX1 (Chemicon). For apoptosis detection, an APO-Brdu TUNEL Assay Kit (Invitrogen) was utilized. All appropriate species-specific secondary antibodies were AlexaFluor 488 or 594 conjugates (Molecular Probes) and diluted 1/200 in 5% NGS. Slides were cover-slipped with ProLong Gold anti-fade reagent with 4',6-

diamidino-2-phenylindole (Invitrogen) to counter stain nuclei and preserve fluorescence. Negative controls were incubated without primary antibodies and positive controls were sections of normal newborn pig pancreas. All slides were visualized with an Axioscope II equipped with AxioCam MRC and analyzed with Axiovision 4.6 (Carl Zeiss, Gottingen, Germany). Insulin, PCNA, glucagon positive, and double stained cells were quantified using ImageJ software (http://rsb.info.nih.gov/ij/download.html)

Static incubation assays previously described (Korbutt & Pipeleers, 1992) were used to determine glucose responsiveness in islets cultured in control media and maturation media for 20 and 11 days. Islets were allowed to gravity settle, collected in representative aliquots, and incubated in 1.5 mL RPMI supplemented with 2.0 mM L-Glutamine and 0.5% w/v BSA in either low 2.8 mM or high 20.0 mM glucose solution at 37°C for 2 hours. Tissue was separated by gravity settling and media was assayed for respective insulin content by a porcine insulin immunoassay (Mesoscale Discovery). Insulin content secreted in media was normalized to that of the total cellular insulin content. To assess total cellular insulin content, islets from representative aliquots were lysed and centrifuged to remove cellular debris. Cellular insulin content and DNA was measured using porcine insulin immunoassay. Stimulation indices were calculated by dividing the amount of insulin released at 20.0 mM glucose by that released at 2.8 mM glucose. Percentage insulin content is reported as insulin secreted at 2.8 mM and 20.0 mM glucose divided by total cellular insulin content.

2.2.3 RT-PCR

Islets were preserved in Trizol at -80°C in preparation for RT-PCR experiments. RNA was extracted with RNeasy Mini kit (QIAGEN). cDNA was synthesized and 1 μL was amplified for 35 cycles using Platinum Taq polymerase (Invitrogen) according to previously run protocols (Seeberger 2006). Quantitative PCR was performed using TaqMan gene expression Assay (Applied Biosystems) with validated primer sets: PDX1 (Ss03373351_m1), insulin (Ss03386682_u1), glucagon (Ss03384069_u1), Pax6 (Ss03397583_m1), NKX6.1 (Ss03373352_m1), Sox9 (Ss03392406_m1), pancreatic polypeptide (Ss03375477_u1), somatostatin (Ss03391856_m1), and glyceraldehyde-3-phosphate dehydrogenase (Ss03375629_u1) as a housekeeping transcript. Results were normalized between samples to glyceraldehyde-3-phosphate dehydrogenase.

2.2.4 Transplantation and Metabolic Follow-Up

After 20 days of culture in control or maturation media, islets were transplanted under the left kidney capsule of anesthetized streptozotocin (STZ)-induced knockout mice (B6.129s7Rag1<tm1Mom>/J, Jackson Laboratory). Representative aliquots of each preparation consisting of ~2000 IEQ were aspirated into polyethylene tubing (PE-50), pelleted by centrifugation, and gently placed under the kidney capsule using a micromanipulator syringe. Once tubing was removed, capsulotomy was cauterized with a disposable high-temp cautery pen (Aaron Medical Industries).

Transplanted mice were monitored for blood glucose levels once a week between 8:00 AM and 11:00 AM. When blood glucose level was ≤15.0 mmol/L, graft was deemed successful. At post-transplant week 20, an oral glucose tolerance test (OGTT) was

performed on recipients with normalized basal glycemia after an overnight fast. D-glucose (3 mg/g body wt) was administered intragastrically as a 50% solution. Blood samples were obtained from tail veins at 0,15, 30, 60, and 120 mins.

2.2.5 Characterization of Harvested Islet Grafts

After 20 weeks post-transplant, recipients underwent a nephrectomy of graft bearing kidney for morphological analysis or to determine insulin contents of harvested grafts. Certain selected recipients nephrectomized recipients were subsequently monitored to confirm return of hyperglycemia. Sections were stained for presence of insulin. For hormone extraction, organs were homogenized and sonicated at 4°C in 10 mL of 2 mmol/L acetic acid. After 2-h at 4°C, tissue homogenates were resonicated, centrifuged (8000 g, 20 min), then supernatants were collected and pellets further extracted by sonication in a an additional 8 mL of acetic acid. Second supernant was collected after centrifugation, combined with the first supernatant, total volume was measured, and samples were assayed for insulin content.

2.2.6 Statistical Analysis

Data is expressed as mean ± SE of *n* independent observations. Statistical significance of differences was calculated with one-way ANOVA, Wilcoxon-Rank sum test, or student t-test.

2.3 Results

2.3.1 Cellular Composition of Neonatal Porcine Islets

Approximately 10 images representing 1 islet per image were taken of the control and matured pancreata and calculated for percentage of insulin, glucagon, PCNA, and CK7 positive staining using ImageJ software (Table 1). Representative images of immunohistochemical analysis confirmed such findings (Figure 5a and b). Long-term 20-day culture of islets in maturation media resulted in a 2-fold increase in β-cell and α-cell composition compared to control media (41.8% vs 29.6%, 17.4 vs. 10.2, P<0.0001) as evidenced by insulin and glucagon staining. Additionally, a 3-fold increase in insulin-PCNA and 2-fold increase in CK7-PCNA double positive cells were observed in the matured islets compared controls (2.7% vs. 0.8%, 5.6 vs. 2.8 P<0.0001 and P=0.0017 respectively). Less TUNEL was observed in matured islets compared to controls. PDX-1, which is a marker for β-cell precurors, stained more intensely control islets compared to matured. However, positive cells were not quantified. More CK7 was observed in matured islets compared to controls as was seen in Table 1. Similar intensity of amylase was observed in both matured and control islets. Again, positive cells were not quantified. Short-term 11 day culture in Nic + Ex4 showed similar results to long-term, however, not as prominent (Table 1). There was a 1.5 fold increase in β-cell composition (29.6% vs. 17.9%, P<0.0001). There appeared to be more amylase and CK7 observed in control compared to mature islets. PDX-1 was observed on the periphery of the matured islet. When comparing long-term maturation vs. short-term maturation, there was a 1.4-fold increase in insulin composition observed (41.8% vs. 29.6%, P<0.0001), and 3-fold

increase in insulin-PCNA composition in long term maturation compared to short-term maturation (3.8 vs 1.2, P<0.0001).

Table 1 Cellular Composition of Neonatal Porcine Islets during Tissue Culture control and maturation media.

Culture Period and Condition	*n*	Insulin	Glucagon	PCNA	Insulin+ PCNA	CK7	CK7+ PCNA
20 Days							
Control	10	20.0 ± 1.2	10.2 ± 2.6	25.6 ± 1.9	0.8 ± 0.1	16.7 ± 4.6	2.8 ± 0.6
Mature	10	41.8 ± 2.0*	17.4 ± 0.9*	40.9 ± 2.0*	3.8 ± 0.4*	36.6 ± 2.8*	5.6 ± 0.6*
11 Days							
Control	4	17.9 ± 1.2	9.1 ± 0.8	15.7 ± 1.6	1.5 ± 0.2	30.2 ± 2.0	6.1 ± 0.8
Mature	4	29.6 ± 1.4§¶	13.7 ± 0.9§¶	16.8 ± 1.0	1.2 ± 0.2¶	43.0 ± 2.2§	4.7 ± 0.4

Results are expressed as a percentage of the total cell number. Values are expressed as mean ± SEM of *n*. Cell composition was determined using immunohistochemistry and percent insulin, glucagon, CK7 and PCNA was determined using Image J software as described in Methods. Statistical difference was calculated by one-way ANOVA and student t-test.

* **20 day control vs mature** : p<0.0001, p=0.0004 for ck7, p=0.0017 for CK7+PCNA
§ **11 day control vs mature :** p<0.0001
¶ **20 mature vs 11 mature :** p<0.0001 for insulin, p=0.016 for glucagon, p<0.0001 for insulin+PCNA

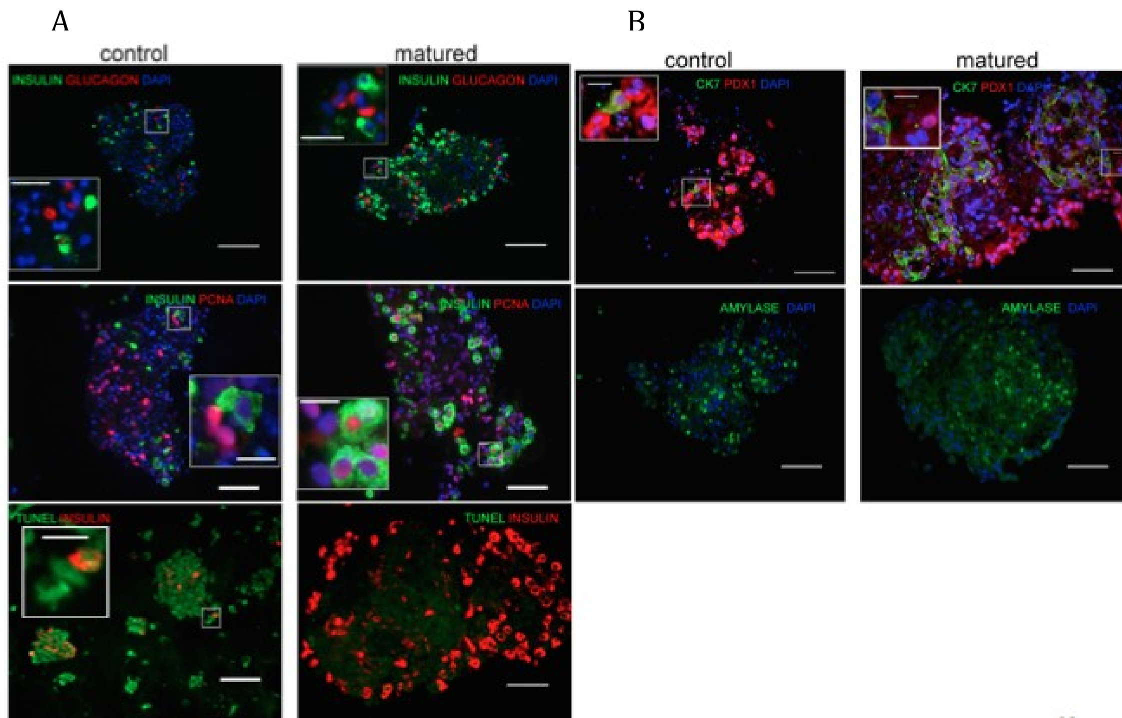


Figure 5 Immunohistochemistry of islets grown in control media and maturation media in 20-day protocol. A) immunostaining for insulin and glucagon, insulin and PCNA, and insulin and TUNEL B) Immunostaining for PDX-1 and CK7, and Amylase. Size bar = 10 to 50 µm

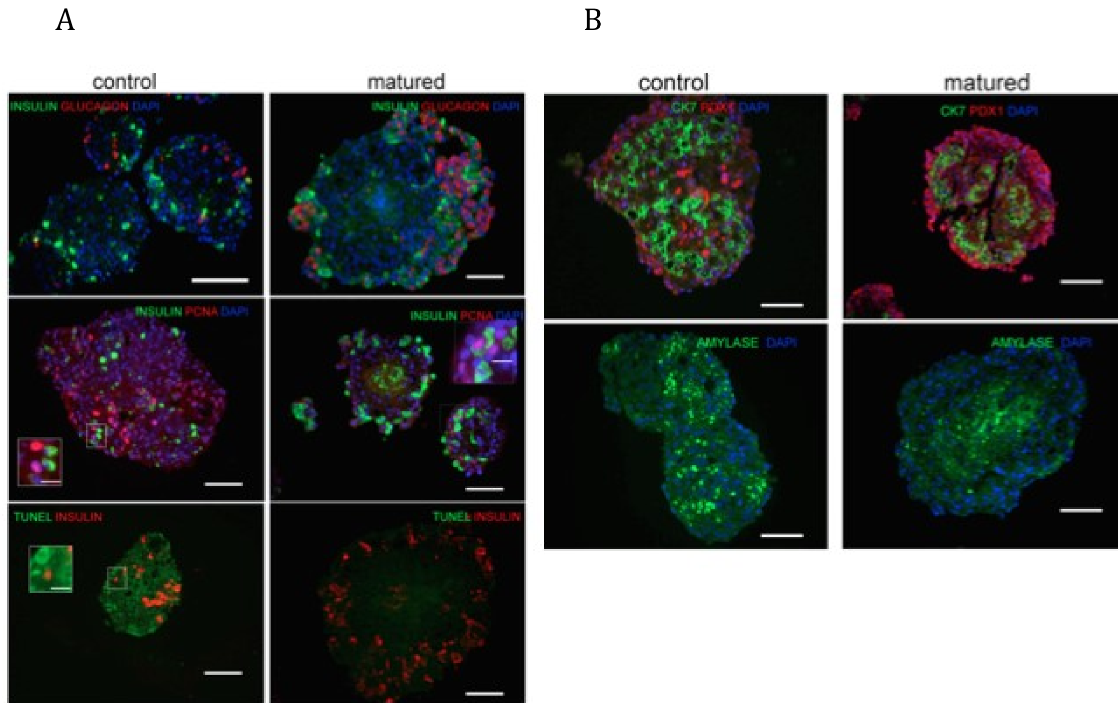


Figure 6 Immunohistochemistry of islets grown in control media and maturation media in 11-day protocol. A) immunostaining for insulin and glucagon, insulin and PCNA, and insulin and TUNEL B) Immunostaining for PDX-1 and CK7, and Amylase. Size bar = 10 to 50 µm

2.3.2 Preparation of Islets

After a 20-day long-term culture, a 10-fold increase in insulin was recovered per pancreas cultured in maturation media (101.7vs 11.7 p=0.0002). Culture in maturation media also resulted in less DNA recovered per pancreas (163.7µg vs. 322.8µg, P=0.023). Cell composition in preps was determined by dividing number of cells (obtained from DNA assay) by the percentage of β-cells (determined from Image J). There were no significant differences in cell composition between control islets and matured islets in 20-day culture. In short-term 11-day culture, there were no significant differences in insulin content or DNA between control islets and matured islets. However, there were differences in cell composition with control islets demonstrating less β-cells (11.7 vs 5.7,

P=0.02) and α-cells (5.7 vs 2.9, p=0.01) than matured islets. When comparing long-term versus short term maturation, more insulin was recovered in long-term matured islets (101.7 vs. 36.6, P=0.05)

Table 2 Preparation of Neonatal Porcine Islets

Culture Period and Condition	n	Recovery per pancreas Insulin μg	% control	DNA μg	% control	Cell Composition Endocrine Cells ß-cells (10^6)	α-cells (10^6)
20 Days							
Control	10	11.7 ± 1.8	100	322.8 ± 0.01	100	9.2 ± 2.6	5.0 ± 2.0
Matured	10	101.7 ± 19.7*	705.2 ± 90.3	163.7 ± 26.0*	57.1 ± 9.6	8.9 ± 1.8	4.1 ± 6.3
11 Days							
Control	4	24.7 ± 5.7	100	249.8 ± 48.0	100	5.7 ± 0.6	2.9 ± 0.1
Matured	4	36.6 ± 8.5¶	159.6 ± 31.2	288.8 ± 43.5	128.8 ± 28.0	11.7 ± 2.2§	5.7 ± 0.1§

Results are expressed as a percentage of the total cell number. Values are expressed as mean ± SEM n experiments. In each experiment, NPIs were prepared from 10 pancreata for 20-day protocol, and 4 pancreata from 11-day protocol. Statistical significance was calculated using the student t-test.

*** <u>20 day control vs mature</u>**: p=0.0002 for insulin, p=0.023 for DNA
§**<u>11 day control vs mature</u>** : p=0.02 β-cells, p=0.01 α-cells
¶ **<u>20 vs 11 mature:</u>** p=0.0477 for insulin

2.3.3 Insulin Secretory Capacity

Secretory capacity of islets was determined by comparing percentages of cellular insulin released at low glucose (2.8 mmol/L) or high glucose (20 mmol/L) (Table 3). Statistically significant differences were observed in the amount of insulin secreted at low glucose concentration (8.8 vs 5.7, P=0.019) among 20 day cultured islets, but not high. Incubation in 2.8 mmol/L and 20 mmol/L glucose significantly increased secretory rate in 11-day matured islets (2.9 vs. 0.7, P=0.029). When comparing matured and control islets in 20 day and 11 day protocols, incubation in 2.8 mmol/L and 20 mmol/L glucose significantly increased secretory rate of 20 maturation protocol treated islets However, calculated stimulation indices after incubation of 2.8 mmol/L and 20 mmol/L glucose showed no statistical significance across all culture conditions and length of culture time.

Table 3 Effect of maturation media on Insulin Secretory Capacity of Neonatal Porcine Islets after 20 days or 11 of culture.

Condition	*n*	Insulin secretory activity (% release)		
		2.8 mmol/L glucose	20 mmol/L glucose	Stimulation indices
20 Days				
Control	10	5.7 ± 0.5	9.2 ± 0.9	1.7 ± 0.2
Mature	10	8.8 ± 0.9*	14.7 ± 2.2	1.6 ± 0.1
11 Days				
Control	4	0.7 ± 0.1	0.8 ± 0.1	1.2 ± 0.1
Mature	4	2.9 ± 0.8§¶	4.2 ± 0.1§¶	1.7 ± 0.3

Values are expressed as mean ± SEM of *n* experiments. In each experiment, NPIs were prepared from 10 and 4 pancreata, and cultured for 20 or 11 days respectively in HAMS F-10 media (control) and maturation media (20-day mature) or Nic+Ex-4 (11-day mature). Stimulation indices were calculated by dividing amount of insulin released at high glucose (20 mmol/L) by that released at low glucose (2.8 mmol/L). Statistical significance of differences was calculated using Wilcoxin-Rank Sum Test.

* **20 day control vs mature**: p=0.019 for 2.8 mM
§ **11 day control vs mature:** p=0.029 for 2.8 mM, p=0.029 for 20 mM
¶ **20 mature vs 11 mature:** p=0.0072 for 2.8 mM, p= 0.0047 for 20 mM

2.3.4 Expression Levels of Islet-Specific Genes

In the RT-PCR experiments, NPI samples were used as calibrators. Consequently, expressions levels of the samples were set to 1. Because graph plots' gene expression levels were set to Log10 values, expression level of calibrators appeared as 0. Since the relative quantities of the target genes are normalized against the relative quantities of the endogenous control, the expression level of the endogenous control is 0, and the are no bars for GAPDH. With this technique we measured changes induced by the different

culture conditions on hormones glucagon, insulin, somatostatin, and pancreatic polypeptide, as well as transcription factors - Nkx6.1, Pax6, SOX9, and PDX1.

In the 20-day matured islets, there was a 3-fold increase in insulin expression (p=0.0025) and 5-fold increase in glucagon expression (p=0.0002) (Figure 7). Somatostatin expression increased 9-fold (p=0.0002) in control islets, in addition to PDX-1 (p=0.0032) and PAX-6 (p=0.0002) increasing 1-fold each. SOX-9 (p=0.0072) increased 1.5 fold in matured islets. In the short-term 11-day matured islets, somatostatin (p=0.02) and pancreatic polypeptide (p=0.02) were increased 3 –fold and 1.4-fold respectively in control islets (Figure 8). However, Nkx6.1 (p=0.04) was increased 50% in expression among matured islets. No other hormones values were statistically significance.

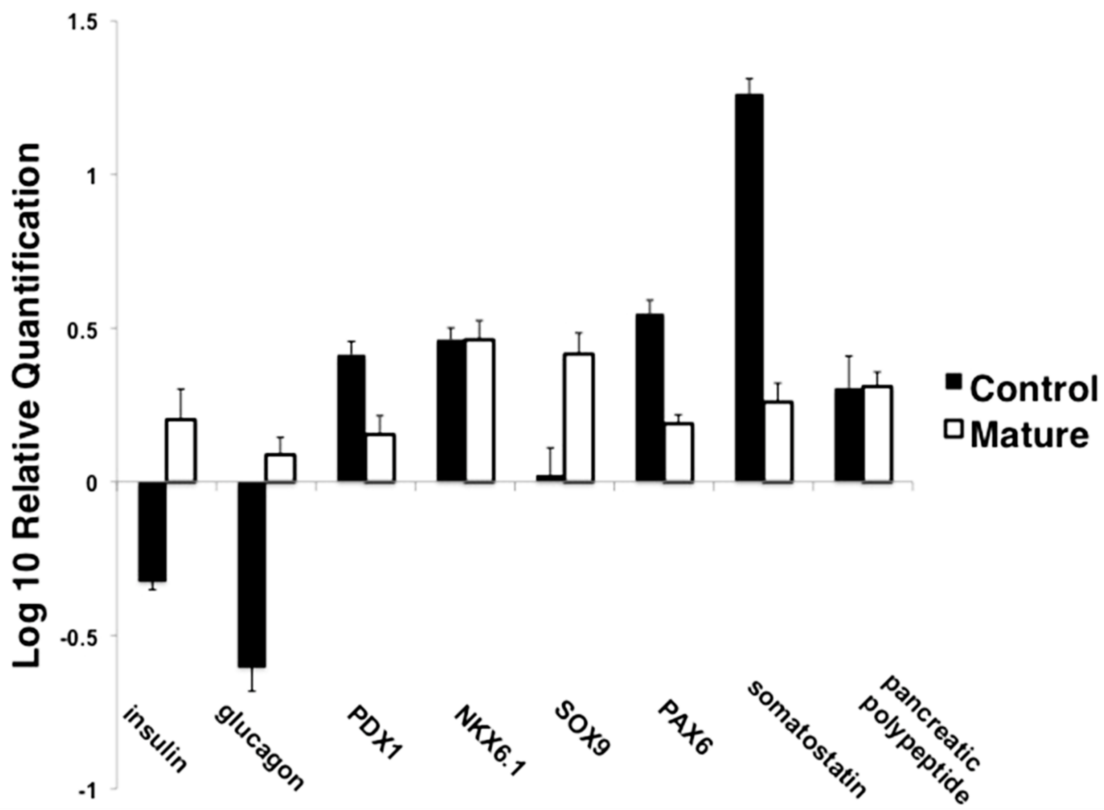


Figure 7 Expression levels of islet-specific genes in islets cultured in control media and maturation media for 20 days. NPIs were used as calibrators and expression level was set to 0 upon plotting log10 values. Expression levels of endogenous control (GAPDH) is also set to 0. N = 10 control, 10 mature.

20 day control vs mature: Insulin – p = 0.0025, Glucagon – p=0.0002, PDX1 – p=0.0032, Sox9 – p=0.0072, Pax6 – p=0.0002, Som – p=0.0002

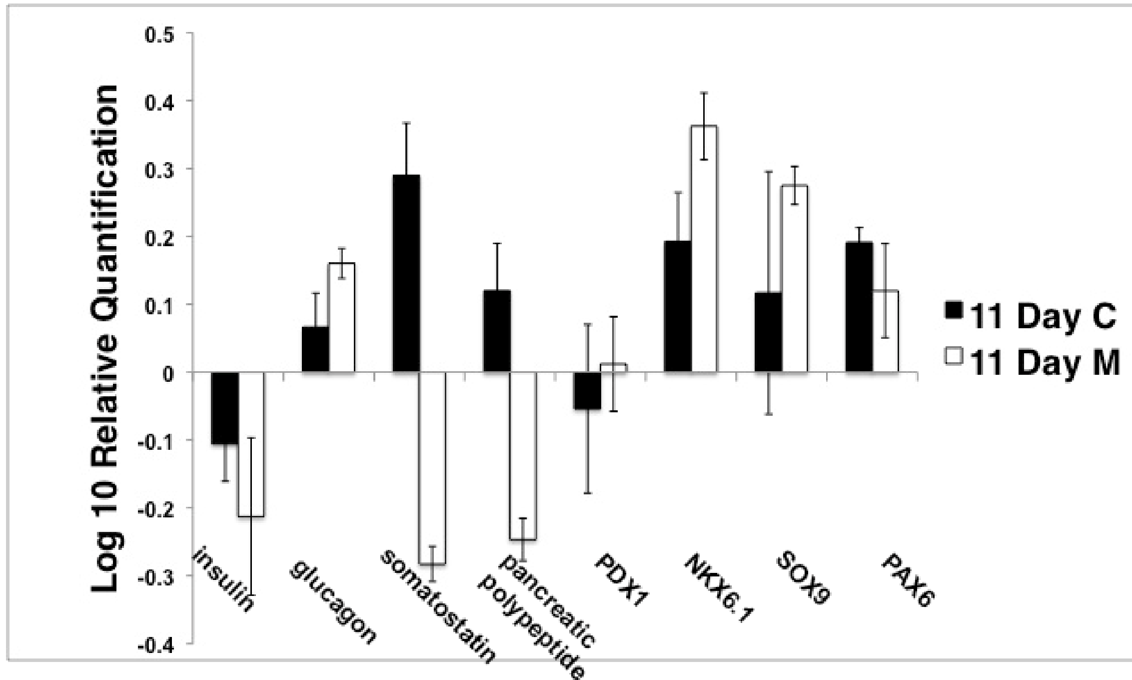


Figure 8 Expression levels of islet-specific genes in islets cultured in control media and maturation media for 11 days in Nic+Ex4. NPIs were used as calibrators and expression level was set to 0 upon plotting log10 values. Expression levels of endogenous control (GAPDH) is also set to 0. N = 10 control, 10 mature.

<u>**11 day control vs mature**</u>: Nkx6.1 – p=0.0433, Som – p=0.0209, PPP - p=0.0209

2.3.5 Transplantation of Control and Matured Islets Into Diabetic Mice

After STZ administration, all 20 (10 control, 10 matured) transplant recipients exhibited blood glucose levels above 20 mmol/L. Both groups (n=6 for control group, n=10 for experimental group) survived until sacrifice except for four mice from control group. All animals were transplanted with approximately ~3000 islet equivalents. Not all controls normalized to ≤15 mmol/L within 20 weeks post-transplant. However, all experimental mice transplanted with matured islets normalized. Comparison with control mice indicated that recipients treated with matured islets exhibited significantly lower blood glucose levels at weeks 18 and 20 ($p<0.05$). Not all mice receiving control islets had

normalized (Figure 9b). In matured transplanted groups, removal of the graft-bearing kidney for morphological examination or insulin extraction was followed by a rapid return to diabetic state. This indicated that the islet grafts were responsible for the normoglycemic state. Glucose tolerance tests were performed on normoglycemic mice 20 weeks post-transplant. When compared to control mice, recipients exhibited significantly lower glycemic values at 20, 60, and 120 min.

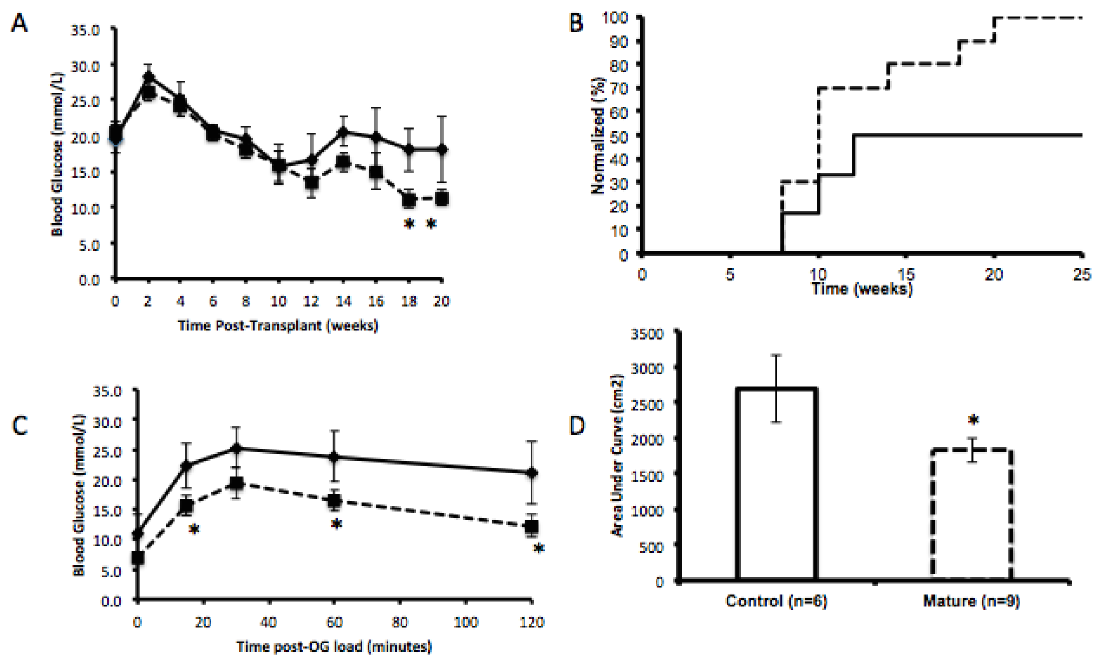
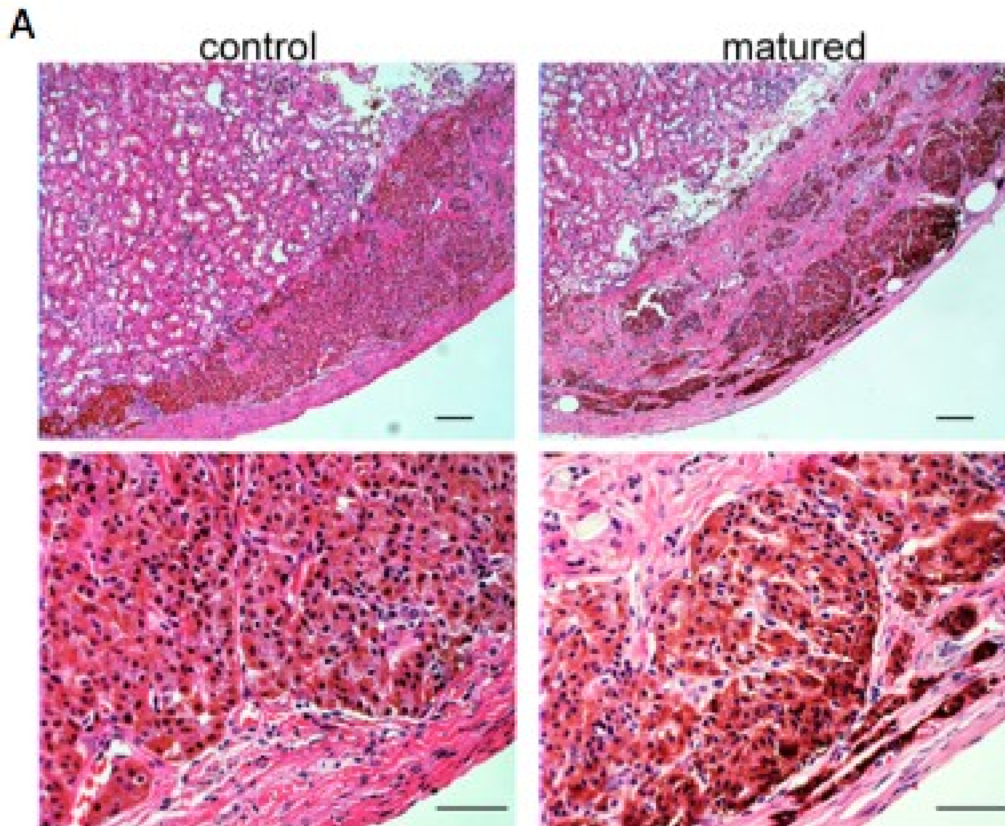


Figure 9 Transplantation of NPIs cultured in control media and maturation media for 20 days and 11 days into diabetic mice. (A) Blood glucose levels over 20 weeks time (B) Percentage normalized curves for transplanted mice that normalized (blood glucose of 15 mmol/L considered normalized) (C) Oral glucose tolerance test (OGTT) (D) Area under curve (AUC) of subsequent OGTT result. Solid line = control, Dotted line = mature, micro-dotted line = 11 days Nic + Ex4. *p<0.05 (20-day control vs. mature)

2.3.6 Composition of Islet Grafts

Post-transplantation, grafts were immunostained for insulin which was positive in both experimental groups, but more so in matured islets (Figure 10a). On the basis of graft composition, there were no significant differences in insulin extracted (Figure 10b) and cell composition (Table 4) between the two groups.



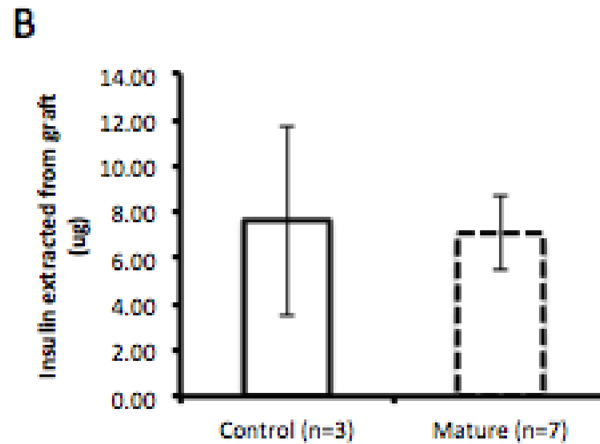


Figure 10 (A) H&E + Insulin staining of NPI graft under kidney capsule in control vs. mature transplanted mice. Last recorded blood glucose values: control – 10.3 mmol/L, mature – 9.6 mmol/L. Size bar = 10 and 50 µm. (B) Hormone content after transplantation of NPIs.

2.4 Discussion

Due to the relative immaturity our current NPIs, we examined the possibility of accelerating maturation that would improve functionality and subsequently lead to improved clinical transplant outcomes. The present data indicates that long-term culture of NPIs in maturation media improves functionality in vitro and in vivo. We also demonstrated that in most cases, longer culture time in maturation media could improve islet function compared to a short-term culture and abbreviated maturation method. Initially, risks of long-term culture were concerning as multiple media changes lead to cell loss, which can be observed after 3 to 9 days (Korbutt et al., 1996). However, we previously reported culture times up to 24-28 days in human infant islet preparations with similar cell loss and improved function (Fox et al., 2013) indicating that these

preparations are rid of dead cells and are substantially more pure. Based on the results reported, 20 days culture is an optimal time frame for NPI maturation.

We observed a generous increase in insulin among matured islets after 20 days culture, and a modest increase of insulin positivity in 11-day cultured matured islets according to cell composition studies. This can be attributed to nicotinamide's ability to increase DNA and insulin content in NPIs (Korbutt et al., 1996) as well as in human fetal islets (Korsgren et al., 1993). Exedin-4 has been reported to induce β-cell proliferation in NPIs (Yoon et al., 1999), which explains the enhanced PCNA activity in matured islet preparations. The same increase in insulin-PCNA positivity between 11-day mature and control was not seen even with exendin-4 supplementation, which could indicate an established schedule for maturation and needs further exploration. We sought to understand which other cell types were PCNA activity in the matured islets and co-stained for CK7. CK7-PCNA positivity was observed more in 20-day matured islets, but not 11-day control and matured islets. This demonstrates that not only are remaining PCNA positive cells most likely pancreatic ductal precursor cells, but long-term culture in enhanced maturation media is also beneficial to this cell type.

It was interesting to see no apoptotic activity in 20 day and 11 day matured islets. NPIs exhibit a natural resistance to apoptosis even under hypoxic conditions (Emamaullee, Shapiro, Rajotte, Korbutt, & Elliott, 2006) due expression of high levels of X-linked inhibitor of apoptosis protein (XIAP)- a potent endogenous anti-apoptotic protein. However, it unclear why then control islets produced a large volume of apoptotic cells as

evidenced by TUNEL positivity. It is possible that long-term culture methods had a detrimental effect on islet stability, and that TGF-β1's ability to maintain islet morphogenesis (Miralles et al., 1998) and Oncostatin M's repair mechanisms following pancreatic injury (Bouwens, 2006) prevented apoptosis in matured islets.

PDX-1 is a developmental transcription factor found in multi-potent progenitors that give rise to β-cells. It is first detected at embryonic day 8.5 in mouse endoderm of the gut, and later spreads to the pancreas (Guz et al., 1995). More PDX-1 staining was observed in 20-day control and 11-day matured islets, which may represent the accelerated maturation process of the islets. Conflicting viewpoints subsist regarding the existence of precursors in the postnatal pancreas as some believe the postnatal pancreas develops with a pre-determined number of β-cells (Solar et al., 2009), while others believe epithelial cells of adult exocrine duct may have a source of insulin producing cells (X. Xu et al., 2008). Nonetheless, these results provide some insight into how porcine islets develop. However, to draw any specific conclusions comparing positivity between matured and control islets, PDX-1 positive cells require an in-depth quantitative analysis.

After 20 days and 11 days culture, we recovered significantly more insulin from matured islets compared to their respective controls. Even at the expense of cell loss in 20 days culture (163.7 μg vs 322.8 μg DNA), insulin content was still maintained in matured islets. Others reported similar results where a concomitant decrease in DNA was paralleled with an increase in insulin content in NPIs cultured in combination nicotinamide, butyrate, FCS, and IGF-1 for 21 days (Lopez-Avalos et al., 2001;

Otonkoski et al., 1999). Interestingly, there were no differences in β-cell or α-cell composition between preparations in 20-day culture, and an almost 2-fold difference was observed in 11-day culture, which demonstrates not only enhanced functionality with long-term culture, but also a β-cell phenotype we hoped to achieve in this experiment.

Unfortunately, we did not observe any significant differences in insulin secretory responsiveness among matured and control islets in either culture method according to stimulation indices. We previously reported NPIs cultured in nicotinamide for 9 days producing indices up to 5.5 (Korbutt 1996) with others reporting similar SI values (Lamb et al., 2014). Lamb et.al experimented with a specific low-dose collagenase enzyme in hopes of reducing endocrine cell loss. However, it should be noted that static incubation itself is not a sole determinant of islet function and a more dynamic assay should be used. Continuous flow perifusion would deliver an accurate determinant of function and can perform simultaneous experiments on multiple islet preparations resulting in high throughput readouts.

Based on differing expression profiles of 20 day and 11 day cultured islets, it is possible that we achieved a high level of maturity and function in our matured islet preparations. Matured islets expressed more insulin and glucagon, while control islets had similar or greater pancreatic developmental gene expression. Interestingly, somatostatin was highly expressed in control islets, demonstrating that the islets composed of mostly δ-cells, and somatostatin's tonic inhibitory effect on insulin and glucagon secretion (Hauge-Evans et

al., 2009; Strowski, Parmar, Blake, & Schaeffer, 2000) as demonstrated by the vastly decreased insulin expression in controls. SOX9 was also highly expressed in matured islets and similar findings have been reported in chondrocytes exposed to dexamethasone (Sekiya et al., 2001). One study reported that expression of porcine insulin, PDX-1, Beta2/NeuroD, Nkx6.1, and Pax6 can be maintained until the end of 9 weeks and slowly ta (Tsuchiya, Tsuchiya, Iwami, & Ohgawara, 2003). Expression profiles we report are at 2 and 3 weeks culture only, and it should be noted that these assays were run separately and cannot be compared. Thus, subsequent experiments should focus on combining 20-day and 11-day samples in one assay to visualize any differences in expression profiles.

Mice transplanted with matured islets had significantly lower glycemic values than mice transplanted with control islets at weeks 18 and 20. This improvement was consistent with the OGTT and percent normalization curves, which reported lower glycemic values and better normalization rates. It was also consistent with increased H&E-insulin staining and β-cell composition in matured grafts. Insulin content recovered from the graft did not show any significant differences, which can be attributed to a lower sample size among the control group. In our opinion, normalization times were still longer than expected and have been shown to be much quicker in other animal models other than mice. Porcine and human insulin have relatively poor bioactivity in mice due to a four amino acid difference between mouse and porcine insulin (Pepper, Gall, Mazzuca, Melling, & White, 2009). The difference occurs at a significant binding residue where a substitution of Serine in porcine insulin for Proline in mouse at residue 9 of the B chain causes a non-fit into subsequent GLUT receptors. This could be the major cause of

porcine insulin's lack of efficacy in rodent models, and was observed in other similar studies (Bonner-Weir 2001, Otonkoski 1999). Animal models are one method to demonstrate the functionality of NPIs, particularly from a translational medicine perspective. Patch clamp electrophysiology can measure the exocytotic capacity of islets via membrane capacitance, and as mentioned before, continuous flow perifusion assesses insulin secretory capacity in a more dynamic fashion. Both are equally as effective in assessing islet function.

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Chapter 3: General Discussion

3.1 Discussion

Many Canadians suffer from type I diabetes with rates expecting to increase annually worldwide in the pediatric population. Insulin has remained the mainstay of therapy whereby daily assigned regimens reduce blood sugar levels and chances of secondary complications. However, it is not a cure as patients are forced to prick themselves several times a day for accurate blood glucose readings. If left untreated, complications of both macrovasuclar and microvascular – such as strokes, retinopathy, nephropathy, and neuropathy - can ensue. Additionally, missing a single dose could result in diabetic ketoacidosis, which can lead to death and incurring costs on the health care system.

Clinical islet cell transplantation involves transplanting insulin producing β-cells in the form of islets into type I diabetic recipients. With over 750 successful human islet transplants worldwide and 44% cure rate (CITR registry, 2011), it is continually evolving from an experimental strategy into a standard clinical therapy. However, due to cadaver shortage and poor stability in human islets, extensive research into an alternative source of graft tissue has been researched extensively. Porcine islets in the form of xenotransplants have the advantage of solving the graft tissue supply problem, and have similar biological activity to human insulin, which makes it an ideal alternative for clinical transplantation. Moreover, neonatal porcine islets contain β-cell precursors, which give it the ability to proliferate and differentiate in the recipient. However, several issues must be resolved before clinical translation of any porcine islet therapy.

Preventing graft rejection, ensuring adequate graft oxygenation, nutrient diffusion, and optimal site of transplantation are being extensively researched in surgical labs. This thesis has focused mainly on enhancing NPI function using long-term and short-term culture methods in maturation media.

We observed substantial increases in insulin positivity, actively proliferating β-cells and CK7 positive ductal tissue in matured islets that were also resistant to apoptosis. Other groups observed similar immunohistological findings in maturation protocols in terms of insulin positivity (Korbutt et al., 1996; Lopez-Avalos et al., 2001; Otonkoski et al., 1999). However, a more in-depth quantitative analysis was performed to differentiate between matured and control islets. Matured islets also exhibited less PDX-1 staining, which could mean an accelerated maturation process was taking place. Higher insulin content was also observed even with a decreased cell volume recovered, demonstrating a more functionally enhanced preparation. This translated to faster normalization times among diabetic mice transplanted with matured islets. However, due to generally poor bioactivity of NPIs in the mouse model, there was no profound reduction of time to correct diabetes. Poor performance could also be attributed to site of transplantation. Merani et.al demonstrated that sustained islet compaction in ependorf tubes pre-transplant can result in poor graft outcome in mice due to increase apoptosis (Merani et al., 2006). A similar situation may exist within kidney capsules as islets become compacted when injected. More insulin and less developmental gene expression was also observed among matured islets, which was confirmed in previous studies (Lopez-Avalos et al., 2001). The goal in mind for developing a short-term maturation protocol was to

reduce cell loss and this was achieved as evidenced by the DNA recovered. In long-term culture, more DNA was recovered in control islet preparations with less β-cells and insulin content. Therefore, long-term culture still proved to be more beneficial for matured islets. Visually, islet preparations in matured media showed less debris and better morphology than controls under stereomicroscopy.

The evidence described in chapter 2 has provided further insight into the documented mechanistic action of growth factor supplements and understanding of NPI viability in long-term culture. The combination of growth factor supplementation in culture media has been shown to be beneficial for insulin recovery and increasing β-cell composition. These experiments, however, demonstrated an additional robust islet phenotype capable of resisting apoptosis as evidenced by TUNEL staining. Our group previously demonstrated human islets co-cultured with bone marrow-derived mesenchymal stem cells prevented β- cell apoptosis after cytokine treatment (Yeung et al., 2012). Similar findings have been reported with heme oxygenase in mouse islets (Pileggi et al., 2001) and pretreatment with growth hormone-release hormone in rat islets has also been described (Ludwig et al., 2012). It would be interesting to see if our matured islets are capable of exhibiting the same effect in such conditions, which would hopefully produce an islet that is less immunogenic and amendable to quicker translation.

While the evidence in chapter 2 provided us the enhanced functional capabilities of NPIs, more in-depth experiments are needed. First, gene expression profiles of hormones and pancreatic developmental genes of matured islets in long-term and short-term culture

showed the importance of timing of transcription factor expression. To make an accurate comparison between both culture times, preparations need to be ran on the same assay, which it wasn't in this case. Secondly, further transplantation experiments should focus on more complex animal models such as porcine and non-human primates, which will hopefully demonstrate better glycemic responses with our matured islets. In-vitro experiments such as patch clamp electrophysiology and continuous flow perifusion can provide an overall assessment of exocytotic function and dynamic secretory response of the islets. Finally, repeating transplantation experiments with 11-cultured control and mature islets will provide a complete picture.

3.2 Conclusion

The popularity of islet transplantation in the surgical field has increased the demand for adequate tissue. Our lab has focused on neonatal porcine islets in the form of xenotransplants to solve the cadaver shortage issue in human islets. An attempt was made to augment NPIs through maturation using a novel media containing growth factors added in a stepwise fashion in 20 days and 11 days of culture time. In vitro matured islet preparations produced more insulin and contained a higher β-cell composition than control islets. This has also translated to better transplant outcomes in diabetic mice. NPIs are a valuable alternative tissue source to human islets. However, the challenges of reducing immunogenicity and determining optimal sites of transplantation still lie ahead. Nonetheless, we are steps closer to developing a cure for type 1 diabetes.

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