Translational Strategies for the Clinical Transplantation of Neonatal Porcine Islets

by

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Abstract

The development of the Edmonton protocol was an important milestone in the process of establishing islet transplantation as a cure for diabetes. However, many challenges must still be overcome, including the shortage of donor organs. To address this issue, we believe that neonatal porcine islets (NPIs) are a clinically applicable, unlimited source of cells. In order for NPIs to be translated to the clinic, we believe two important issues must be addressed. The first aim of this thesis is to develop an alternate site. The second aim of this thesis focuses on developing a clinically applicable protocol for the isolation, culture, and delivery of NPIs. For the first aim, based on the understanding of the interactions between islets and extracellular matrix proteins, a type one collagen-based matrix is developed containing chitosan, chondroitin-6-sulfate, and laminin to support NPI viability and function both *in vitro* and *in vivo* in Chapter 2. Therein, we demonstrate that such a matrix has the capacity to deliver islets to an increasingly vascularized subcutaneous site. We further developed this collagen-based matrix in Chapter 3 by altering the crosslinking concentration and observing the effects on the matrix microstructure and other outcomes, including *in vivo* vascularization after subcutaneous transplantation. Our results indicate that this matrix could be modified to be applicable for many cell delivery systems.

Chapters 4 and 5 address the second aim of this thesis, beginning with the development of a clinically applicable and scalable protocol for the isolation and culture of NPIs. Using an automated chopper, a general caspase inhibitor, and a protease inhibitor cocktail during culture in one petri dish instead of four resulted in islets with improved insulin content, β cell mass, and glucose responsiveness both *in vitro* and *in vivo* in a murine model. This modified protocol allows for the isolation of high quality islets from more pancreases in compliance with Good Manufacturing Practice (GMP), and may be further improved by replacing all reagents with those with GMP certification. Chapter 5 describes a protocol for cold storage of whole neonatal porcine

pancreases as a means to transport organs from a designated pathogen free source animal housing facility to a GMP cell manufacturing facility.

Finally, in Chapter 6 we explore some of the ethical and regulatory issues that must be considered to justify xenotransplantation clinical trials in Canada. We propose that NPIs have demonstrated efficacy to justify clinical trials; the safety data gathered from these clinical trials would then be extremely valuable for Canadian citizens to decide in a deliberative democratic model whether clinical trials for other cell or tissue xenotransplantation can also be justified. We conclude that there is a clear expected benefit for xenotransplantation as an unlimited source of donor tissue, particularly in the case of islet transplantation.

Collectively, these studies demonstrate that NPIs have the potential to be utilized as a clinical cell therapy for type 1 diabetes, isolated and cultured following a clinically applicable protocol and delivered in a collagen-based matrix that can be further modified as required. This matrix could also be readily retrieved if necessary. Furthermore, sharing of expertise and expenses across centers is feasible with a successful cold storage protocol, further improving the translational relevance of NPIs. In summary, this thesis advances the applicability of the NPI cell therapy as a treatment for type 1 diabetes.

Preface

Chapter 2 of this thesis has been published as Cara E. Ellis, Branka Vulesevic, Erik Suuronen, Telford Yeung, Karen Seeberger, and Gregory S. Korbutt, "Bioengineering a highly vascularized matrix for the ectopic transplantation of islets," *Islets*, vol. 5, issue 5, 1-10. I was responsible for the data collection and analysis as well as the manuscript composition. B. Vulesevic, E. Suuronen, and T. Yeung assisted with the data collection and contributed to manuscript edits. K. Seeberger contributed to manuscript edits. G.S. Korbutt was the supervisory author and was involved with concept formation and manuscript composition.

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ABC – avidin-biotin complex
aFGF – acidic fibroblast growth factor
bFGF – basic fibroblast growth factor
BM – basement membrane
BSA – Bovine Serum Albumin
C – Collagen matrix
CAM – cellular adhesion molecular
CC – collagen and chitosan matrix
CCC – collagen, chitosan, and chondroitin matrix
CCCL – collagen, chitosan, chondroitin and laminin matrix
cGMP – current good manufacturing practice
CS – chondroitin sulfate
DPF – designated pathogen free
ECM – extracellular matrix
EDC – 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
FBS – Fetal Bovine Serum
GAG – glycosaminoglycan
GM – genetically modified
GSIS – glucose stimulated insulin secretion
GTKO - α1,3-galactosyltransferase gene-knockout
GTT – glucose tolerance test
HBSS – Hank's Balanced Salt Solution
hESC – human embryonic stem cell
HS – heparan sulfate
HSA – human serum albumin
IBMX – isobutylmethylxanthine
ICC – islet cell cluster
IEQ – islet equivalent
iPSC – induced pluripotent stem cell
IXA – International Xenotransplantation Association
JDRF – Juvenile Diabetes Research Foundation
LCT – Living Cell Technologies
LPA - lysophosphatidic acid

MSC – mesenchymal stem cell
NHS – N-hydroxysuccinimide
NPI – neonatal porcine islets
PERV – porcine endogenous retrovirus
PIM – Prodo islet media
RGD – a tripeptide composed of L-arginine, glycine, and L-aspartic acid
ROCK – Rho kinase
RPMI - Roswell Park Memorial Institute medium
SDF-1 - Stromal cell-derived factor-1
TGF-β – transforming growth factor β
UW – University of Wisconsin cold storage solution
VEGF – vascular endothelial growth factor
vWF – von Willebrand factor
WHO – World Health Organization

Chapter 1: Introduction

1.1. DIABETES MELLITUS

Diabetes mellitus is a demoralizing chronic disease that affects more than 220 million people across the planet (1). In Canada it is expected that by 2020 3.7 million people will suffer from diabetes, or 9.9% of the projected national population; this vast increase is driven by the recent and alarming rise in obesity, inactive lifestyles of an aging population, and the higher prevalence of diabetes in immigrating ethnic groups (2). Beyond the micro- and macrovascular complications and the near-certainty of a decreased lifespan, diabetes causes a marked decrease in quality of life; the prevalence of comorbid depression in diabetics is double that of non-diabetics (3). Furthermore, diabetes is a massive financial cost to healthcare systems worldwide, requiring nine to fifteen percent of the total health care budget in first world countries (1). In Canada, the economic burden of diabetes is projected to approach $16.9 billion by 2020 (2). Clearly, diabetes merits the attention of the scientific community to a search for a cure. The ability to synthesize insulin, discovered in 1921, has significantly decreased diabetic mortality, as well as delaying and allowing some management (although not full prevention) of the associated nephropathy, neuropathy, retinopathy and cardiovascular disease through intensive insulin therapy, but with this treatment comes risk of serious hypoglycemia (1,4). Although advances in closed loop insulin pump technologies are promising (5), consistent and precise technology for sensing glucose *in vivo* that could be utilized by implanted insulin pumps or other secreting devices does not yet exist. Research must focus on alternative therapies for treating and eventually curing diabetes. The transplantation of tissue that produces insulin, whether a whole pancreas, isolated islets, or stem cell derived β cells, could be such a therapy.

1.2. ISLET TRANSPLANTATION FOR DIABETES

1.2.1. History

In 1889, Josef, Baron von Mering and Oskar Minkowski first discovered the correlation between the pancreas and diabetes when experiments in pancreatectomizing dogs at the University of Strasbourg revealed increased propensity of uncontrolled urination wherein the urine had high sugar content in previously housetrained animals (1). In July 1916, Frederick Charles Pybus, a surgeon of Newcastle-on-Tyne, attempted to reverse diabetes in two male patients, the first 37, the second, 32, both with "grave and intractable" disease, by grafting a donor pancreas divided into thin slices into the subcutaneous tissue of the abdominal wall. Both patients died, the first after three months and the second after three years. In one case the grafts became infected and in the other they degenerated, however Pybus observed a temporary decrease in sugar excretion in one of the patients (6). In 1920 Sir Frederick Grant Banting and his student Dr. Charles Herbert Best found that ligation of the canine pancreatic duct ameliorated the recovery of the "internal secretions" of the pancreas. Further investigations revealed that these internal secretions were released by the islets of Langerhans as postulated by Gustave-Edouard Laguesse, and, verifying what was found by Laguesse, these secretions control the level of glucose in the blood (7). Subsequent exploration of these secretions resulted in the discovery of insulin, revolutionizing the clinical treatment of diabetes (8).

As the mortality of insulin-treated patients was deferred and thus lifespan increased due to the insulin therapy, it was increasingly evident that diabetes mellitus caused secondary complications. When it became clear that insulin alone was insufficient in preventing these complications, pancreatic tissue transplantation was once again considered (1). In 1966 the first vascularized pancreas transplant was attempted at the University of Minnesota by the team of Drs. Kelly, Lillehei, Merkel,

Idezuki and Goetz; the patient had immediately decreased blood sugar levels but succumbed to a pulmonary embolism three months later (9). However, further investigation in pancreatic transplantation resulted in high rates of morbidity and mortality; speculation that transplanting islets only instead of the vascularized pancreas would decrease complications led to study of islet transplantation in a rodent model (1,10). Evidence from various studies suggested that chemical diabetes could successfully be reversed using islet isografts in the liver via the portal vein (1). The same experiments repeated in large animal models were not as successful, however, as it was difficult to obtain adequate numbers of islets by isolation. Therefore, as a substitute for isolated islets, pancreatic fragments were transplanted in an attempt to increase islet mass; these experiments were not successful, and it was clear that further investigation was necessary in order to increase the purity and efficacy of islet isolations (1). Despite such improvements as the Ricordi digestion chamber, the COBE continuous purification system, the use of collagenase to control pancreatic distention, and increased purity of enzymes with low endotoxin levels, there was no spectacular improvement in clinical results; from 1974 to 1999, more than 450 type 1 diabetic patients underwent islet allotransplantation, with less than 10% of these patients maintaining insulin independence after one year (1). It was posited that the failure of these grafts was due to insufficient islet mass for the graft, poor prevention of allograft rejection, and toxic immunosuppressive drugs (1).

Finally, in 1999, in Edmonton, Alberta, Canada, a team of researchers developed a new protocol resulting in seven type 1 diabetic recipients achieving stable insulin independence for longer than a year; this protocol achieved clinical success because the patients received the islets from two donor pancreases, and the antirejection therapy was more potent but less diabetogenic (1). As of 2012, at the University of Alberta where the Edmonton protocol was developed, the clinical islet transplantation team has performed over 300 intraportal infusions in over 150 patients, most requiring two

separate transplantations to receive enough islets. Of these 150, 15% remain insulin independent after 5 years; 70% of recipients maintain some islet function with detectable C-peptide secretions for 8 years post-transplantation (11).

1.2.2. Indications for Transplantation

Transplantation in diabetes is not always an acceptable treatment because of the toxic effects of immunosuppression; if patients with type 1 diabetes can achieve adequate glycemic control with insulin therapy, or patients with type 2 diabetes with lifestyle changes with or without drugs, then the benefits of tissue transplantation do not outweigh the risks (10). However, if a patient has already received a kidney transplant and is therefore currently undergoing an immunosuppressive regimen, incapacitating clinical and psychological problems are associated with the use of insulin therapy, the insulin treatment results in frequent hypoglycemic events, or if the insulin treatment is insufficient to prevent the acute complications related to diabetes, then islet or pancreas transplant is a viable option (10). Recent studies have illustrated that the transplantation of the vascularized pancreas consistently returns the glycemia of the patient to acceptable therapeutic levels for the long term, thereby improving quality of life and potentially ameliorating some of the complications related to diabetes (1).

1.2.3. Challenges

Despite the revolutionary success of the Edmonton Protocol, there are still many areas for improvement before islet transplantation can become the "gold standard" therapy for diabetes. Because more than one donor pancreas is required for insulin independence, it is apparent that there is some factor impairing graft function; it is desirable to improve function such that a single donor pancreas is all that is required (10). Suggested factors impairing graft function in the recipient include a pathological autoimmune response, the diabetogenic immunosuppressive regimen, and brain death of the donor, which results in hemodynamic instability and a requirement for high

doses of inotropic support, which itself has inherently negative consequences for organ perfusion; additionally circulating brain-derived inflammatory peptides are directly toxic to the islets (1). This situation could be ameliorated with further improvements in immunosuppressive drugs and advances in the techniques used to obtain the pancreas from the deceased donor (1). Following the success of single-donor islet transplantation, a future challenge is to increase the supply of islets so that more patients can receive the therapy. Segmental pancreas grafts are a viable option that could be obtained from living donors as opposed to a whole pancreas transplant, reducing the risk to the patient; however, because it is only a portion of the pancreas, it will be difficult to transplant a sufficient islet mass (1). Another viable alternative is xenotransplantation, a nearly unlimited source of islet cells; these cells can be isolated from transgenic donors that have been genetically engineered to express human proteins, decreasing to some extent the need for the toxic immunosuppressive drugs, although the problem of heavy immunosuppression has still not been solved (1), (12). These islets could potentially be "designed" to match the transplant recipient; however, as with any genetic engineering, the risks associated such as zoonotic viral transmission must first be drastically reduced.

Great efforts have also been made to prolong islet graft survival (13-15). The low availability of viable islets is seriously exacerbated by the failure of the graft to subsist in recipients. It is estimated that even without immune response greater that 60% of the cells transplanted are lost via apoptosis (15). Detrimental factors include hypoxia and hypoxia-reoxygenation, as well as recurrent ß-cell autoimmunity, allograft rejection, metabolic exhaustion, chronic islet toxicity of immunosuppressive drugs, poor revascularization of the islets, and moreover the limitations of the intra-portal site ; transplantation of islets into the portal vein has been associated with life-threatening intraperitoneal bleeding (16), portal vein thrombosis and hepatic steatosis, the abnormal retention of lipids in the liver (17, 18). Possible alternate sites for islet transplant, such as

the subcutaneous space, the omentum, and intra-muscular sites, are less than ideal as they are poorly vascularized and thus result in poor islet survival. It is therefore highly desirable that a vascularized site be found, or manufactured. Additionally, the hypoxic period can last up to two weeks after the transplant before the graft site becomes revascularized, at which time the islets suffer a reoxygenation insult, and β cells are known to be especially sensitive to injury via hypoxia and hypoxia-reoxygenation (19). Culturing the islets prior to transplant helps to prevent this apoptosis, as well as recovery of some cell surface molecules lost or damaged during isolation and increasing the purity of the eventual graft, decreasing the risk of an immune response (20).

1.2.4. Alternate sources for islet transplantation

Donor organ shortages prevent islet transplantation from being a widespread solution as the supply cannot possibly equal the demand. Porcine islet xenotransplantation has the potential to address these shortages, and recent pre-clinical and clinical trials show promising scientific support for xenotransplantation being a possible cure. It is therefore important to consider whether the current science meets the ethical requirements for moving towards clinical trials. Xenotransplantation is a therapy with great potential to benefit many people and improve global health equity. Despite the potential risks and the scientific unknowns that remain to be investigated, there is optimism regarding the xenotransplantation of some types of tissue, and enough evidence has been gathered to ethically justify clinical trials for the most safe and advanced area of research, porcine islet transplantation. Researchers must make a concerted effort to maintain a positive image for xenotransplantation, as a few well publicized failed trials could irrevocably damage the public perception of xenotransplantation, and because all of society carries the burden of risk it is important that the public be involved in the decision to proceed. As new information from pre-clinical and clinical trials develops, policy decisions should be frequently updated and if

at any point evidence shows that xenotransplantation is unsafe, or alternatives are created that are superior, then clinical trials will no longer be justified and they should be halted. However, as of now the expected benefit of an unlimited supply of islets, combined with adequate informed consent, justifies clinical trials for islet xenotransplantation.

1.3. ISLET ARCHITECTURE

1.3.1. Islets of Langerhans

The islets of Langerhans are small, discrete clusters of endocrine cells dispersed throughout the pancreas, a continuous medium of exocrine tissue. Named after Paul Langerhans, a German pathological anatomist who identified the structures in 1869, islets are primarily composed of five distinct cell types. Alpha (α) cells produce glucagon; β cells secrete insulin; δ cells secrete somatostatin; ε cells produce ghrelin; and PP or γ cells secrete pancreatic polypeptide. Islets comprise 1-2% of the pancreatic volume, with β cells comprising 15-20% of the islet in humans; there are approximately 1 million islets in an adult human pancreas. However, the size of islets remains fairly consistent between species, ranging from approximately 100 to 200 μm. This suggests that islets have an optimal size that does not vary between species, or that islet architecture in larger species is more efficient (21). Despite variations in pancreas size, the islet size distributions are remarkably similar between species (21). However, greater numbers of islets with diameters greater than 100 μm are found in human pancreases compared to murine or porcine pancreases (21).

The dispersal of each cell type within the islets varies between species, and it is likely that this arrangement is important for intra-islet regulatory processes. In human islets, α, β and δ cells are interspersed irregularly throughout the structure (22). Because the cells are in close proximity, they are able to influence the function of other endocrine cells; for example, somatostatin powerfully inhibits insulin and glucagon secretion,

insulin inhibits glucagon secretion, and glucagon stimulates insulin secretion. This disorganized architecture has also been suggested to allow human islets to respond to lower concentrations of glucose than prototypical rodent islets (22). Mouse islets consist of a β cell islet core surrounded by a peripheral mantle of α and δ cells. Porcine islets tend to have architecture between that of human and mouse islets, with some α cells within the core. There are fewer large islets in porcine pancreases, and the overall architecture tends to be less compacted (21).

It is important to note that while islets are only 1-2% of the pancreatic volume, they receive 15-20% of the blood supply (23). Each individual islet is highly vascularized by direct arteriole blood flow; arterioles form fenestrated networks within the islet, promoting bidirectional exchange between endocrine cells and blood (23). Islet architecture likely has an important relationship with the microcirculatory pattern for efficient signaling; for example, blood flow in the mantle-core architecture of murine and porcine islets can occur via three mutually exclusive models. The mantle to core model suggests that non-β cells first sense glucose concentration, then subsequently inhibit or stimulate the core β cells. Alternately, the core to mantle model postulates that the β cells first respond to changes in glycemia, and then the insulin signal is modulated by the mantle cells. A final model, the artery to vein model, implies that blood enters the islet from an artery and drains to the vein, regardless of cell composition. All three of these models have been observed in murine islets, with the core to mantle model predominant; this implies that islet architecture does not control microcirculatory patterns and thus that the relationship is likely complex (21).

The islets of Langerhans are also highly innervated by both sympathetic and parasympathetic nerves that regulate hormones and may have trophic functions (23). Parasympathetic stimulation powerfully enhances both α and β cell secretion; sympathetic nerve stimulation strongly inhibits insulin and enhances glucagon secretion, for example during exercise. Adult islet cells are coupled by gap junctions

and cell adhesion molecules (CAMs) such as integrins, neural CAMs, and E-cadherin; these connections allow signal transduction regulating islet development, glucose sensing, and insulin secretion (23).

Adult human islets are partially encapsulated by a single layer of fibroblasts and associated collagen fibers; this capsule is associated with the periinsular basement membrane (BM), a specific type of extracellular matrix (ECM) associated with the epithelium and endothelium (23). The BM is comprised mostly of laminin and nonfibrillar collagens, linked by interactions with entactin, a glycoprotein (23). The composition, depth, and continuity of the ECM on the islet periphery vary between species, and there is also a significant BM associated with pervading microvasculature that varies between species (23). Peripheral ECM of mature human islets is mostly composed of laminin and type IV collagen, likely emerging from the pancreatic ducts during development, but fibronectin and collagens I, III, V, and VI are also detected (23). Human islets have two distinct BM layers surrounding the islet microvasculature, with a separate peri-islet BM layer surrounding perivascular BM (23), whereas murine islets only have perivascular BM and porcine islets have minimal coverage, with cell-cell interactions predominating at the exocrine/endocrine interface (23). The endocrine tissue in a human islet is almost all in contact with the perivascular BM, while murine β cells are nearly all in contact with the perivascular BM. These BM interactions influence islet health and function through both direct adhesion interactions and maintenance of islet vasculature; laminin provides a substrate for endothelial cell attachment and migration (23), and proteoglycans act as a molecular sieve to sequester growth factors (24).

The islet-ECM interactions are key for islet viability and function, but these interactions are complex and not fully understood as it is challenging to study them in situ; it is difficult to discern differences between the results of specific binding events and culturing cells in two or even three dimensions (23). Additionally, islets are

complex cell clusters with many interacting cell types and each have many receptors for ECM components; each receptor may bind to multiple ligands, and each ligand may have multiple cell binding domains, with each combination resulting in different cellular events (23). There are also differences in both receptors and matrix compositions as islets develop and mature. However, the importance of ECM has been recognized since the 1980s (23-26). In mature, intact islets, natural or synthetic ECMs regulate survival, insulin secretion, proliferation, and islet morphology; similar results are seen in purified β cell preparations (23). β cells are protected from anoikis, apoptosis that only occurs in unattached cells, by ECM components including laminin (27). The NF-κB pathway in β cells, essential for proper glucose stimulated insulin secretion, relies on β cell-ECM interactions (27, 28). However, the peripheral ECM is almost completely lost due to the enzymatic and mechanical stresses during islet isolation, and then replaced by a layer of matrix proteins after several days in culture that differs from the native islet ECM (29). Perivascular BM also likely lost during isolation by the same mechanisms, as well as due to the disruption of the vascular supply (23). The perivascular BM is never restored to its native state, as demonstrated by changes in the microvasculature of transplanted islets after implantation (30-33). Therefore, a comprehensive knowledge of islet ECM composition and interactions is integral to designing a biomaterial scaffold that may preserve islet viability and function after transplantation.

1.3.2. Islet Extracellular Matrix Composition

An ECM, or its equivalent, can be identified in all organisms from prokaryotes to humans; for example, biofilms produce extracellular polymeric substance through secretion and cell lysis (34). The composition of the ECM, as well as its interactions will cells, varies. However, the main components of the ECM are the same proteins, glycoproteins, and proteoglycans. The arginine, glycine, and aspartic acid tripeptide,

known as RGD, is an important domain for islet cell adhesion; RGD-dependent adhesion inhibits apoptosis in mature human islets and is critical for cell migration in early islet development (23). This domain is also common to many, but certainly not all, ECM polymers, and can adopt variable conformations in different proteins, suggesting that the receptor specificity may be dependent on conformational flexibility (35). The main components of the ECM of islets are collagens, glycoproteins, laminins, and proteoglycans.

1.3.2.1. Collagens

Collagens are structural ECM proteins that typically form right handed triple-helical domains of three α chains. These chains can either be identical, forming a homotrimer, or different, forming a heterotrimer (34). Typically, glycine repeats as every third amino acid residue, in a Gly-Xaa-Yaa triplet; Xaa and Yaa are often proline and hydroxyproline, respectively. Glycine is essential for tight packaging of the α chains (36), because glycine lacks the bulky sidechains of other amino acids; each polypeptide chain has a left-handed helical conformation, with three residues per turn (37). Proline and hydroxyproline are also integral to the structure of the polypeptide chains. These cyclical iminoacids distort the α chain backbone, causing glycine to always be located close to the axis of the superhelix, leaving proline and hydroxyproline exposed on the surface and thus able to interact with other molecules (37). The Gly-Xaa-Yaa triplet repetition, as assembled in the superhelix, is identified as a collagenous domain; other proteins also contain this domain, so collagens can form supramolecular structures within the ECM (34). Collagens contain both collagenous and non-collagenous, non-helical domains.

Currently, twenty eight human collagens have been identified, and are organized into subgroups based on structure. The fibrillar collagens, including types I, II, III, V, XI, XXIV and XXVII are the most abundant proteins within vertebrates; collagens comprise

approximately 25% of the dry weight of mammals (36). Fibrillar collagens are stabilized by electrostatic interactions between the exposed sidechains of adjacent superhelices. As previously mentioned, fibrillar collagens I, III, and V are present in the human islet ECM (23). There are many types of non-fibrillar collagens, but the only two known to be present in the pancreas are collagens IV and VI. Collagen IV is a hexagonal collagen, forming chicken-wire structures; this structure is possible due to the long telopeptide extensions at the collagen monomer termini interacting with the non-helical domains of other collagen monomers (37). Collagen IV forms tetramers, with four triple helical collagens linking through disulfide bonds and hydroxylysine-lysine interactions due to the cysteine and lysine rich termini; collagen IV is heavily glycosylated, lending resistance to collagenases (38).

Collagens are produced in the cell as procollagens, containing noncollagenous domains in the N- and C- termini (39). The C-terminus is essential for the formation of the superhelix by trimerizing the three α chains in the C-to-N direction; after secretion from the cell, these propeptide domains are cleaved by specific proteinases (39). Short, non-collagenous telopeptide extensions remain at the ends of both terminals of the collagenous domains (37). Collagens also undergo hydroxylation of proline and some lysine residues in the Yaa position; crosslinking between groups of triple helices is through the condensation of the hydroxlysine and lysine side chains, forming a hydration shell around the fibrils that stabilizes them through hydrogen bonding (37).

Collagens interact with integrins through both helical and non-helical domains (37). Collagen IV interacts indirectly with islets by binding with other ECM proteins, such as heparin sulfate proteoglycans and laminin through the intermediary glycoprotein entactin (23). The RGD domains are usually inactive in collagens in their normal conformations because they are covered by the helicity of the protein as glycine is at the center of the helix; these domains are also presented to the cells at lower curvatures than the flexible loop conformations of fibronectin and entactin (23).

Collagen type IV is highly prevalent in the peripheral ECM and BM of human islets, but its role in islet physiology is unclear; despite the close proximity, it is possible that islets have limited direct interaction with collagen. However, *in vitro* studies demonstrate that whole islets have improved survival and function when cultured on collagen IV compared to collagen I, while purified β cells have decreased insulin production and secretion in the same conditions (23).

1.3.2.2. Glycoproteins

Fibronectin is a dimeric glycoprotein found in both a soluble and fibrillary form; the fibrillar form is a common component of ECM in many tissues (23, 24). Fibronectin creates noncovalent adhesion of other ECM components, including collagen, heparin, chondroitin sulfate proteoglycans, specific receptors, and itself, through several domains. Cell adherence is through an extended, flexible RGD loop between two β strands (23). A secondary binding sequence, PHSRN, is close enough to the RGD loop to be recognized by the same integrin; other binding sequences are also present, but likely are not relevant in islets (23). In islets, fibronectin is found in the adult islet periphery, the islet ductal pole, and in perivascular areas; fibronectin is associated with collagens I, III, IV, and laminin. Fibronectin may play a role in controlling anoikis via the $\alpha_5\beta_1$ integrin pathway in mature human islets (23, 24).

Entactin, also known as nidogen-1, is a globular glycoprotein found in basement membrane, responsible for linking perlecan, collagen IV, fibulin, and laminin; the globular domains bind to the protein core of perlecan, the short arm of the laminin trimer, and collagen. Similar to fibronectin, cell adherence is via an RGD loop in the central domain of entactin and the $\alpha 5\beta 1$ and $\alpha 5\beta 1$ integrins. The role of entactin in β cell and islet function is unclear (24).

Vitronectin is a glycoprotein found in both cell plasma and ECM. Its three domains have separate functions, including regulating proteolysis by binding and

stabilizing plasminogen activator inhibitor-1, cell adherence through an RGD sequence, and binding heparin following its activation by thrombin-anti-thrombin III complex. In humans, vitronectin is only found in fetal islet tissue and is produced by the pancreatic duct cells. Vitronectin likely plays an important role in islet morphogenesis, as the integrin that interacts with this glycoprotein, $\alpha_v\beta_1$, is also upregulated in fetal β cells. This integrin is essential for the spread and migration of fetal β cells grown on vitronectin (24). However, non-RGD interactions with other ECM proteins such as laminin are likely more important for creating and maintaining mature islet architecture (23).

1.3.2.3. Laminins

Laminin is a cross-shaped, heterotrimeric glycoprotein with three polypeptide chains linked by disulfide bonds (23). There are at least 12 different laminin isoforms, but their specific expression and distribution in islets is not thoroughly understood (23). Collagen and laminin form stabilized polymer networks, linked by entactin and the GAG chains of perlecan. The three short arms of the cross-shaped trimeric laminin protein bind to other laminin molecules, and the long arm binds to cell membranes (24). Laminin can interact with both integrin, including α_6, α_3, β_1, and non-integrin receptors, including dystroglycan protein complex, Lutheran blood group glycoprotein (Lu), and laminin receptor-1, so the interactions between islets and laminin may not be integrin based (23, 24). Lu localizes to the surface of adult human islet cells that interact with the BM and microvasculature, while laminin receptor-1 is able to bind to elastin and collagen IV if it has the same secondary conformation as laminin (24). Like collagens, many laminin isoforms contain RGD sequences but they are generally inaccessible; these "cryptic" domains may be implicated in tissue repair or remodeling as they can become exposed after degradation or denaturation. In the developing pancreas, laminin is colocalized with α_6 integrins (40). Laminin is highly prevalent in mature islet ECM;

pancreatic endocrine cells interact with laminin associated with the islet microvasculature (24).

1.3.2.4. Proteoglycans

Proteoglycans are heavily glycosylated glycoproteins that are an important component of ECM. Glycosylation is the post-translational attachment of long carbohydrate side chain to the core protein (24); these glycosaminoglycan (GAG) side chains are negatively charged under physiological conditions, allowing the ECM to bind and sequester growth factors and cytokines (24). Additionally, the strongly anionic GAGs attract cations and water molecules, allowing for hydration and therefore resistance to compressional forces (41). There are two main groups of proteoglycans, characterized by the type of sulfated GAG, are synthesized by almost all eukaryotic cells and present in almost all tissues in mammals (41). Heparan sulfate (HS) proteoglycans with relevance to the islet ECM include perlecan, syndecan, glypican, betaglycan, and agrin, where chondroitin sulfate (CS) proteoglycans include versican. HS proteoglycans are decorated with linear chains of alternating N-acetylglucosamine and glucuronic acid; heparin is a similar molecule, but contains 80% or more N-sulfated glucosamine compared to heparin sulfate's less than 50%, and is less sulfated (41). CS proteoglycans are instead glycosylated with different GAG side chains, alternating N-acetylgalactosamine and uronic acid (41). They are major components of cartilage, as well as regulating neuronal development and differentiation in the central nervous system (24). Limited CS proteoglycans have been studied in the pancreas, but they are found in the periislet BM as well as in the vasculature, at least in rats (42).

Perlecan is found in all BM, cartilage, and in several mesenchymal tissues during human development (24). Perlecan has a very large core protein with five domains, four of which share structural homology with proteins such as agrin, laminin, collagen XVIII, low density lipoprotein-receptor, and neural cell adhesion molecule (24, 41).

Because of these five different domains, perlecan is functionally diverse; it can self-associate or interact with other macromolecules via its protein core, GAG chains, or sequestered growth factors, including laminin and collagen IV (24, 41); an important function of perlecan is improving stability of the ECM network due to this panoply of interactions. The strongly anionic GAG side chains also grant cellular control of local effector molecule gradients due to the charge selective permeability barrier for proteins (24, 41). Perlecan may modulate angiogenesis through controlling the binding of growth factors (24). The function of perlecan is islets is not well understood, but because of its presence in the capillaries inside islets, it may be a protective barrier for the islets in the chronically inflamed milieu of the diabetic pancreas (24); perlecan expression is lost in the diabetic murine pancreas (43).

Syndecans are transmembrane HS proteoglycans, occasionally containing CS chains, which modulate the interactions of cell surface receptors with ligands such as growth factors and other ECM components (24). Syndecan can interact with collagens I, III, IV, V, and VI, but has the highest affinity for collagen V; it may also bind to the heparin-binding domain of fibronectin (24). Syndecan is a component of focal adhesions, specialized sites of cell attachment linking the ECM to the actin cytoskeleton (35), and therefore participates in downstream intracellular signaling (24). Syndecans also participate in leukocyte recruitment by creating a chemokine gradient at the endothelium surface; this gradient modulates permeability of the vascular endothelium, thereby allowing leukocyte extravasation, and through integrin activation (24). The HS and CS side chains of syndecan also inhibit leukocyte binding to E-selectins and collagen I, which also protects endothelial cells from apoptosis (44). Syndecans also plays a role in modulating inflammation and promoting wound healing through the degradation of the HS, enhancing the bioavailability of basic fibroblast growth factor (bFGF), a growth factor important for angiogenesis (45). The impact of syndecan on islet cells is not clear, but it may ameliorate binding to collagen and laminin (24).

Glypicans are cell surface proteoglycans that, like syndecan, can bind to fibronectin through HS chains to the heparin-binding domain (24). In mammals, the six isoforms are mostly expressed during development; therefore, glypicans may regulate GF sequestering during pancreatic development, regulating islet morphogenesis (24). Betaglycans, transmembrane HS and CS proteoglycans, function as co-receptors for transforming growth factor β (TGF-β), a family of cytokines that helps regulate cell proliferation, migration, differentiation and apoptosis (24); betaglycans thereby upregulate the TGF-β signaling pathway, while soluble betaglycans in the serum act as antagonists for TGF-β and decrease ECM and TGF-β expression (24). Unlike most proteoglycans, betaglycans bind TGF-β through interactions with the protein core, and not the GAG side chains (24). Betaglycans also induce cytoskeleton re-organization and inhibit cell migration in epithelial cells (46). While betaglycans have not been studied in islets, TGF-β is essential for islet morphogenesis and protective against diabetes; therefore, betaglycans may play a role in maintaining normal islet function (24). Finally, agrin is a HS proteoglycan with both CS and HS side chains that is critical for postsynaptic differentiation at the neuromuscular junction; the function of agrin in the pancreas has not been studied (24), but agrin mRNA has been detected in murine islets (47).

Versican is a CS proteoglycan found in adult human pancreatic exocrine acini, ducts, and blood vessels, but not in islets (42). Despite versican's lack of an RGD domain, it is able to bind to integrin β1, increasing focal adhesion and decreasing oxidative stress-induced apoptosis (48). Versican can bind to fibulins, fibrillin, and collagen I, as well as decreasing cell adhesion to fibronectin by sequestering fibronectin's RGD domains (24).

1.3.3. Islet Integrin Composition

The integrin receptor family are well studied cell adhesion receptors that play integral roles in development, immune response, leukocyte migration, and hemostasis (49). Found in all metazoa from sponges and cnidarian to humans, integrins in mammals are composed of eight β and 18 α subunits that combine into 24 distinct integrins, each with specific, non-redundant functions (49). Their general functions include regulating adhesion to ECM ligands or counter receptors on adjacent cells and acting as transmembrane mechanical links to the cytoskeleton (49). The interaction of integrins with their many ligands induces a plethora of signal transduction events, including modulating proliferation, apoptosis, cell shape, polarity, motility, gene expression, and differentiation. Integrin signaling is coupled with growth factor receptor pathways; for example, lysophosphatic acid (LPA), a serum factor normally secreted into a wound to stimulate cell contraction and subsequent wound closure, is dependent on cell adhesion to a substrate via integrins (35, 49). Integrins are also necessary for angiogenesis, and can play both pro- and anti-angiogenic roles (50).

Adult human islets have cells positive for the α3, α5, αv, and β1 subunits, while intraislet endothelial cells express α3, αv, β1, and β5 integrin subunits, as well as the laminin receptors α-dystroglycan and Lu glycoprotein (23). Fetal β cells likely rely on α1β1 integrin interactions with collagen IV for motility during development to form correct islet architecture, then later on αvβ5 integrin interactions for adhesion and maintenance of their differentiated form; αvβ3 and αvβ5 may be important for the differentiation of β cells from the islet cell progenitors in the ductal epithelium (51, 52). In the adult islet, β1 maintains islet architecture (49).

There are variations between in integrin expression species, likely due to differing complexities in islet cell and ECM composition (23). For example, as described in section 1.3.1, human islets have both a periislet BM layer in direct contact with endocrine tissue and a perivascular layer and are therefore more dependent on binding

interactions, especially with laminin; porcine islets depend almost exclusively on cell-cell adhesion. Porcine islets do not express integrin β1, an important receptor for laminin, but do express α2, an integrin subunit with superior binding to the fibrillary collagens I, II, III rather than non-fibrillar collagen IV (53), possibly indicating more of a reliance on interactions with peripheral ECM. Additionally, integrin expression changes after islets are isolated from the whole pancreas. For example, integrin α5 expression is markedly decreased across all species after isolation, and isolated porcine islets begin to express integrin αv, possibly reflecting an increased affinity for RGD-based binding (29). However, it is difficult to speculate about the specific consequences of integrin-based cellular interactions in islets because some integrins differ in their binding affinities across different cell types (23). It is clear, however, that the structural changes observed in islets post-isolation have consequences for cell survival and function (29), and restoration of native islet integrin expression would be advantageous.

1.4. ANGIOGENESIS

1.4.1. Definition

Angiogenesis, the growth of new blood vessels from pre-existing blood vessels, has potential to restore ischemic tissue and increase the viability of tissue transplantations. Although vasculogenesis, wherein new blood vessels form spontaneously, is similar in some ways to angiogenesis, it does not contribute to repair and disease in the postembryonic period of life (54). Angiogenesis is a key morphogenetic process for reproduction, wound healing, bone repair, rheumatoid arthritis, ischemic heart disease, ischemic peripheral vascular disease, diabetic retinopathy, and tumor growth and metastasization. Therapeutic angiogenesis is in general directed at re-establishing blood perfusion to ischemic tissue via the use of cytokines, growth factors, or cells (55).

1.4.2. Relevance to Islet Transplantation

The portal vein of the liver is the gold standard site for human islet transplantation due to its ready access to the portal venous system, where insulin is physiologically secreted from the pancreas, and the ease of access. However, this site has some remaining problems, as described above, that lead to gradual attrition of graft function. Therefore, establishing a vascularized graft that could be implanted in a non-vascularized site would be ideal for islet transplantation.

Although much successful research has been conducted to develop surface optimization technologies the natural endothelium remains the optimal surface for contact with blood (56). Therefore, attempts have been made to induce endothelial cell growth on synthetic surfaces and to tissue engineer blood vessels. In order to create an autologous tissue-engineered blood vessel, the endothelial cells of the donor must be harvested via a biopsy, and then cultured on a biodegradable scaffold in a bioreactor; the scaffold acts as a physical support system for the forming extracellular matrix and guides the shape and size of the new blood vessel. This process often takes longer than eight weeks, and is highly costly; because the culture period is so long, there is sufficient time for phenotypic or genotypic changes in the cells or bacterial contamination, rendering the autologous blood vessel useless. The isolation and purification of islets from whole pancreas by necessity results in a loss of the original vasculature, meaning the graft must be revascularized by the endothelial cells and microvessels *in situ* after transplantation (31). Because of the challenges of tissue engineering blood vessels combined with the absolute requirement for vasculature for islet survival, other alternatives to inducing angiogenesis are necessary.

1.4.3. Mechanism of Angiogenesis

Angiogenesis is influenced by the activity of a panoply of cells, such as monocytes, macrophages, mast cells, lymphocytes, connective tissue cells, pericytes, endothelial

cells, and tumor cells, which release soluble angiogenic and anti-angiogenic molecules such as TGF and proteolytic enzymes. Although the mechanism of activation for these cells is not entirely understood, it has been postulated that hypoxia due to insufficient vasculature may be a driving force for angiogenesis by the expression or secretion of angiogenic molecules (54). Vascular endothelial growth factor (VEGF) has increased expression in hypoxic environments; hypoxia also stimulates the expression of platelet-derived growth factor (PDGF) B-chain in endothelial cells, and secretion of the active form of transforming growth factor-β_1 (TGF- β_1) from smooth muscles cell (54). Despite the lack of detailed understanding of how hypoxia is sensed by cells, or induces the secretion of angiogenic molecules, there is evidence that hypoxia and other factors associated with poor oxygen concentration or inflammation, such as acidic pH or high lactate concentrations, has some effect on angiogenesis (54). However, hypoxia is by no means the only driving force for angiogenesis; fibroblast growth factor (FGF) is secreted *in vivo* during angiogenic process, and has been noted to be released upon mechanical insult, ischemic damage, and from damaged cells. PDGF B-chain and TGF-β and released by thrombin and heparin, plasmin, thrombin and thrombospondin respectively, and both can bind to ECM substrata (54). The changes that cause cells to become angiogenic and to begin angiogenesis are those that cause a "switch to an angiogenic phenotype" (54).

Angiogenesis, like most functions, is controlled by balancing angiogenic and antiangiogenic factors, and neovascularization will not occur unless homeostasis is disturbed and the positive regulators are more plentiful than the negative regulators. A great number of angiogenic regulators have been characterized, and others have been observed to affect angiogenesis but have yet to be fully understood. It is not possible to separate the direct effects of factors on vascular cells and the recruitment or stimulation of inflammatory and connective tissue cells that release their own angiogenic molecules; however, direct angiogenic or antiangiogenic molecules are defined as those that induce

an effect on angiogenesis *in vivo* as a result of exogenous dosing or overexpression; have an effect on endothelial cells *in vitro* that congruent with angiogenesis; cause a response in relevant vessels *in vivo*, with relevant receptor expression; and/or have an established role in angiogenesis in a process or disease (54). Antiangiogenic molecules include polypeptides and peptides such as thrombospondin, angiostatin, somatostatin, platelet factor 4, interferon-α, and the 16-kDa N-terminal fragment of prolactin; angiogenic steroids such as medroxyprogesterone and 2-methoxyestradiol; angiogenic retinoids; inhibitors of vascular reassembly such as GPA 1734 and metalloproteinase inhibitors; antagonists of integrin $\alpha_v\beta_3$; and other compounds such as fumagillin derivative AGM-1470, taxol, pentosan, and thalidomide. Angiogenic molecules include acidic FGF (aFGF), basic FGF (bFGF), angiogenin, angiotropin, erythropoietin, gangliosides, heparin and other simple sulfated polysaccharides, hepatocyte growth factor, insulin-like growth factor I (IGF-I), interleukin-8 (IL-8), platelet-activating factor, platelet-derived endothelial growth factor, platelet-derived growth factor-BB, proliferin, SPARC and SPARC-derived bioactive peptides, TGF-α, TGF-β, tumor necrosis factor-α, VEGF, and 2-Deoxy-D-ribose. Additionally, nitric oxide (NO), haptoglobin and other molecules have a role in angiogenesis as yet not fully characterized (54).

Physiologic angiogenesis begins with vasodilation and increased capillary permeability due to the secretion of intercellular adhesion molecules including vascular endothelial (VE)-cadherin and platelet endothelial cell adhesion molecule, regulated by NO and VEGF. These initial steps result in secretion of plasma proteins, which is in turn downregulated by angiopoietin-1. Smooth muscle cells then detach, and the surrounding perivascular matrix degrades via the action of angiopoietin-2 and metalloproteinases, allowing endothelial cells to migrate into the newly available space and increasing the secretion of endogenous growth factors such as FGF-2 and VEGF from the ECM. Endothelial cells proliferate and migrate to sites as determined by the presence of VEGF, FGFs, angiopoietin-1, and PDGFs, where they assemble into cords

and tubules, forming a lumen, and express relevant cell adhesion molecules such as VE-cadherin. The cords and tubules may remain as they are or proliferate, branch, and recruit more endothelial cells, becoming mature functional vessels (55).

1.4.4. Experimental Models for Angiogenesis

The activity of angiogenic factors is modified by proteases, such as plasminogen activators and matrix metalloproteinases (MMPs) which also modulate migratory activity, contribute to adult blood vessel formation, and control inflammation. A variety of strategies have been developed to induce neovascularization in ischemic tissue. In wound repair, where macrophages play a particularly important role, angiogenesis is stimulated by the release of growth regulatory molecules that regulate neovascular growth and tissue remodeling and repair (54). Many studies have demonstrated improved wound healing and decreased scarring upon the addition of angiogenic molecules. For example, PDGF-BB, TGF- β_1, and bFGF affect the deposition of the ECM and angiogenesis to accelerate dermal wound healing in a rabbit model. Additionally, PDGF, EGF, and bFGF increase the thickness of the granulation tissue, the vascularized, fibrous connective tissue that replaces a fibrin clot during wound healing, in guinea pig wounds. TGF- β_1 and bFGF, and PDGF and TGF-α, accelerate wound healing in diabetic rates and mice respectively. In humans, chronic pressure ulcers heal more quickly upon the exogenous administration of high doses of PDGF-BB, and duodenal ulcers (a model for non-healing chronic wounds) with the administration of FGF (54). Antiangiogenic factors such as AGM-1470 have been shown to inhibit angiogenesis and joint inflammation in a rat model of immune rheumatoid arthritis (54).

Similarly, many studies have shown that angiogenesis can be induced in ischemic myocardial tissue, although no clinical benefit has yet been shown (55). The addition of FGF to ischemic porcine and healthy canine hearts has shown that angiogenesis and collaterallization encouraged by FGF quickly evolves, resulting in the healing of

ischemic tissue and myocardial pump function (54). Intramyocardial FGF-1 has also been used to induce angiogenesis, resulting in a small local increase in collateral blush along the left anterior descending coronary artery; intracoronary administration of FGF-2 demonstrated a slight improvement in ischemic tissue and myocardial vascularization. Alternatively, the delivery of VEGF genes has been shown to result in improvements in ischemia, albeit small (54).

1.5. BIOMATERIALS

1.5.1. Biomaterials and Tissue Engineering

Tissue engineering is a relatively novel science with great potential to regenerate or replace damaged tissues and organs. Because tissue engineering combined with biomaterials can create not only customized cells but a nanostructure resembling that of extracellular matrices, there is the possibility to mimic the local environment of tissues, enhancing the survival of the transplant (57). The vast majority of tissue cells, particularly islets, are surrounded by three dimensional matrices with structure at the nanoscale, including other cells and extracellular matrices. Within the extracellular matrices there are ligands such as collagens, fibronectin, and laminin, joining cells and providing a mechanism to transport oxygen, hormones, nutrients, and wastes (57). Particularly with β cells, it is clear that the extracellular matrix is a key component of the microenvironment (58).

As an environment for pre-transplantation culture, a nanostructured biomaterial could provide a more representative milieu. Cells that are cultured in a two dimensional surface do not experience the biomechanical forces, interactions, and diffusion gradients present in tissue in organisms, and may modify their signal pathways accordingly. Additionally, these cells are bereft from the same chemical and physical signals that their 3D counterparts experience; chemical cues include the molecules that bind to the cell membrane receptors, while physical cues are the micro-

and nanoscale changes to the three dimensional extracellular matrix, including the cell's recognition of topographical changes such as ridges and grooves (57). This recognition combined with the ability to control nanoscale features has the potential to grant precision control of cell directionality and migration, as well as improving cell adhesion and proliferation (57). Additionally, in 2006 an important study showed that mesenchymal stem cells can be induced to differentiate into osteoblasts, neurons, or myoblasts depending on the mechanical properties, such as stiffness, of the synthetic matrix in which they are embedded (57).

Beyond altering the composition and mechanical properties of a biomaterial, there exists the possibility of biofunctionalizing the materials with biochemical cues to, for example, induce angiogenesis (57).

1.5.2. Biocompatibility

Biocompatibility is defined as the ability of a material to perform with an appropriate host response in a specific application. This broad definition has some inherent complexities as a material may be biocompatible for one purpose and completely inappropriate for another; for example, PTFE is excellent as a material for coating catheters because it has a low coefficient of friction and is highly hydrophobic and therefore prevents unwanted cell adhesion, however despite these properties if causes a massive inflammatory response due to polymer fragmentation when used in a metal-on-plastic hip replacement (59). Biocompatibility depends not only on the material characteristics but on the biological system in which the material will be used, and therefore is a systems property, not a material property. Following transplantation of a bioengineered device, there is an immediate host response from immune cells, target cells, and other cell populations (Figure 1.1). The immune response is inevitable, but may be modulated by growth factors, drugs, or transplanted cell populations. Cells may initially interact with the implanted material through mechanotransduction

pathways, cell adhesion, interactions with degradation byproducts of the material, or protein adsorption (59). This host response progresses over time and may have unacceptable clinical outcomes such as chronic inflammation, hyperplasia, thrombosis or calcification, or the response may be resolved through modifications to the above pathways (Figure 1.2).

Considerations on biocompatibility are important, particularly from a regulatory perspective. Materials may be labeled as non-toxic and biocompatible and therefore less stringent testing would be required to use this material in a different purpose; as seen with the PTFE example above, a material cannot have the property of biocompatibility and should never be labeled as such. In this thesis, the collagen-based matrix studied will not be described as a biocompatible material, but rather its ability to be modified to be utilized in a variable of systems will be considered.

1.5.3. Types of Biomaterials

There are a large variety of biomaterials that may be used in for tissue engineering purposes. Usually a polymer of some sort, these biomaterials emulate the extracellular matrices found in any tissue, providing sites for cell adhesion, control the tissue structure and shape, regulating cell function, and allowing diffusion of nutrients, metabolites, growth factors, and cellular products in or out of the matrix (60). It is far beyond the scope of this thesis to consider all types of biomaterials, particularly with the recent advances in nanobiomaterials, so only those with some relevance to islet transplantation or angiogenesis will be discussed below.

1.5.3.3. Hydrogels

Hydrogels are defined as chemically stable or degradable hydrophilic polymer networks with the potential to absorb from ten percent to many thousands of times of their dry weight in water (61). They are similar in structure to the macromolecular-based components found in the body (60). There are two main categories of hydrogels –

reversible or physical gels, and permanent or chemical gels. Physical gels are gels in which the network is bound by molecular entanglements and possibly secondary forces such as ionic, hydrogen bonding or hydrophobic forces. These gels are heterogeneous with clusters of molecular entanglements or hydrophobically or ionically associated domains causing defects, as well as free chain ends or chain loops. Combining a polyelectrolyte with a multivalent ion of opposite charge results in a physical hydrogel known as an ionotropic hydrogel, for example calcium alginate. When polyelectrolytes of opposite charges are mixed, depending on concentration, ionic strength, and pH of the solution, a gel may result known as a complex coacervate or polyelectrolyte complex. Physical gels may also solidify due to biospecific interactions, for example Concanavalin A with a polymeric sugar. The gelation of physical gels is not permanent, and can be reversed by changes in ionic strength, pH, temperature, stress concentrations, or the addition of solutes competitive for the affinity site on the protein (61). Additionally, the ions used for crosslinking could be exchanged with ions in aqueous solutions, changing the chemical composition and thus the mechanical properties and stability of the hydrogel (60).

Permanent or chemical gels are hydrogels with covalently crosslinked networks, either by copolymerization, crosslinking of water soluble polymers, or by conversion from hydrophobic to hydrophilic polymers plus crosslinking to form a network. When crosslinked, hydrogels reach an equilibrium swelling level in aqueous solutions dependent on the crosslink density and estimated as the molecular weight between crosslinks. Permanent hydrogels are also not homogenous, containing regions with high crosslink density and subsequent low water swelling within areas of low crosslink density and high water swelling, possibly a result of the aggregation of hydrophobic crosslinking agents. Phase separation can be another source of heterogeneity, depending on solvent composition, temperature and solids concentration during gel formation; in these areas of phase separation it is possible that water-filled voids can

form. Additionally, free chain ends and chain loops and entanglements are other network defects that do not contribute to the elasticity of the hydrogel (61). Some of the chemicals used to crosslink the chemical hydrogels are prohibitively toxic, and because the crosslinking is non-degradable some applications of tissue engineering, such as eventual replacement of the graft with natural tissue, are impossible with permanent hydrogels (60).

Interestingly, another option for crosslinking hydrogels is available because of the property of all polymers, the lower critical solution temperature. A small temperature change at this temperature results in a phase transformation from a polymer in solution to a gel. Much research has been conducted to develop a lower critical solution temperature that is close to body temperature so that the hydrogel could solidify *in situ*. It is important to note, however, that the crosslinking reaction tends to be somewhat exothermic so care must be taken to control this property (60).

For both physical and chemical hydrogels, a variety of macromolecular structures exist, including crosslinked or entangled networks; polyion-polyion, polyion-multivalent ion, or H-bonded complexes; hydrophilic networks with increased stability due to hydrophobic domains within the main structure; and interpenetrating networks or physical polymer blends (61). Hydrogels can be manufactured in a variety of physical forms, including molds such as contact lenses, pressed powder matrices such as pills or capsules, microparticles such as bioadhesive carriers, coatings such as those on implants or catheters, membranes or sheets such as patches from transdermal drug delivery, encapsulated solids as for osmotic pumps, or liquids that form gel on heating or cooling (61).

As hydrogels are so dependent on water for their physical shape and mechanical properties, the volume and location of water in a hydrogel also contributes significantly to the permeability with respect to nutrients into and cellular products out of the gel. The initial water molecules absorbed by the hydrogel, the primary bound water,

hydrates the most polar and hydrophilic groups, causing swelling and the exposure of hydrophobic groups. These newly exposed groups interact further with more water, resulting in hydrophobically-bound secondary bound water, together with the primary bound water referred to as the total bound water. Once all the polar and hydrophobic sites are occupied by bound water, the network accepts more water via the osmotic driving force of the network chains, approaching infinite dilution. The resultant swelling is resisted by the crosslinks, resulting in an elastic network retraction force and the equilibrium swelling level. This last water that is absorbed in termed free or bulk water, and fills the spaces between network chains, the centers of the larger pores, and any macropores or voids. Swelling, if the gel is degradable, results in disintegration and dissolution at a rate dependent on composition (61).

Pores may form during the synthesis of a hydrogel due to phase separation or as a result of entropy within the network. Tortuosity is defined as the interaction between average pore size, pore size distribution, and pore interactions, and importantly defines the diffusion path length across a hydrogel film barrier along with the film thickness and the pore volume fraction, factors that are influenced by the composition and crosslink density of the hydrogel polymeric network (61).

Because of their excellent biocompatibility with a wide variety of applications, combined with their ability to transport nutrients and cellular products, potential for modification for biofunctionalization, and injectability, hydrogels are an exciting option for tissue engineering. Additionally, they may be designed with pores large enough for living cells, or, similar to the hydroxyapatite matrix used in bone regeneration, could be designed to biodegrade and create pores for living cells to penetrate and proliferate, providing an *in situ* scaffold for the growth of new tissue (61). Because they are hydrophilic, hydrogels easily allow the incorporation of cell membrane receptor peptide ligands, providing stimulation for cell adhesion and the proliferation of these

cells. However, hydrogels in general have low mechanical strength and difficulties with sterilization (61).

In order for hydrogels to be utilized for tissue engineering there are many design criteria that must be met, including stability, mechanical strength, biological performance parameters such as cell adhesion, and biocompatibility. Biocompatibility is defined as the ability of a material to be placed inside the body without inducing a detrimental effect, such as inflammation, scarring, or cell damage, for a specific purpose (59). For hydrogels *in vivo*, an inflammatory response can elicit a stronger immune response to the transplanted cells, and this in turn can worsen the inflammatory response to the hydrogel in an unfortunate cycle. It is advantageous, then, to use natural polymers such as collagen for hydrogels, as synthetic polymers may cause a significant immune response from the body (60).

1.5.3.4. ECM components for tissue engineering

Collagen in particular is an excellent biomaterial as it is highly biocompatible in many applications and does not in general stimulate an immune response. It is the most plentiful protein in the human body - a component of skin, bone, cartilage, tendon, and ligament - therefore a collagen matrix would be an excellent representative for the natural extracellular matrix, and, unlike the self-assembling peptide scaffold, it can have specific interactions with cells and tissue because it is composed of recognizable and degradable sequences of amino acids (60). Additionally, it can utilize biomolecules, such as cytokines and cell adhesion molecules, in order to stimulate tissue growth (62). There is also only a small decrease in the Young's modulus, a measure of the stiffness or elasticity of an isotropic material, with the incorporation of these molecules (62). Cell adhesion can also be modified by the addition of such materials as fibronectin, chondroitin sulfate, or low levels of hyaluronic acid (60). However, there are inherent issues with using collagen for tissue engineering. Collagen is very hydrophilic and

swells upon hydration, thus becoming highly deformable and rapidly losing its mechanical strength. Purified collagen also tends to have low strength and some negative immune response, as well as being expensive (60). Collagen is also biodegradable, and this degradation can result in restoration of tissue structure and function possibly due to interactions with the cryptic RGD domains and because its degradation byproducts chemotactically attract human fibroblasts (36). It would be therefore advantageous to develop the means to adjust these properties of collagen without sacrificing its biocompatibility for the purpose of delivering islets (62).

One such method commonly used to ameliorate collagen to make it more suitable for use *in vivo* is chemical cross-linking. This is commonly done with gluteraldehyde as this chemical efficiently produces the best mechanical properties. However, gluteraldehyde is associated with some cytotoxicity and calcification, unfortunately increasing toxicity of the biomaterial (63). An alternative chemical to gluteraldehyde is the water soluble carbodiimide, carbodiimide 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide hydrochloride (EDC). EDC is potentially less cytotoxic than gluteraldehyde, as well as being more transparent; additionally, it grants the collagen a much higher Young's modulus, and perhaps improved biological interactions (64). EDC activates the carboxyl groups of aspartic acid and glutamic acid residues, which then react with primary amine groups to form stable, zero length amine bonds. The EDC crosslinking reaction is ameliorated by the presence of N-hydroxysuccinimide (NHS), a nucleophile that increases the rate and degree of crosslinking. In the crosslinking reaction, water can act as a nucleophile, which may result in the hydrolysis of the O-acylisourea group, subsequently resulting in a substituted urea and carboxylic acid group. Then, the highly reactive O-acylisourea group can rearrange to a stable N-acylurea group, an undesirable end-stage product. NHS instead converts the O-acylisourea group to an NHS-activated carboxylic acid group, which is less susceptible to hydrolysis in a rapid reaction (65) (Figure 1.1).

A third option for cross-linking the collagen includes physical methods, such as heat or dehydrothermal treatment, or ultraviolet or γ irradiation; however, these methods cause some protein denaturation and therefore induce instability (63). An alternative to these methods is photochemical cross-linking; in this process, light and a photosensitizing chemical are used to enhance the compressive strength and long-term stability without causing toxicity. Additional benefits to photochemical cross-linking include reduced platelet adhesion and fibrin mesh formation, two key steps in the formation of a thrombosis (63).

Collagen can also be electrospun to create complex, seamless three dimensional shapes. One study demonstrated that collagen I dissolved in 1,1,1,3,3,3 hexafluoro-2-propanol, electrospun, then cross-linked in glutaraldehyde vapour for twenty four hours resulted in a biomaterial with structural, material and biological properties that create a "nearly ideal" tissue engineering scaffold; electrospun collagen nanofibers promote cell growth and penetration of cells into a matrix (66).

Chitosan is another potential material for hydrogels, gelling by ionic or chemical crosslinking with gluteraldehyde. It is produced via N-deacetyleation of chitin, a glucose derivative that makes up the exoskeleton of crustaceans and insects. It is biodegradable, structurally comparable to glycosaminoglycans found naturally, and has low toxicity. Although chitosan is soluble in acids, it unfortunately is insoluble in solvents of neutral pH and organic solvents because of its high crystallinity (due to strong hydrogen bonding in the network) and the presence of amino acids. Chitosan can be used with sugar residues such as fructose or galactose, or with proteins such as gelatin, albumin and collagen (60). Additionally, chitosan has been observed to accelerate wound healing, by acting as a stimulant for the migration of polymorphonuclear leukocytes into the wound, then causing biodebridement by these cells; it also has antimicrobial properties because, during the phagocytotic activity of the polymorphonuclear leukocytes, bactericidal toxic oxygen species are produced,

sterilizing the wound (67). Cross-linking chitosan to collagen can also increase the time the matrix resists enzymatic degradation (68).

Chitosan is polycationic, and can stabilize the structure and improve the mechanical strength of collagen-based matrices by creating ionic bonds between its many amine groups and the carboxyl groups of collagen (36). Chondroitin sulfate is a heavily glycosylated proteoglycan; its core protein is decorated with long carbohydrate side chains, attached post-translationally and negatively charged under physiological conditions (69). This negative charge allows a matrix containing chondroitin sulfate to bind and sequester growth factors and cytokines (24, 69). Laminin, when incorporated into a collagen-based matrix in the presence of a proteoglycan such as chondroitin sulfate, form stabilized polymer networks bound together by GAG side chains.

1.5.4. Use for Islet Transplantation

It has been observed that at least two-thirds of islets transplanted in the clinic are dysfunctional following transplant. To ameliorate this poor performance, it has been suggested that biomaterials can provide a support system mimicking the ECM, protecting the islets from cellular stress that impairs β cell function and viability (70). Islets undertake a "perilous journey" between organ procurement from the donor to final engraftment in the recipient, with stresses induced by isolation and transplantation into a foreign environment (23). As described above, the complex peripheral ECM and BM is almost completely lost during isolation, and therefore attempts to restore the islet microenvironment through a matrix seems intuitive (24). Achieving the goal of making a biomimetic scaffold with specific surface modifications would allow specific control of islet cellular events; providing sites for cell adhesion is particularly important as cell attachment is a precursor for growth, differentiation, viability, and motility (24).

Divers techniques have been attempted to create such a scaffold, using natural ECM proteins, synthetic polymers, or combination of the two. Synthetic polymers offer

the advantages of complete control over mechanical and chemical properties and lower manufacturing costs (71, 72); however, natural polymers offer significant advantages of their own, including the ability of their degradation by-products to be metabolized (71, 72). Additionally, these natural polymers can be engineered to mimic properties of the natural extracellular matrix to support various cell types, including islets and recruited recipient cells (24). Matrices of collagen I or IV have been shown to maintain glucose responsiveness of islets in vitro (73), while islets seeded onto scaffolds with non-specifically adsorbed collagen IV reversed diabetes earlier and with superior glucose responsiveness up to 10 months post-transplantation in a murine model (74). However, scaffolds coated with fibronectin, laminin, and serum proteins did not demonstrate the same effectiveness (74). Another study demonstrated that a combination of collagen IV and laminin improves islet survival (75). Human islets cultured embedded in collagen I demonstrated cell viability up to 8 weeks compared to 14 days in normal media, as well as maintaining glucose responsiveness for longer than the controls (76). The addition of fibronectin increased cell adhesion and spreading but the impact on glucose responsiveness and insulin production varies between species (77). Porcine islets do not increase insulin production in response to fibronectin, but human islets have improved maintenance of architecture and insulin content distribution (77). The addition of soluble fibronectin to isolated islets also helps maintain islet integrin expression, as well as maintaining β cell mass and function (78).

Synthetic polymers for are also utilized to develop devices for islet transplantation, often in combination with natural ECM proteins (24). A common technique is to utilize a synthetic mesh with a collagen scaffold. A polyethylene terephthalate mesh bag containing a collagen sponge and bFGF in gelatin microspheres was transplanted intermuscularly in diabetic rats; prevascularization occurred within 10 days, then rat islets embedded in 5% agarose were added to the site (79). These devices demonstrated the ability to restore normoglycemia within 3 days, and

transplanted islets showed a normal glucose response during an intravenous glucose tolerance test (GTT) after 35 days (79). A stainless steel mesh with polytetrafluoroethylene (PTFE) stoppers coated with acidic FGF and containing rat islet isografts was also prevascularized; all rats that received an intraperitoneal device with 10 μL of acidic FGF became normoglycemic (80).

These studies and others have clearly demonstrated the potential for a bioengineered matrix to support islet viability and function in a transplantation setting. However, limitations remain. The source of the ECM or BM proteins is an important consideration, as animal sources may be immunogenic. As these proteins are purified, their bioactivity may be altered or lost due to the removal of ECM molecules or denaturing, and the native resistance to enzymatic degradation and mechanical strength are lost (24). While resistance to biodegradation and strength may be restored by processing such as chemical crosslinking, the orientation and conformation of key side chains may be transformed. There is a clear need for understanding the interactions between islets and bioengineered scaffolds if a vascularized ectopic site is to be developed.

1.6. NEONATAL PORCINE ISLETS

Porcine islet transplantation is by no means a new idea. In 1994, Groth et al. reported the transplantation of fetal porcine islet-like cell clusters (ICC) in ten insulin-dependent diabetic kidney-transplant patients (81). These ICC secreted detectable levels of porcine C-peptide in urine up to 400 days, and in one case insulin and glucagon staining was detectable in a renal-graft biopsy. In 2006, two groups published results demonstrating the survival of functional neonatal porcine islet grafts in diabetic non-human primates for longer than 3-6 months with immunosuppression (82, 83), and subsequent studies with transgenic porcine islets have shown islet survival for more than one year (84). Encapsulation of islets in an alginate matrix without

immunosuppression has also demonstrated effectiveness in non-human primates (85) and humans (86). Neonatal porcine islets are a focus as a source because they are easy to isolate and can proliferate after isolation, they are more resistant to injury than adult islets (87), and genetically engineered islet-source pigs could be utilized within a few days of birth, greatly decreasing housing costs in perhaps prohibitively expensive DPF facilities (88). Based on these pre-clinical and clinical trials, there is demonstrated scientific justification to expect a benefit from transplanting porcine islets into human diabetic subjects, particularly those with episodes of hypoglycemic unawareness who are currently eligible for allogeneic islet transplantation (89) as there is a limited supply of high quality human islets for transplantation in these patients. The therapy appears to be safe with no evidence of zoonotic infections, and because cells are being transplanted instead of whole organs there is likely to be a lesser immune response compared to whole tissues.

These pre-clinical data were considered enough evidence of benefit for the New Zealand Ministry of Health to approve a phase I/IIa clinical trial in 2009 for the transplantation of porcine islets in human subjects based on perceived safety as the source animals were DPF and housed in a biosecure barrier facility, the islets were produced in a GMP facility, and there is an accredited diagnostic laboratory for viral follow-up. The primary objectives for these clinical trials are to demonstrate safety and efficacy through an improvement in blood glucose levels. Although the New Zealand clinical trial is not yet completed, the most recent results show safety with an absence of PERV transmission, a reduction in the number of unaware hypoglycemic episodes, and an up to 30% reduction in exogenous insulin dosage (86).

One key risk for xenotransplantation is zoonosis, the cross-species transmission of a porcine disease to humans, either directly or through a third party. Therefore, microbial status of the source pigs is a major aspect of regulatory oversight (90). Sentinel animals can be tested regularly and frequently to ensure no infectious agents

are present in the cells and tissues to be transplanted (91). With careful screening and designated pathogen free (DPF) breeding and housing, all potentially zoonotic pig microorganisms should be removed, but there may also be unknown endogenous retroviruses that would not be identified until after transmission to the patient (92). The only known endogenous retrovirus is the porcine endogenous retrovirus (PERV), and studies have shown that subtypes of PERV can infect human cells (93). The AIDS pandemic has demonstrated the disastrous effects of zoonotic retroviruses, so it is extremely important that a similar situation be avoided for porcine xenotransplantation (94). There have been no reported transmission of PERVs in the past decade of porcine to human xenotransplantations, or in pre-clinical animal models (see (92) for a review), but because of the serious potential for harm to not only the patient receiving the transplantation but humanity as a whole, strategies are being developed to prevent the transmission of PERVs after xenotransplantation (91).

To further decrease the demonstrably low risk of zoonotic infection, careful handling of the source animals and tissue will be very important. In order to have DPF status, the herd must be free of a specified and comprehensive list of bacteria, fungi, protozoa, and viruses (95), and there must be meticulous documentation and standard operating procedures to maintain this status (96). If the animals are housed in a biosecure barrier facility, DPF status can be maintained and the donor organs and cells will be less likely to cause infectious complications than their allogeneic counterparts (90). Feed restrictions should also be incorporated into the animal husbandry procedures, as although prion disease is rare in pig species one suspected albeit unproven case has been documented (97). Thus, the feed should be free of mammalian material, as well as herbicides and pesticides, and the source animal should be on this certified feed for more than two generations before being considered as donors for xenotransplantation (96).

Once the donor tissues are procured from the source herd in a controlled, sterile operating room, the manufacturing of the xenotransplantation product must be carefully handled so that a therapeutic product can be made safely and reproducibly (98). Manufacturing facilities must therefore comply with established current Good Manufacturing Practices (cGMP), and only organs that comply with stringent acceptance criteria should be considered (98). The tissues must be transported to the manufacturing facility or the clinical site under conditions such that the product remains sterile, viable, and potent. A sample of the final product should be tested for sterility before transplantation and PERV expression using *in vitro* co-culture with human cells or other techniques (99). Particularly for cells that require extra handling for isolation and subsequent culturing, such as pancreatic islets, the media and solutions used must have endotoxin levels below a safe, specified value (98). A sample of the final product should be characterized and tested for viability and potency, and samples should be stored against the possibility that future analysis is necessary (98). Although not all tests may be conducted prior to transplantation if the need for the xenograft is urgent, it is still important to maintain documentation that the product is well characterized and safe according to international and national regulations.

1.7. OVERVIEW OF MY THESIS

The development of the Edmonton protocol revolutionized the transplantation of islets, and elevated islet transplantation into a viable permanent cure. However, many challenges must still be addressed before this cure is a reality. One such challenge is the shortage of donor organs. To address this issue, we believe that neonatal porcine islets (NPIs) are a clinically applicable source of cells. In order for NPIs to translate to the clinic, we further believe two important issues must be addressed. The first aim of this thesis is to develop an alternate site must be developed with the potential to be retrieved should it prove necessary. The second aim of this thesis focuses on developing

a clinically applicable protocol for the isolation, culture, and transport of NPIs. For the first aim, the use of biomaterials, combined with cell and protein based factors for angiogenesis, offers an extremely exciting means to solve many of the associated problems with islet transplantation, including but not limited to the toxicity of the transplant site, damage to the cells upon transplant, immune response, and poor function of the graft. Based on the understanding of the interactions between islets and ECM proteins, a collagen-based matrix is developed that supports NPI viability and function both *in vitro* and *in vivo* in Chapter 2. Specifically, this matrix contains polymers that encourage vascularization as well as being biomimetic for the natural islet ECM. This study shows that such a matrix has the capacity to deliver islets to a subcutaneous site, as well as to become increasingly vascularized over time in a murine model.

In Chapter 3, this collagen-based matrix is further developed, with a focus on determining the effect of altering the crosslinking concentration on the matrix microstructure and other material properties, including *in vivo* vascularization after subcutaneous transplantation. We demonstrate that higher crosslinking concentrations modify the nanostructure of the matrices and modulate angiogenesis in a murine model while having no negative effects on NPI survival, indicating that this matrix could be modified to be applicable in many cell delivery systems.

For the second aim of this thesis, in Chapter 4 we explore a clinically applicable and scalable modified protocol for the isolation and culture of NPIs. We demonstrate that utilizing an automated chopper, a general caspase inhibitor, and a protease inhibitor cocktail during culture in one petri dish instead of four resulted in islets with improved insulin content, β cell mass, and glucose responsiveness both *in vitro* and *in vivo* in a murine model. This modified protocol allows for the isolation of islets from minimally 12 pancreases by employing automated tissue chopping, collagenase digestion in a single vessel and tissue culture and media changes in 75% fewer petri

dishes. The positive results of this clinically applicable may be further improved by increasing compliance with cGMP by replacing all reagents with those with GMP certification.

As it is unlikely that one center would have access to both a cGMP and a DPF source animal housing facility, in chapter 5 we develop a protocol to store whole neonatal porcine pancreases for 24 hours at 4°C in UW solution. This protocol would allow transport of organs from the DPF facility where the pancreatecomy would occur to the cGMP facility for isolation. We show that this is feasible, with no differences between islets isolated from cold stored pancreases and those isolated from pancreases immediately post-surgery.

Finally, in Chapter 6 we explore some of the ethical and regulatory issues that must be considered to justify xenotransplantation clinical trials in Canada. We discuss the 2001 Health Canada citizen's jury consultation with Canadians as our knowledge of the risk and efficacy of xenotransplantation with respect to porcine islet transplantation has advanced in the past 14 years. We propose that NPIs have sufficient demonstrated efficacy to justify clinical trials with sufficient informed consent; the data gathered from these clinical trials would then be extremely valuable for Canadian citizens to decide in a deliberative democratic model whether clinical trials for other cell or tissue xenotransplantation can also be justified. We conclude that there is a clear expected benefit for xenotransplantation as it is a potentially unlimited source of donor tissue, and therefore we should move forward with biotechnologies that have been previously demonstrated to be safe and efficacious.

Collectively, these studies demonstrate that NPIs have the potential to be utilized as a clinical cell therapy for type 1 diabetes, isolated and cultured following a clinically applicable protocol and delivered in a collagen-based matrix that can be further modified as required. This matrix could also be readily retrieved if necessary. Furthermore, sharing of expertise and expenses across centers is feasible with a

successful cold storage protocol, further improving the translational relevance of NPIs. In summary, this thesis advances the applicability of the NPI cell therapy as a treatment for type 1 diabetes.

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1.9. FIGURES

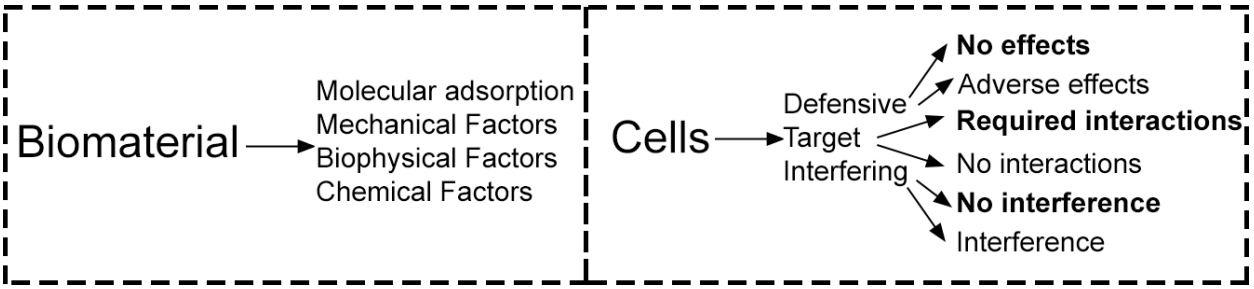

Figure 1.1. A summary of the biocompatibility pathways involving interactions between biomaterial and host cells, resulting in both acceptable and unacceptable clinical outcomes. Adapted from (59).

Figure 1.2. A generic biocompatibility paradigm, showing the progressive host response from initial contact to clinically acceptable resolution or an unacceptable clinical outcome. Adapted from (59).

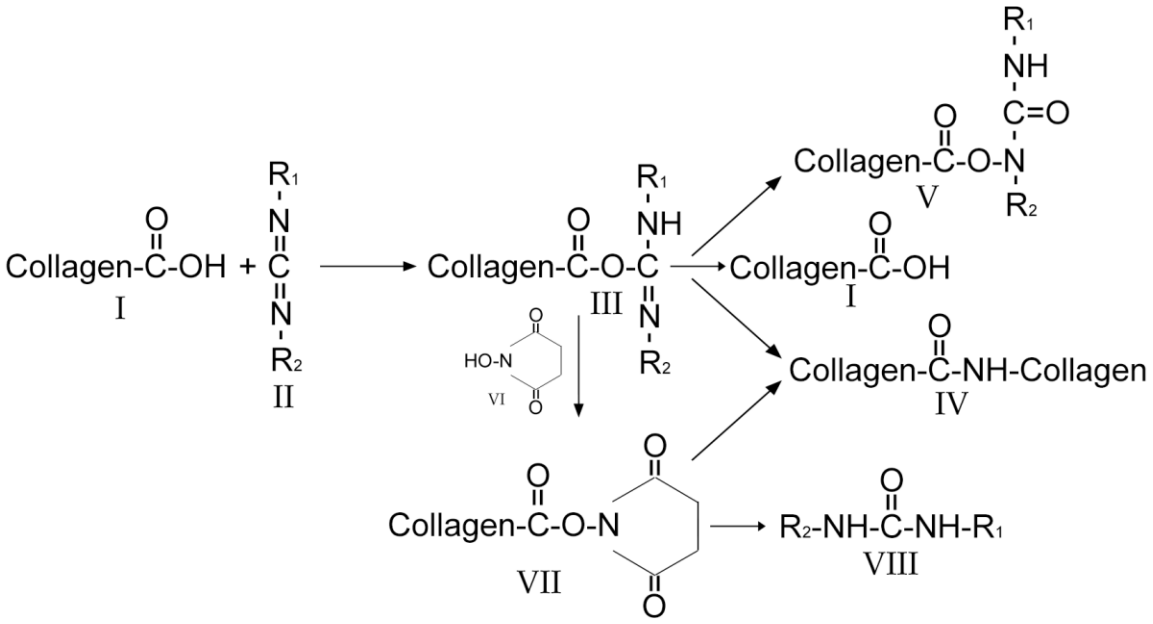

Figure 1.3. The chemical reactions of crosslinking collagen with EDC and NHS.
I represents collagen with carboxylic acid groups of glutamic or aspartic acid residues; II EDC; III a highly reactive O-acylisourea group; IV crosslinked collagen, the desired product of the reaction; V an undesirable stable N-acylurea group; VI NHS; VII the NHS-activated carboxylic acid group; and VIII an EDC-related water soluble end product, 1-ethyl-3-(3-dimethyl aminopropyl) urea. Adapted from (65).

Chapter 2: Bioengineering a highly vascularized matrix for the ectopic transplantation of islets

2.1. ABSTRACT

Islet transplantation is a promising treatment for Type 1 diabetes; however limitations of the intra-portal site and poor revascularization of islets must be overcome. We hypothesize that engineering a highly vascularized collagen-based construct will allow islet graft survival and function in alternative sites. In this study, we developed such a collagen-based biomaterial. Neonatal porcine islets (NPIs) were embedded in collagen matrices crosslinked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide containing combinations of chondroitin-6-sulfate, chitosan, and laminin, and compared to controls cultured in standard media. Islets were examined for insulin secretory activity after 24 hours and 4 days and for apoptotic cell death and matrix integrity after 7 days *in vitro*. These same NPI/collagen constructs were transplanted subcutaneously in immunoincompetent B6.Rag$^{-/-}$ mice and then assessed for islet survival and vascularization. At all time points assessed during *in vitro* culture there were no significant differences in insulin secretory activity between control islets and those embedded in the collagen constructs, indicating that the collagen matrix had no adverse effect on islet function. Less cell death was observed in the matrix with all co-polymers compared to the other matrices tested. Immunohistochemical analysis of the grafts post-transplant confirmed the presence of intact insulin-positive islets; grafts were also shown to be vascularized by von Willebrand factor staining. This study demonstrates that a collagen, chondroitin-6-sulfate, chitosan, and laminin matrix supports islet function *in vitro* and moreover allows islet survival and vascularization post-transplantation; therefore, this bio-engineered vascularized construct is capable of supporting islet survival.

2.2 INTRODUCTION

Since 2000, when the "Edmonton Protocol" achieved insulin independence in seven patients by using islets from multiple donors and steroid-free anti-rejection therapy, islet transplantation has been seen as an increasingly promising treatment for patients with type 1 diabetes (1-4). Despite remarkable progress in clinical islet transplantation, the liver implantation site remains far from ideal (5-9). Although alternative sites have been suggested, including subcutaneous and intramuscular spaces, poor blood supply in these sites results in delayed graft revascularization and thereby loss of islet cell mass and function (6). Our group has previously used collagen-based biomaterial matrices to facilitate cell delivery resulting in revascularization of ischemic tissue (10). Such a collagen-based matrix could potentially be used to deliver islets to an alternate site, then support islet survival and function through rapid neovascularization.

Collagen is a natural extracellular matrix protein that has been widely examined as a potential biomaterial for vascularization and islet delivery. Collagen and other polymers are commonly considered because they can be engineered to have similar properties to the extracellular matrix, allowing vascularization and cell support. Various approaches have been explored, including using a polyethylene terephthalate mesh bag around a collagen sponge to deliver Sprague-Dawley rat islets intramuscularly in Lewis rats (11); a polytetrafluoroethylene "solid support" in collagen delivering isogenic rat islets in a peritoneal site (12); and polyurethane foam filled with heparin and collagen delivering syngeneic rat islets in the subcutaneous space (13). These devices utilize a combination of natural and synthetic polymers, which is advantageous because of the ease of manufacturing and the control of mechanical properties of the synthetic polymers. Polymers are widely used as biomaterials for cell delivery and inducing vascularization because of their potential to mimic the natural extracellular matrix.

However, natural polymers, compared to synthetic polymers, are generally less immunogenic as whole polymers and have non-toxic degradation by-products. The ideal device for islet delivery should then be based on natural polymers such as collagen but also have the mechanical properties of synthetic polymers.

The poor mechanical properties resulting from the purification process of collagen necessitate modifications in order to restore mechanical strength. Two common methods to achieve this goal are chemical crosslinking and the addition of copolymers. The crosslinker 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) combined with N-hydroxysuccinimide (NHS) has been used to achieve this with minimal negative impact on cell viability (10, 14, 15). The addition of copolymers such as chitosan, chondroitin and laminin to the matrix can also lead to improved mechanical strength and local angiogenesis (15, 16). Chitosan, prepared from the deacetylation of chitin, is a natural polymer that has been shown to accelerate wound healing and vascularization (16). Crosslinking chitosan to collagen may improve the resistance of the collagen-based matrix to enzymatic degradation. Chitosan also possesses unique tissue-adhesive and antimicrobial properties that could improve the properties of the matrix (16). Collagen-chitosan constructs are successful cell delivery vehicles that promote neovascularization (16). Chondroitin-6-sulfate is a sulfated glycosaminoglycan that interacts with functional proteins to bind growth factors into the biomaterial (15). Chondroitin additionally ameliorates the matrix structure by regulating matrix elasticity, allowing collagen-chondroitin-6-sulfate gels to be utilized as potential islet delivery devices (16-18). Laminin, an important basement membrane protein, may aid in the retention of transplanted islets *in vivo* in addition to acting as a guidance molecule for vascularization and islet survival (19, 20).

The objective of this study was to examine the effect of various collagen-based matrices on the *in vitro* function of NPIs and their survival following transplantation in the subcutaneous space of mice. Neonatal porcine islets (NPIs) were used because they

are a potential clinical source of islets for treating patients with type 1 diabetes (21, 22).

2.3. MATERIALS AND METHODS

2.3.1. Preparation of neonatal porcine islets

Porcine pancreases were obtained from 1 to 3 day old Duroc neonatal piglets from the University of Alberta Swine Research Centre (1.5-2.0 kg body weight), and the islets were isolated and cultured for 5-7 days as described previously.[22] Briefly, the retrieved pancreases were cut into 1 to 3 mm tissue fragments, then exposed to 2.5 mg/mL collagenase (type XI; Sigma, C7657), filtered through a 500 μm nylon screen, washed in Hank's Basic Salt Solution (HBSS) (Gibco, H6136) supplemented with 0.25% BSA (fraction V; Sigma-Aldrich, A9543), 10 mM HEPES (Sigma-Aldrich, H4034), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Lonza Walkersville, Inc., 09-757F). NPI were then cultured in non-tissue culture treated petri dishes containing Ham's F10 tissue culture media purchased from Sigma-Adrich (N6635), and supplemented with 14.3 mM sodium bicarbonate (Fisher, S233), 10 mM D-glucose (EM Science, DX0145-3), 2 mM L-glutamine (Sigma-Aldrich, G8540), 0.25% BSA (fraction V; Sigma-Aldrich, A9543), 50 μM isobutylmethylxanthine (IBMX) (Sigma-Aldrich, I5879), 10 mM nicotinamide (Sigma-Aldrich, N0636), 1.6 mM calcium chloride dihydrate (Sigma-Aldrich, C7902), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Lonza Walkersville, Inc., 09-757F). The islets were cultured at 37°C for 5-7 days, with the medium changed the first, third and fifth days after isolation.

2.3.2. Preparation of collagen matrices

Neonatal porcine islets (NPIs) were embedded in collagen-based matrices utilizing rat tail collagen I (BD Biosciences, Inc., 354249) crosslinked with 30 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Sigma-Aldrich, E6383) and 30 mM N-hydroxysuccinimide (NHS, Sigma-Aldrich, 56480) containing combinations of

chondroitin-6-sulfate (Wako Pure Chemical Industries, 034-14612), chitosan (Sigma-Aldrich, C3646), and mouse laminin (BD Biosciences, Inc., 354232), as outlined in Table 1. In Table 1, the acronym C refers to gels with collagen, CC collagen with 5% v/v chitosan, CCC collagen with 5% v/v chitosan and 40% w/v chondroitin, and CCCL collagen, chitosan, chondroitin and 20 μg/mL laminin. Following embedding in the various collagen matrices, islets were cultured overnight in supplemented Ham's F10. Controls included non-embedded islets cultured in supplemented Ham's F10 (NPIs).

2.3.3. In vitro assessment of islets and matrices

A static incubation assay[22] was used to assess glucose stimulated insulin secretion of collagen embedded and control NPIs following 1 and 4 days culture. Each condition was incubated in duplicate at 37°C for 2 hours in 1.5 mL RPMI (Roswell Park Memorial Institute medium) supplemented with 2.0 mM L-glutamine, 0.5% w/v BSA and either 2.8 mM (low) or 20.0 mM (high) glucose. The media was then separately collected and assayed for respective insulin contents by a rat insulin immunoassay that quantitatively detects porcine insulin (Meso Scale Discovery, K152BZC). Stimulation indices (SI) were calculated by dividing the amount of insulin released at 20.0 mM glucose by that released at 2.8 mM glucose. The stimulation indices were subsequently normalized to the non-embedded control values and expressed as a percentage of the control SI.

Matrices containing NPIs were cultured for 7 days in Ham's F10 culture media supplemented as above, then dark field images were taken to identify changes in mechanical integrity. The matrices were then collected and fixed in Shandon Zinc Formal-Fixx (Thermo Fisher Scientific, 6764255) if possible; if the matrices did not have sufficient integrity to be collected on their own, the liquid matrix and NPIs were collected, fixed for 24 hours in 1% formalin in PBS, and then embedded in agarose plugs. Apoptosis of the NPIs in the matrices was assessed using a commercial TUNEL assay kit (Invitrogen Molecular Probes A23210). After rehydration, antigen retrieval was

performed in sodium citrate buffer (pH 6.0), then slides were incubated according to the kit instructions. The slides were mounted with ProLong Gold Antifade Reagent with DAPI (Invitrogen Molecular Probes P36935). To quantify the percentage of TUNEL positive cells, five images of TUNEL and DAPI staining were taken then the images were combined in ImageJ (National Institutes of Health, Bethesda, Maryland, USA). Separate images of the TUNEL and DAPI positive channels were altered to black and white, then the number of particles counted. Each particle was confirmed to be TUNEL or DAPI positive visually and the brightness of the colour threshold adjusted to only include positive cells. This process was repeated by two independent viewers.

2.3.4. In vivo assessment of matrices and islets with matrices

Based on the *in vitro* data only the CCCL matrices were assessed in subsequent *in vivo* experiments. First, in order to examine whether CCCL matrices alone can be vascularized *in vivo* CCCL matrices were prepared without islets and transplanted subcutaneously in naïve BALB/c mice. Weekly up to 28 days these grafts were retrieved and examined for evidence of vascularization. Secondly, C, CC, and CCCL embedded NPI were cultured for 24 hours with supplemented Ham's F10 culture media to ensure no excess crosslinker remained in the matrices that could cause a negative reaction upon transplantation. These matrices were then transplanted subcutaneously in naïve immunoincompetent B6.Rag$^{-/-}$ mice. Pre-transplantation, at 4 days, and then weekly up to 28 days these grafts were retrieved and assessed for gross morphology, evidence of vascularization and islet survival. For histological assessment, retrieved collagen-based matrices were fixed in Shandon Zinc Formal-Fixx (Thermo Fisher Scientific, 6764255) then embedded in paraffin and 5 μm sections were prepared. To assess vascularization, von Willebrand Factor (vWF) staining was utilized to visualize arterioles and capillaries and islet survival, and sections of the grafts were also stained for insulin, hemotoxylin and eosin. After rehydration, antigen retrieval for vWF was performed in sodium citrate

buffer (pH 6.0). All immunohistochemical samples were blocked with 20% normal goat serum for 20 minutes (NGS, Jackson ImmunoResearch Laboratories Inc., 0005-000-121). Slides were also stained with a guinea pig anti-insulin antibody (Dako, A564) diluted at 1:1000; a mouse anti-glucagon antibody (Sigma G2654) or a rabbit anti-vWF antibody (abcam, ab6994) diluted at 1:500. Slides were visualized with an Axioscope II microscope equipped with an AxioCam MRC and analyzed with Axiovision 4.6 software (Carl Zeiss, Gottingen, Germany). To quantify the degree of vascularization five images of vWF staining were taken from each of two retrieved matrices and the images were combined for particle analysis in ImageJ (National Institutes of Health, Bethesda, Maryland, USA). Particles were counted if they were between 50 and 10000 μm^2 in size, with a circularity greater than 0.2. Each particle was confirmed to be a capillary visually and excluded by hand if mistakenly included by the software.

2.3.5. Statistical Analysis

All statistics were performed using the Kruskal-Wallis one-way analysis of variance with the Šidák correction; the level of significance was considered to be α=0.05. All statistical analyses were performed with STATA 11 (StataCorp LP, College Station, TX). Results are presented as mean±SEM.

2.4. RESULTS

2.4.1. In vitro assessment of islet:collagen matrices

Prior to conducting the transplant studies we assessed the glucose stimulated insulin secretion of collagen embedded NPIs in order to examine if the matrices had an adverse effect. The insulin secretory capacities of NPIs embedded in the different collagen-based matrices and cultured for 1 and 4 days were tested by comparing the amount of cellular insulin that was released at low glucose (2.8 mM) and high glucose (20.0 mM). No statistically significant differences were noted in the amounts of insulin

secreted at low or high glucose when comparing all collagen conditions with control NPIs. Similarly, when the stimulation indices were calculated no significant differences were observed in any conditions at either one day or four days post culture (Figure 2.1). The stimulation indices shown in Figure 2.1 are typical of immature NPIs.

Differences in the mechanical properties were noted between the various matrices. When NPIs were added to matrices containing collagen alone (C) or collagen and chitosan (CC), the matrices did not crosslink completely. Culture media added on top of these C and CC matrices accelerated the degradation of the matrices after 7 days, leaving small pieces of matrices and loose NPIs (Figure 2.2A and B). The CCC matrices did crosslink with the addition of NPIs and appeared similar to the CCCL matrices after 7 days in culture (Figure 2.2C and D).

To determine the effect of the matrices on islet survival, matrices containing NPIs were also cultured for 7 days then stained for insulin and using a TUNEL assay for apoptosis (Figure 2.3). Since the C and CC matrices did not adequately crosslink, we embedded the NPIs with the remaining separated pieces of the matrix in agarose. While all matrices showed intact islets, the CCCL matrix (Figure 2.3D) had significantly fewer apoptotic cells with 4.3±0.2% of the DAPI positive cells staining for TUNEL compared to the C matrix at 9.9±0.4 % ($p<0.05$), the CC matrix at 7.3±0.2% ($p<0.05$), and the CCC matrix at 7.2±0.2% ($p<0.05$) (Figure2.3A, 2.3B, and 2.4C, respectively). Some of the cells stained positively for TUNEL but not for DAPI, likely indicating apoptotic cells with extremely fragmented DNA (23).

2.4.2. In vivo assessment of islet:collagen matrices

Based on the above data, the C and CC matrices were determined to be inferior and were no longer considered as potential biomaterials for islet transplantation. The CCCL matrix exhibited no adverse effects on mechanical stability, islet survival, or islet function. Chondroitin-6-sulfate binds functional proteins (16), chitosan improves

vascularization, and laminin enhances vascularization (19) and islet survival (20); therefore, the following *in vivo* transplant studies were conducted using the CCCL matrix. In a first series of experiments we implanted the CCCL matrices without NPIs subcutaneously into BALB/c mice in order to examine their potential to become vascularized. Upon retrieval, the matrices were adhered to the hypodermis and maintained their size and shape over the 28 day period. A thin, transparent membrane formed around the transplanted matrix by four days after transplantation. Retrieved matrices were fixed and paraffin embedded, and sections were stained for von Willebrand factor (vWF) to evaluate the potential for vascularization of the matrix. At 7 days, vWF positive endothelial cells began to penetrate the periphery of the matrix (Figure 2.4A). Two weeks post-transplantation, small capillaries with a vWF positive lining were visible, migrating towards the interior of the matrix (Figure 2.4B). After quantification of capillary size, these early capillaries were found to have a mean size of 53.54±0.75 μm^2. At 3 weeks, the capillaries increased in size to a mean area of 75.24±2.32 μm^2 as well as in number, and some appeared to be combining into larger structures (Figure 2.4C). After 4 weeks, the capillaries were widespread throughout the matrix (Figure 2.4D) and had grown to a significantly larger mean size of 221.68±5.63 μm^2 ($p<0.05$). These data clearly demonstrate that the CCCL matrix can become vascularized following subcutaneous implantation.

After confirming the ability of the CCCL matrix to become vascularized, C, CC, and CCCL matrices containing NPIs were transplanted subcutaneously in naïve immunoincompetent B6.Rag$^{-/-}$ mice and retrieved after 4 (CCCL only), 7, 14, 21, and 28 days and examined for gross morphology (Figure 2.5). The CCCL matrices were transplanted using a microspatula, but because the C and CC matrices did not crosslink sufficiently to be manipulated, they were injected using a large orifice pipette. Prior to implantation, NPIs were distributed throughout the CCCL matrix (Figure 2.5A). Following transplantation, blood vessels were observed in the periphery of the matrices

as early as four days (Figure 6B) as well as at 7 and 28 days (Figures 2.5C and 2.5D). However, following implantation NPIs were only observed at 4 days since the matrixes become overgrown with connective tissue making it difficult to detect the NPIs within the matrix. At 7 days and later, no C or CC matrices were found; it is likely that these matrices degraded quickly due to their lack of mechanical integrity at the time of transplantation.

We therefore conducted another series of CCCL:NPI transplants in non-diabetic B6.Rag$^{-/-}$ mice and collected the grafts for histological assessment. It should be noted that since NPIs are not fully developed islets the number of insulin and glucagon positive cells are lower than that observed in adult islets. When examined for the presence of insulin and glucagon positive cells, the proportion of insulin and glucagon positive cells tended to increase at 4 (Figure 2.6C and D) and 7 (Figure 2.6E and F) days post-transplantation, as compared to those prior to transplantation.

2.5. DISCUSSION

This study demonstrates that our collagen-based matrix has the potential to create a site for islet transplantation. The insulin secretory capacity of NPIs seeded into the CCCL matrices was not adversely affected and when transplanted subcutaneously the NPIs were shown to survive as the matrix became more vascularized. When compared to controls cultured in Ham's F10, insulin secretion of NPIs in all tested collagen matrices was comparable; the stimulation indices of the collagen embedded NPIs tended to be higher than controls most notably in CCCL, however this was not significantly different. Nonetheless these data demonstrate that the matrices did not have an adverse effect on NPI viability as indicated by comparable stimulation indices. The transplanted CCCL matrices were also shown to support NPI survival *in vivo* for up to 28 days as evidenced by the positive insulin and glucagon staining at each time point. There were no signs of inflammation or adverse reaction to the matrices, and the matrix

maintained its size and shape over the 28 day period indicating a slow biodegradation rate. Islet survival over a longer term must still be investigated further.

A critical feature of using a collagen-based matrix for islet transplantation is being able to maintain mechanical stability. Mechanical stability is essential for physical manipulation of the matrix in order to successfully place it into the transplant site. Although this collagen based matrix is similar to that described previously by Suuronen et al. (19, 24) to recruit and deliver endothelial progenitor cells, our matrix required alteration to support NPIs. In this study we observed that collagen alone and collagen with chitosan matrices degraded quickly during culture upon the addition of NPIs before crosslinking and thereby lost their mechanical stability. Therefore, we had to improve the integrity of the CCCL matrix and its ability to crosslink upon the addition of NPIs. Differences from the previously described matrix (19, 24) include using pure rat tail collagen instead of porcine collagen; using a higher concentration of chondroitin-6-sulfate; using 20 μg/mL of laminin instead of 40 μg/mL; and using 10x DMEM and 10x HEPES as a "collagen buffer" instead of 2-(N-morpholino) ethanesulfonic acid buffer to dissolve the crosslinking chemicals.

When the co-polymer chondroitin-6-sulphate was added these matrices had improved mechanical stability, thus allowing for ease of implantation into the subcutaneous space. Additionally, the CCCL matrix better supported islet viability, as shown by fewer apoptotic cells compared to the C, CC, and CCC matrices. Because chitosan, chondroitin-6-sulfate, and laminin have beneficial properties such as binding functional proteins (chondroitin-6-sulfate) and enhancement of vascularization and islet survival (laminin), we decided to use the CCCL matrix for the subsequent *in vivo* transplant studies. To confirm this result, C and CC matrices were transplanted subcutaneously; as early as seven days, no C matrices were found in any of the mice and only one very small CC matrix was found with no visible islets. This indicates that the C and CC matrices, in addition to being much more difficult to transplant,

biodegrade more quickly than the CCCL matrix and are therefore not suitable as biomaterials for NPI transplantation.

Our data demonstrate that at 4 days post-transplant vWF positive endothelial cells begin to penetrate the periphery of the CCCL matrix and as time progresses capillaries begin to form then subsequently increase in size and number. These data clearly demonstrate that the CCCL matrices can become highly vascularized, allowing for NPI survival. Although it is unknown if these capillaries are sufficient to support longer-term islet survival, other components may be added to the matrix such as vascular endothelial growth factor (VEGF) to further promote the growth of larger blood vessels. VEGF has previously been shown to increase vascularization in collagen-based matrices, and it is known to bind to chondroitin-6-sulfate (13). Stromal cell-derived factor-1 (SDF-1) is an alternative growth factor that has also been shown to improve recruitment of angiogenic cells in a collagen-based matrix *in vivo* (25).

Another potential benefit of a CCCL matrix is that it could theoretically allow for the co-localization of ancillary cells such as mesenchymal stem cells (MSCs) with the islets. MSCs have been shown to promote vascularization in various models, including in a collagen-glycosaminoglycan scaffold wherein the mesenchymal stem cells were observed to adopt an endothelial phenotype, thereby enhancing vascularization (26). Moreover, MSCs have been also shown to have beneficial effects when co-transplanted with islets, such as promoting graft revascularization, prevention of islet allograft rejection and protection of human islets from pro-inflammatory cytokines (27-30). Because the kidney is not an applicable site for clinical islet transplantation and the liver will not ensure close association of the co-grafted cells, an alternative site must be developed. CCCL matrixes may be the solution because they would greatly improve the likelihood that the cells are co-localized.

Although islets can be supported in a collagen-based matrix as it becomes vascularized over time, it would be ideal to transplant the islets into an environment

with an already established vascular system. Two ways to create this environment would be to transplant islets into a prevascularized collagen-based matrix, or to increase vascularization in the surrounding site so that there are more vessels available to grow into the matrix. Both these approaches have successfully been used in other bioengineered devices, with both natural and synthetic polymers, with prevascularization periods ranging from 14 days to 60 days (11, 12, 26, 31-33). Both approaches have also been combined with growth factors such as fibroblast growth factor (FGF) (11, 12, 31) and vascular endothelial growth factor (VEGF) (26). Prevascularizing the matrix could also be achieved by transplanting a stem cell population to promote angiogenesis. One or a combination of these approaches will be investigated further in order to optimize the conditions for islet survival and function.

Although the subcutaneous space may not be a practical site for clinical islet transplantation, it was used in this study to examine the feasibility of using the collagen matrix. A more practical site would be the omental pouch; however, the size of the collagen matrices did not permit us to use this site in mice. The ideal site for clinical islet transplantation should be optimal for islet survival and function. It should allow for portal drainage of secreted hormones without direct exposure to high concentrations of immunosuppressive drugs, host platelets and complement; accommodate a large tissue volume; be easily accessible; and allow for retrieval of the grafted tissue. The omental pouch satisfies all of these criteria and has been used to successfully cure diabetes in both rats and dogs.35-38 This site will be investigated in the future in a larger rodent model, such as the rat.

We have shown that embedding NPIs in a collagen-based matrix containing the co-polymers chitosan, chondroitin-6-sulfate and laminin does not adversely affect NPI function *in vitro;* when these matrices are implanted subcutaneously in mice they induced significant vascularization and the supported the survival of the NPIs. This suggests that the CCCL matrix maybe used to create a site for islet transplantation,

which could include several benefits including prevention of tissue loss and cellular co-transplantation. Further study in diabetic recipients is warranted.

2.6. ACKNOWLEDGMENTS

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2.8. TABLES

Table 2.1: Composition of matrices tested on neonatal porcine islets.

	Matrix Composition			
Material	**C**	**CC**	**CCC**	**CCCL**
Collagen (mg/mL)	6.1	6.0	5.3	5.2
Chitosan (mg/mL)	-	0.2	0.2	0.2
Chondroitin(mg/mL)	-	-	1.1	1.0
Laminin (mg/mL)	-	-	-	0.1
EDC (mM)	31	30	26	26
NHS (mM)	35	34	30	30

A summary of the composition of matrix types tested and their respective polymer concentration. C, collagen; CC, collagen and chitosan; CCC, collagen, chitosan, and chondroitin, and CCCL, collagen, chitosan, chondroitin, and laminin.

2.9. FIGURES

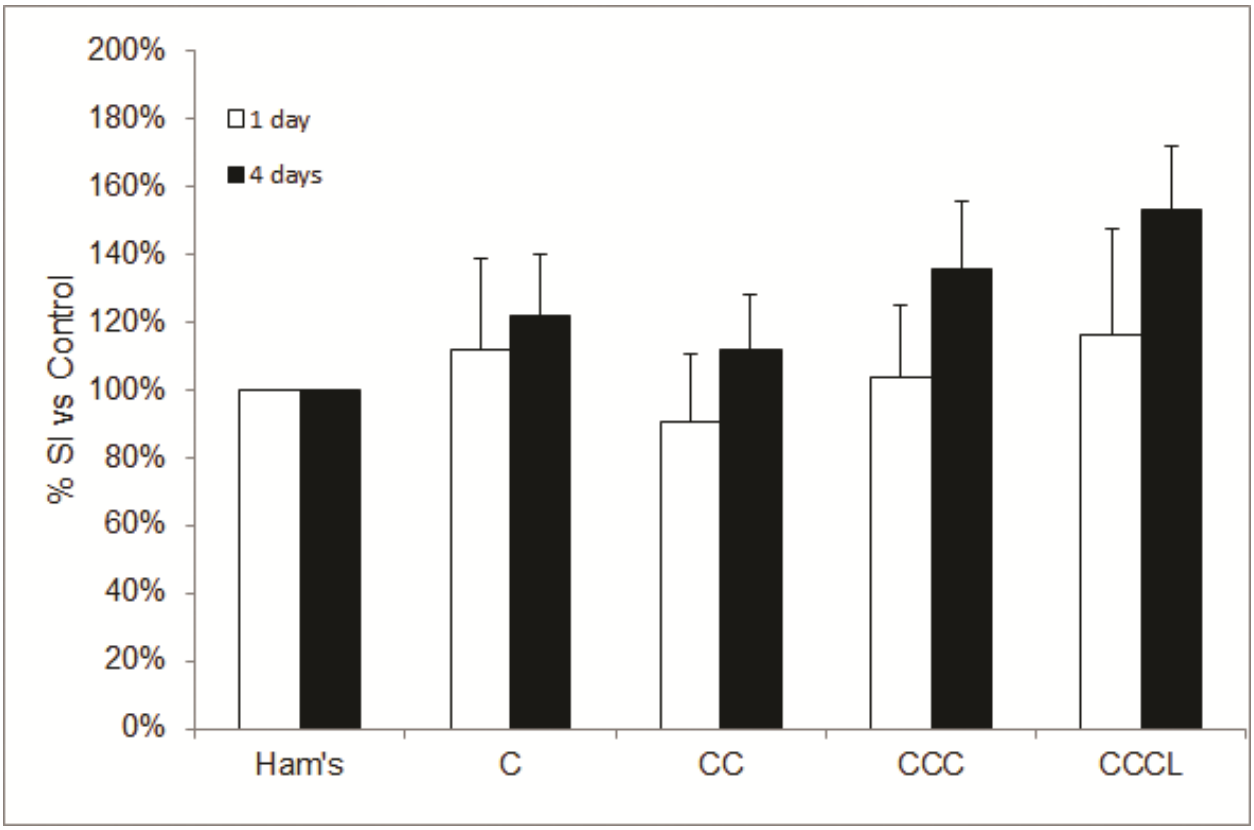

Figure 2.1. Glucose stimulated insulin secretions of control and collagen embedded NPIs were measured following 1 and 4 days in culture.

The stimulation indices (SI) were determined by dividing the amount of insulin released at high glucose (20.0 mM) by that at low glucose (2.8 mM). The stimulation indices were subsequently normalized to the non-embedded control values. The stimulation indices for the control Ham's F10 condition after 1 and 4 days in culture were 1.42±0.48 and 1.05±0.18, respectively. Data are expressed as mean±SEM (n=6 per time point).

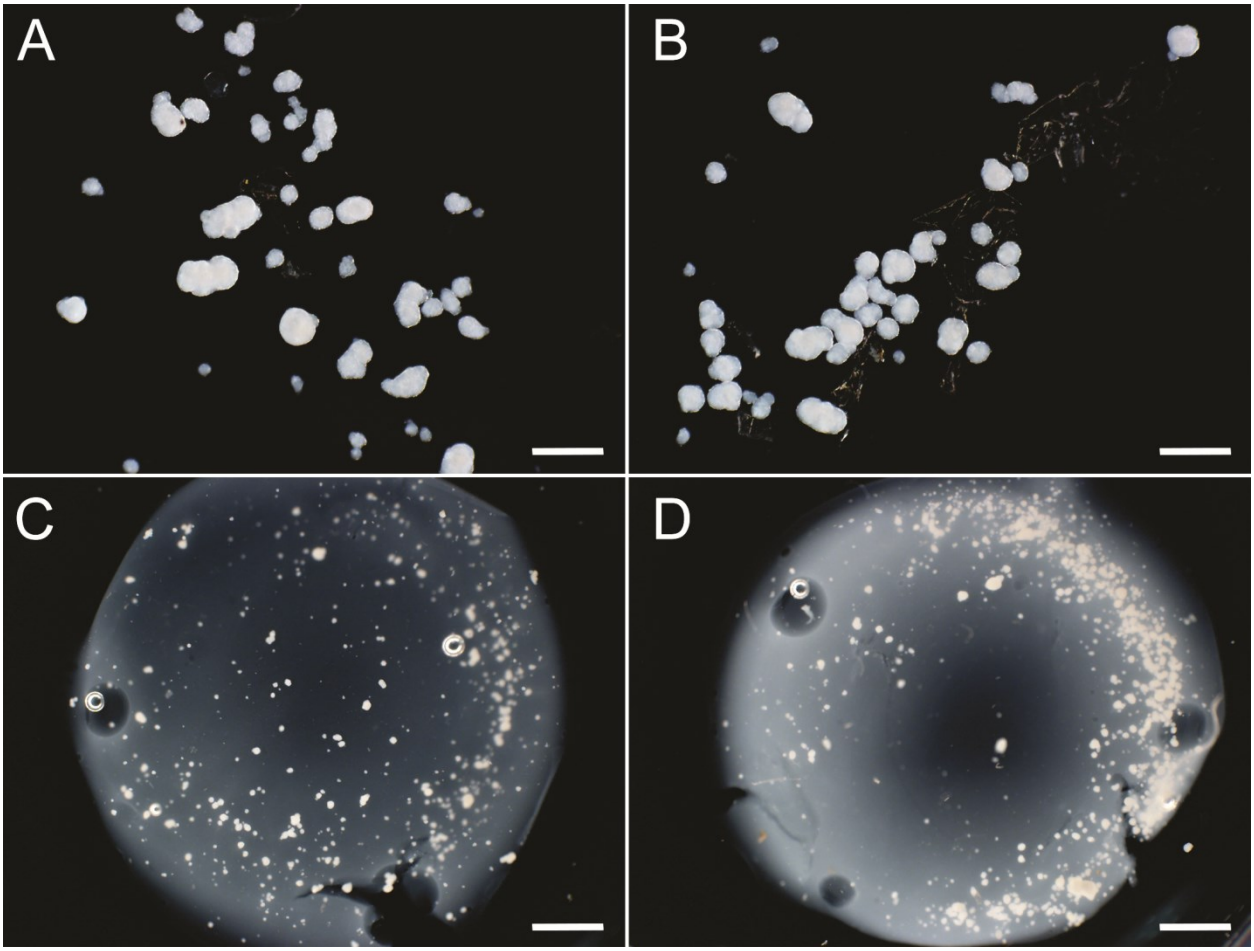

Figure 2.2. Effect of copolymers on matrix integrity with NPIs after 7 days in culture. NPIs embedded in C (A), CC (B), CCC (C) and CCCL (D) matrices were cultured for 7 days then dark field images were taken of the matrices in a 6 well plate. Scale bars are 400 μm for A and B and 1.6 mm for C and D.

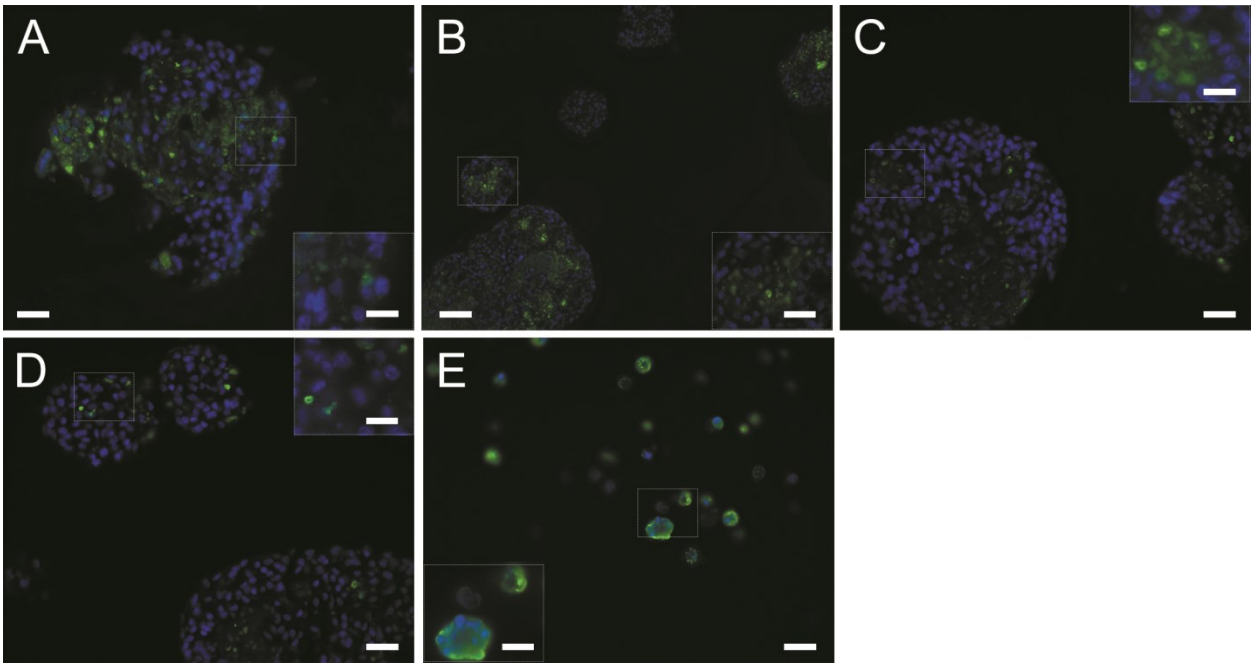

Figure 2.3. Effect of addition of copolymers on apoptosis of NPIs after 7 days in culture. NPIs embedded in C (A), CC (B), CCC (C) and CCCL (D) matrices following 7 days in culture were fixed and paraffin-embedded, then sections were stained for TUNEL and compared to TUNEL positive controls (E). Scale bars of A-E are 20 μm and scale bars of insets are 10 μm.

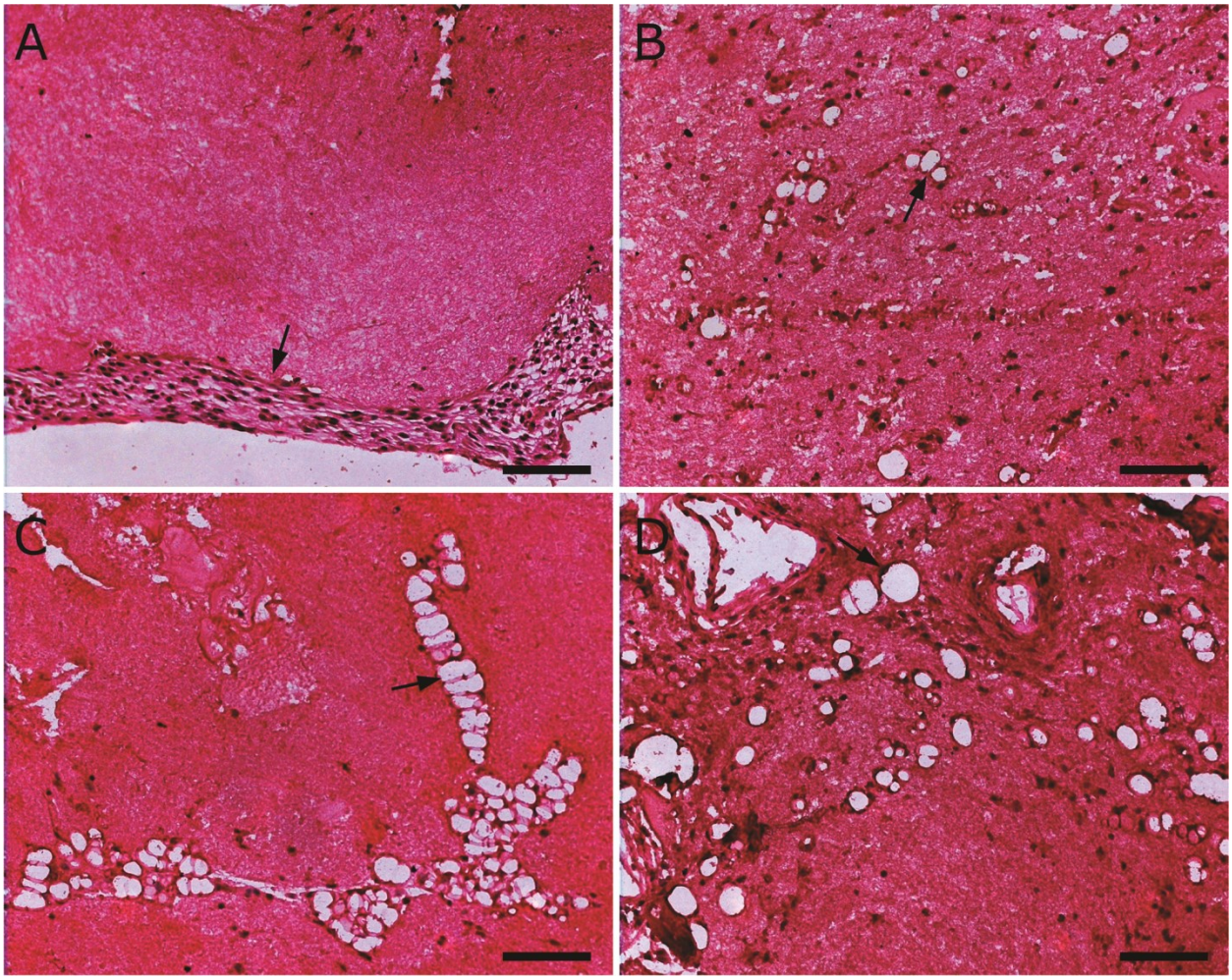

Figure 2.4. Vascularization of CCCL matrices up to 28 days after subcutaneous transplantation.

In order to examine whether CCCL matrices without islets can be vascularized in vivo the CCCL matrixes were implanted subcutaneously in immunoincompetent BALB/c mice and retrieved after 7 (A), 14 (B), 21 (C) and 28 days (D). The grafts were fixed and paraffin-embedded; sections were stained for von Willebrand Factor (vWF), hemotoxylin, and eosin. All scale bars are 100 μm. Arrows indicate vWF positive cells (A) and capillaries (B-D).

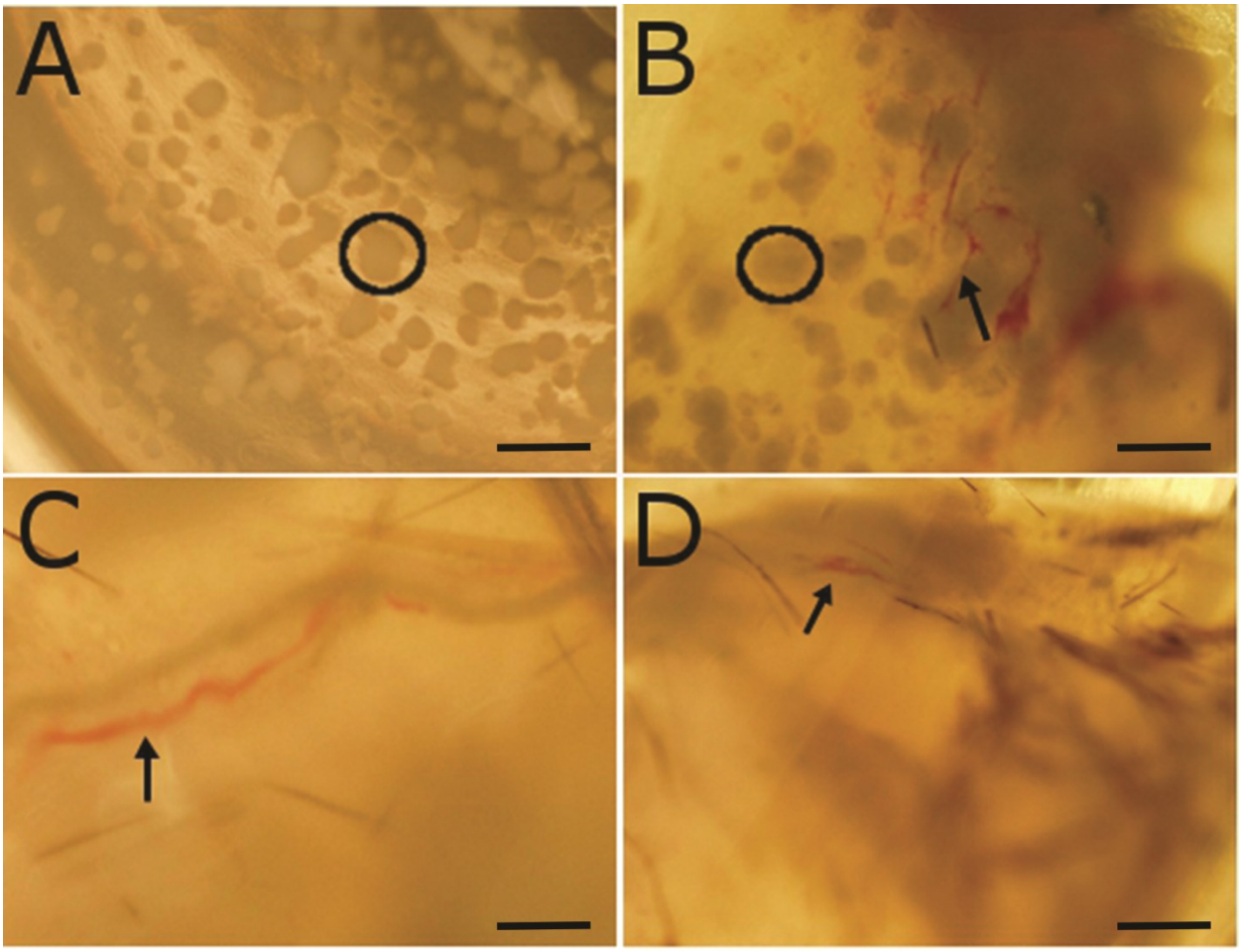

Figure 2.5. Gross morphology of NPIs in the CCCL gels and transplanted subcutaneously in immunocompromised B6.Rag$^{-/-}$ mice.

Grafts were analyzed pre-transplant (A) and after 4 (B), 21 (C) and 28 (D) days. Circles indicate visible islets; arrows indication visible vasculature. Scale bars are 400 μm.

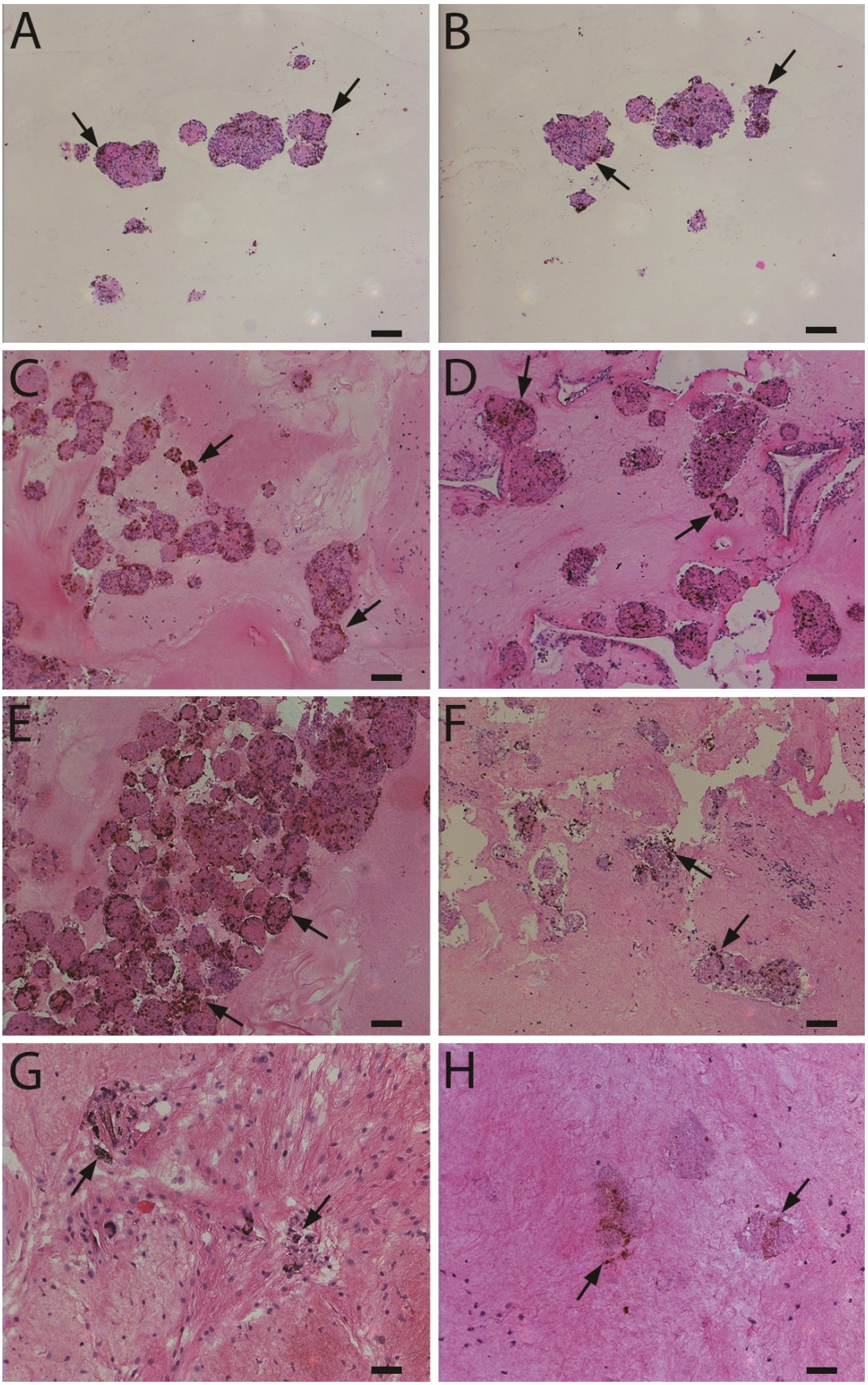
A
B
C
D
E
F
G
H

Figure 2.6. Insulin and glucagon staining reveals NPIs in the CCCL matrices up to 28 days post subcutaneous transplantation in immunocompromised B6.Rag$^{-/-}$ mice. Neonatal porcine islets in the CCCL gels were transplanted subcutaneously and retrieved after 4 (C, D), 7 (E, F), and 28 (G, H) days. Pre-transplantation grafts were also collected (A, B). The grafts were fixed and paraffin-embedded; sections were stained for insulin, hemotoxylin and eosin (A, C, E, G) and glucagon, hemotoxylin and eosin (B, D, F, H). Arrows indicated insulin (A, C, E, G) or glucagon (B, D, F, H) positive cells. Scale bars for A-F are 100 μm and for G and H are 50 μm.

Chapter 3: Development and characterization of a collagen-based matrix for vascularization and cell delivery

3.1. ABSTRACT

Since the development of the Edmonton protocol, islet transplantation is increasingly encouraging as a treatment for type 1 diabetes. Strategies to ameliorate problems with the intraportal site include macroencapsulating the islets in diverse biomaterials. Characterization of these biomaterials is important to optimally tune the properties to support islets and promote vascularization. In this study, we characterize the crosslinker-dependent properties of collagen-based matrices containing chondroitin-6-sulfate, chitosan, laminin, and crosslinked with 7.5 mM, 30 mM, or 120 mM of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide. The swelling ratio was found to be significantly negatively correlated with increasing crosslinker concentration ($p<0.0001$; $R^2=0.718$). The matrix released insulin in a reproducible logarithmic manner (R^2 of 0.99 for all concentrations), demonstrating crosslinker-dependent control of drug release. The matrices with the highest crosslinker concentrations resisted degradation by collagenase for longer than the lowest concentration (58.13±2.22% vs. 13.69±7.67%; $p<0.05$). SEM images taken of the matrices revealed that the matrices had uniform topography and porosity, indicating efficient crosslinking and incorporation of the polymer components. Matrices were transplanted subcutaneously in naïve BALB/c mice and the number and size of vessels quantified using von Willebrand factor staining; matrices with higher crosslinking concentrations had significantly larger capillaries at every time point up to four weeks after transplantation compared to the lowest crosslinker concentration group. CD31 staining visualized the capillaries at each time point. Taken together, these data show that this collagen-based matrix is reproducible with crosslinking-dependent properties that can be optimized to support vascularization and islet function.

3.2. INTRODUCTION

Islet transplantation is a promising clinical cell-based therapy for treatment of type 1 diabetes (1-4). However, despite remarkable progress, the liver implantation site remains far from ideal. Clinical transplantation of islets into the portal vein has been associated with life-threatening intraperitoneal bleeding (5), portal vein thrombosis and hepatic steatosis (6, 7). The liver may also contribute to the gradual attrition of chronic islet graft function (8). Search for a safer alternative site for islet transplantation is therefore desirable and an important issue to address (9). Using biomaterials to deliver islets to an alternate site could be advantageous if vascularization was promoted, particularly if an immune barrier could also be incorporated into the device (9). Divers techniques have been attempted to this end, including utilizing a polyethylene terephthalate mesh bag (10), a polyurethane foam dressing (11), a stainless steel mesh with polytetrafluoroethylene (PTFE) stoppers (12), and gelatin microspheres in a collagen coated polyvinyl bag (13). Most devices for islet delivery are based on synthetic polymers, which offer the advantages of complete control over mechanical and chemical properties and lower manufacturing costs (14, 15); however, natural polymers offer significant advantages of their own, including the ability of their degradation by-products to be metabolized (14, 15). Additionally, these natural polymers can be engineered to mimic properties of the natural extracellular matrix to support various cell types, including islets and recruited recipient cells (16).

Collagen-based biomaterial matrices have been previously used to deliver neonatal porcine islets (NPIs) subcutaneously in a murine model (17, 18). This matrix contains the copolymers chondroitin-6-sulfate, chitosan, and laminin, and is crosslinked with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide to support NPI viability and function. The matrix is formed in a planar shape to improve graft oxygenation (10), and has sufficient mechanical strength to resist the mechanical

stress of the subcutaneous site. This is an attractive approach to creating a highly vascularized site for the implantation of islets, particularly because the matrix could be functionalized with growth factors that promote angiogenesis (10). Our work has demonstrated that this matrix has no effect on glucose stimulated insulin secretion, and can support NPI viability and function *in vivo* (17, 18). It is important to characterize any biomaterial to ensure that the material functions in a way that supports the target cells' viability and function; for a material that is intended for vascularization, these properties would include degradation rate, swelling ratio, and degree of vascularization. Biocompatibility depends not only on the material characteristics but on the biological system in which the material will be used, and therefore it is valuable to be able to alter the material properties to suit the applications (19). Natural polymers can be tuned by various strategies, including the use of co-polymers and the controlling the degree of crosslinking of all the polymers (15). Additionally, it is known that the topography of a material is important as an alternate signaling mechanism to control many properties related to vascularization, including adhesion, migration, and differentiation (15, 16). We have previously optimized the co-polymers in our collagen-based matrix (18, 20, 21), but the most favorable concentration of the crosslinker remains to be determined. In this study, the crosslinker dependent properties of the collagen-based matrix are characterized in order to facilitate the optimization of a biomaterial able to support cellular grafts such as islets for the treatment of type 1 diabetes.

3.2. MATERIALS AND METHODS

3.2.1. Neonatal Porcine Islet Preparation

Donor pancreases were obtained from 1 to 3 day old Duroc cross neonatal piglets from the University of Alberta Swine Research Centre (1.5-2.0 kg body weight), and the islets were isolated and cultured for 5-7 days as described previously (18, 22). Briefly,

the retrieved pancreases were cut into 1 to 3 mm tissue fragments, then exposed to 2.5 mg/mL collagenase (type XI; Sigma, C7657), filtered through a 500 μm nylon screen, washed in Hank's Basic Salt Solution (HBSS) (Gibco, H6136) supplemented with 0.25% BSA (fraction V; Sigma-Aldrich, A9543), 10 mM HEPES (Sigma-Aldrich, H4034), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Lonza Walkersville, Inc., 09-757F). NPI were then cultured in non-tissue culture treated petri dishes containing Ham's F10 tissue culture media (Sigma-Aldrich N6635) supplemented with 14.3 mM sodium bicarbonate (Fisher, S233), 10 mM D-glucose (EM Science, DX0145-3), 2 mM L-glutamine (Sigma-Aldrich, G8540), 0.25% BSA (fraction V), 50 μM isobutylmethylxanthine (Sigma-Aldrich, I5879), 10 mM nicotinamide (Sigma-Aldrich, N0636), 1.6 mM calcium chloride dihydrate (Sigma-Aldrich, C7902), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Lonza Walkersville, Inc., 09-757F). The islets were cultured at 37°C for 5-7 days, with the medium changed the first, third and fifth days after isolation.

3.2.2. Preparation of collagen matrices

Collagen matrices crosslinked with 7.5 mM, 30 mM, and 120 mM of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, Sigma-Aldrich, E6383) and N-hydroxysuccinimide (Sigma-Aldrich, 56480) containing 0.2 mg/mL chondroitin-6-sulphate (Wako Pure Chemical Industries, 034-14612), 1.0 mg/mL chitosan (Sigma-Aldrich, C3646), and 0.1 mg/mL mouse laminin (BD Biosciences, Inc., 354232), were manufactured using previously described methods (18, 22). High concentration denatured rat tail collagen I (BD Biosciences, Inc., 354249) was used. Briefly, all components were mixed together in a 50 mL glass tube on ice, with varying concentrations of crosslinker added as the last time. The liquid matrix was then adjusted to a pH of 6.0 for 5 minutes to initiate crosslinking, then 150 μL of liquid matrix were added to the wells of a 24 well plate; matrices were then crosslinked for 30

minutes at 37°C before continuing on to the *in vitro* or *in vivo* analyses. Prior to transplantation, matrices were cultured for 24 hours in phosphate buffered saline (PBS) to ensure excess crosslinker was removed.

3.2.3. In vitro measurements of matrix properties

The masses of the matrices were measured after a 30 minute crosslinking period. The matrices were then dehydrated for one hour in increasing concentrations of ethanol, from 70% to 100%, in periods of 15 minutes. The masses of the fully dehydrated matrices were measured three times to ensure consistency throughout the test. The swelling ratio (Q) of the matrix was calculated by:

$$Q = \frac{M_c - M_d}{M_c}$$

Where M_c=crosslinked weight and M_d=dehydrated weight.

To ensure that hormones or growth factors can readily diffuse out of the matrix, 34.7 μg (1 UI) of porcine insulin was added to 1000 μL of liquid matrix in triplicate, then PBS without insulin was added on top of the matrices. All of the PBS was removed and fresh PBS added at 30 minutes, 1 hour, 2 hours, 3 hours, 4 hours, and 6 hours to mimic biologically fast acting insulin. The PBS was subsequently assayed for porcine insulin using a commercial mouse/rat insulin assay (Meso Scale Diagnostics, K152BZC).

3.2.4. Scanning electron microscopy

Matrices were also taken for SEM analysis of the microstructure using a technique described by McEwan et al. (20). Briefly, to preserve cell morphology, matrices with cells were fixed in 3% glutaraldehyde (Sigma-Aldrich) buffered with 0.1 M PBS for 30 minutes and then rinsed with PBS three times prior to ethanol washes. 10 mm diameter matrix samples were dehydrated in 70, 80, 90, 95, and 100% ethanol

solutions for 10 minutes each. For cross-sectional viewing, samples were fractured after immersion in liquid nitrogen. Samples were sputtered (Anatech, Hummer VII) with a palladium/gold (60:40 palladium:gold) alloy to form a thin coating (3 nm). SEM images were obtained using an accelerating voltage of 1.0 kV in order to minimize sample damage. Cross-sectional images were obtained using backscattering and secondary electron detectors. Micrographs were evaluated for porosity and pore diameter using Image-J 1.43u software.

3.2.5. In vitro degradation

The masses of matrices with 7.5 mM, 30 mM, and 120 mM crosslinker concentrations were measured, and then the matrices were exposed to 25 U of type V collagenase in PBS at 37°C (n=3 for each matrix type). At 30 minutes, 1 hour, then hourly up to 10.5 hours, the collagenase solution was aspirated and any adsorbed solution removed, then the matrices were weighed to determine the *in vitro* degradation rate.

3.2.6. NPI survival

Apoptosis of the NPIs in the matrices was assessed after 7 days in culture using a commercial TUNEL assay kit (Invitrogen Molecular Probes A23210). The matrices were fixed in Z-Fixx, paraffin embedded, and then slides of sections were prepared. After rehydration, antigen retrieval was performed in sodium citrate buffer (pH 6.0), then slides were incubated according to the kit instructions. The slides were mounted with ProLong Gold Antifade Reagent with DAPI (Invitrogen Molecular Probes P36935). To quantify the percentage of TUNEL positive cells, five images of TUNEL and DAPI staining were taken, then the images were combined in ImageJ (National Institutes of Health, Bethesda, Maryland, USA). Separate images of the TUNEL and DAPI positive channels were altered to black and white, then the number of particles counted. Each particle was confirmed by two independent reviewers to be TUNEL or DAPI positive visually and the brightness of the colour threshold adjusted to only include positive

cells. The number of TUNEL positive cells was subsequently divided by the total DAPI positive cells to determine the percentage of apoptotic cells in each image.

3.2.7. In vivo measurements of matrix properties

Matrices with varying crosslinker concentration were transplanted subcutaneously under the dorsal skin of naïve BALB/c mice. These matrices were retrieved at 2, 3, and 4 weeks. After retrieval, all collagen-based matrices were fixed in Shandon Zinc Formal-Fixx (Thermo Fisher Scientific, 6764255) then embedded in paraffin and 5 μm sections were prepared. For vascularization, CD31 and von Willebrand Factor (vWF) staining was utilized to visualize arterioles and capillaries. After rehydration, antigen retrieval for both CD31 and vWF was performed in sodium citrate buffer (pH 6.0). All immunohistochemical samples were blocked with 20% normal goat serum for 20 minutes (Jackson ImmunoResearch Laboratories Inc., 0005-000-121). Slides were visualized with an Axioscope II microscope equipped with an AxioCam MRC and analyzed with Axiovision 4.6 software (Carl Zeiss, Gottingen, Germany). Five images of vWF staining were taken from each of two retrieved matrices for each crosslinker concentration and the images were combined for particle analysis in ImageJ (National Institutes of Health, Bethesda, Maryland, USA). Vessels were counted if they were between 50 and 10000 μm^2 in size, with a circularity greater than 0.2. Each particle was confirmed to be a capillary-like structure visually and excluded manually if mistakenly included by the software. Images of CD31 staining were also obtained to confirm the vWF staining identify the quality and stability of the vessels.

3.3. RESULTS

3.3.1. Swelling behaviour and drug release are crosslinker dependent

The swelling ratio of the collagen-based matrix was significantly correlated with the crosslinking concentration ($p<0.0001$) with a coefficient of determination of 0.718

(Figure 3.1A). Increasing the crosslinking concentration resulted in a lower swelling ratio, consistent with hydrogel behaviour.

Insulin was diffused from the matrix with all crosslinking concentrations in a reproducible logarithmic manner with a coefficient of determination of 0.99 for all concentrations (Figure 3.1B). By the end of 6 hours, a total of 30.62 μg of insulin was released from the matrices with 7.5 mM crosslinker (88% of the total insulin), 27.89 μg of insulin had been released from the matrices with 30 mM crosslinking or approximately 80% of the total insulin, and 28.90 μg of insulin had been released from the matrices with 120 mM insulin (83% of total). A lower crosslinker concentration seemed to release insulin more quickly, but none of the groups were significantly different.

3.3.2. Increased crosslinker concentration gives resistance to in vitro degradation

The matrices with 7.5 mM crosslinking concentration were significantly more degraded as early as 30 minutes compared to the matrices with 30 mM and 120 mM crosslinking concentration (77.20±3.73% vs. 90.41±0.57% and 86.76±1.64%, respectively; $p<0.001$) (Figure 3.1C). The 7.5 mM crosslinking concentration matrices were also significantly more degraded at 1 hour compared to the matrices with 30 mM and 120 mM crosslinker (43.32±2.59% vs. 86.32±1.63% and 80.73±4.12%, respectively; $p<0.01$), and at 2 hours (18.62±0.23 vs. 72.12±6.65% and 69.52±4.56%; $p<0.01$). These low crosslinking concentration matrices were completely degraded after 3 hours of exposure to 25 U/mL collagenase. There were no significant differences in degradation between the matrices with 30 mM and 120 mM crosslinking concentration at any time point; both groups were too degraded to be measured after 10.5 hours.

3.3.3. Increased crosslinker concentration is associated with increased vascularization

At two weeks post transplantation, there were no significant differences between the capillary sizes in the matrices with 7.5 mM, 30 mM or 120 mM crosslinker

concentration ($p=0.054$) (Figure 3.1D). At three weeks, the matrix with 120 mM crosslinker concentration had significantly larger capillaries (270.38±4.89 μm) compared to the other two crosslinker concentrations (50.82±1.52 μm and 75.24±2.32 μm for 7.5 mM and 30 mM crosslinker concentration, respectively; $p<0.05$), and compared to the mean size of the capillaries at two weeks. Additionally, the matrix with 30 mM crosslinker concentration had significantly larger mean vessel size compared to the matrices with 7.5 mM crosslinker concentration. Similarly, at 4 weeks, the matrix with 120 mM crosslinker concentration had significantly larger capillaries (433.12±5.12 μm) compared to the other two crosslinker concentrations (81.82±1.20 μm and 221.68±5.63 μm for 7.5 mM and 30 mM crosslinker concentration, respectively; $p<0.05$), and compared to the mean size of the capillaries at two weeks. Again, the matrices with 30 mM crosslinker concentration had a significantly larger mean capillary size compared to the 7.5 mM crosslinker concentration matrices ($p<0.05$). All groups had significantly larger capillaries than the earlier time points. These quantitative data are supported by CD31 staining; 2 weeks post transplantation, CD31 positive cells and some small vessels can be observed in the matrices with 7.5 mM (Figure 3.2A), 30 mM (Figure 2B) and 120 mM (Figure 2C) crosslinker concentrations. As the crosslinking concentration increases, larger vessels are visible. After 4 weeks post-transplantation, all matrices exhibited many CD31 positive vessels, with the matrices with 120 mM crosslinker concentration showing the largest and most plentiful vessels (Figure 3.2F) compared to those with 7.5 mM (Figure 3.2D) and 30 mM (Figure 3.2E) crosslinker concentrations.

3.3.4. Nanotopography is crosslinker dependent

Scanning electron microscopy (SEM) analysis revealed that the matrix had uniform topography and porosity, indicating efficient crosslinking and incorporation of the different components. At a micrometer level, geometric changes in the surface of the matrices were observed. Matrices with 7.5 mM crosslinking concentration were

observed to have micropores with a diameter of 166±6.4 μm (Figure 3.3A), while matrices with 120 mM crosslinking had microconvexities with diameters of 148±8.0μm (Figure 3.3C). The nanotopography of the surface of the matrices did not vary with crosslinking concentration and appeared to be very smooth for a depth of 50±4.3 nm. Below this smooth surface, a randomized network of crosslinks was visible. The matrices with 7.5 mM, 30 mM, and 120 mM crosslinking concentrations had significantly different average crosslink densities of 63±2.2%, 70±2.5%, and 82±2.3%, respectively ($p< 0.05$ between 120 mM and 30 mM and 30 mM and 7.5 mM; $p<0.01$ between 120 mM and 7.5 mM) (Figure 3.3D-F). The average crosslink diameters of all three matrices were not significantly different (137.8±12.0 nm).

3.3.5. The matrix supports NPI viability at all crosslinker concentrations

After 7 days of *in vitro* culture with embedded NPIs, there were visible differences between the matrices with the three different crosslinking concentrations. The group with the 7.5 mM crosslinking concentration were the most opaque and maintained sufficient mechanical integrity for manipulation. There appeared to be pores visible under light microscopy (Figure 3.4A, inset). Matrices with 30 mM crosslinking concentration had improved mechanical integrity compared to the 7.5 mM group and were slightly more translucent (Figure 3.4B). Interestingly, the addition of NPIs to the matrices with 120 mM crosslinking concentration caused the matrices to lose mechanical integrity after 7 days such that manipulation resulted in the matrix fragmenting into multiple pieces (Figure 3.4C). The matrices with 120 mM crosslinking concentration were completely transparent. Intact NPIs were visible in all three matrix groups (Figure 3.4 insets).

No TUNEL positive cells were visible in any of the matrices (Figure 3.5), indicating excellent support for the NPIs. The control NPIs cultured in the standard Ham's F10 media (Figure 3.5A) had the most apoptotic cells as one or two cells per

section were TUNEL positive.

3.4. DISCUSSION

These data, combined with our previously described results, demonstrate that this collagen-based matrix has properties reproducibly tunable by the crosslinker concentration, and thus could be adapted for various tissue engineering and cell delivery purposes. Our previous work has demonstrated that the matrix with 30 mM crosslinker concentration promotes survival and function of NPIs *in vivo* (18); data from this study will allow further optimization of the matrix to allow adjustment of the biocompatibility of the system in a variety of situations. All matrices tested have sufficient initial mechanical strength to be easily transplanted and subsequently retrieved if necessary, which may be an important factor when delivering a xenogeneic cell product such as NPIs (10).

The swelling ratio of the matrix was significantly correlated with crosslinker concentration. Controlling the swelling ratio of a hydrogel could offer control of drug release (23); this matrix could be functionalized with additional growth factors such as vascular endothelial growth factor (VEGF), localized immunosuppressive drugs, or anti-inflammatory cytokines or drugs to protect the cell product. This could also be modified by adjusting the concentration of chondroitin-6-sulfate, a glycosaminoglycan (GAG) known to bind and sequester growth factors and cytokines (16), if a specific level of crosslinker concentration was required for another aspect of biocompatibility. Insulin was diffused more quickly in matrices with lower crosslinking concentration, supporting the rationale to adjust crosslinker concentration as a technique for controlling drug release. In particular, the immunomodulatory and anti-inflammatory cytoprotective factors secreted by mesenchymal stem cells (MSCs) have been shown to protect islets from pro-inflammatory cytokines (24); the matrix could have hepatocyte growth factor (HGF), fibroblast growth factor, or MSCs themselves added to further

protect the islet graft. The properties of the collagen-based matrix could then be tuned to support MSC viability and function.

Matrices with higher crosslinker concentrations better resisted collagenase degradation compared to those with lower crosslinker concentrations. This is related to the swelling ratio as less fluid entering the matrix decreases the exposure to the collagenase solution. All matrices showed sufficient resistance to in vivo degradation to allow transplantation (15), but it is valuable to have the capacity to control degradation for diverse applications; for example, to match the natural healing or regeneration processes of the recipient tissues (25). The number and size of vessels also increases with crosslinker concentration, an advantageous property as these higher crosslinking concentration matrices would have a greater neovascular system to support transplanted cells in the long term. As higher crosslinker concentration did not have cytotoxic effects on the NPIs, a matrix transplanted with higher crosslinker concentration transplanted without a cell population could benefit from faster, more functional vascularization of an ectopic site. However, this remains to be tested *in vivo*.

SEM imaging of the matrix with 7.5 mM, 30 mM, and 120 mM crosslinking concentration revealed uniform microstructure throughout the samples. Interestingly, the micropores visible in surface of the 7.5 mM matrix do not correlate with increased vascularization, whereas the microconvexities in the 120 mM matrix are related to faster neovascularization *in vivo.* There appears to be a relationship between higher surface energy from crosslinking and the resultant geometric changes and vascularization. The geometric changes lower the surface energy of the matrices; surface energy is an important characteristic of a material that may be more important than topography for guiding cellular adhesion and proliferation (26). Although the surface energies of these matrices were not directly measured, the microconvexities of the 120 mM crosslinking concentration matrices are likely a result of higher surface energy that allows for improved cell spreading and ameliorated vascularization (27, 28). The dense,

interconnected structure contributes to the mechanical strength of the matrices, and may facilitate cell attachment and intracellular signalling (29). Additionally, the availability of ECM proteins such as laminin and collagen can provide vital signalling cues and allow ECM receptor interactions; degradation of these proteins can provide substrates as well as space for angiogenesis (30). Thus, both the surface energy and topography can potentially be tuned by the crosslinking concentration to support the target cell population, for example to encourage angiogenesis.

The rapid increase in vascularization between 2 and 3 weeks in the 120 mM matrices is likely due to increasing porosity from biodegradation and infiltrating endothelial cells that remodel the matrix (31). SEM imaging of vascularized matrices at varying time points would be helpful in elucidating how the neovasculature forms in the centre of the matrix, with or without the presence of proangiogenic factors such as VEGF. Tuning the porosity to mimic that of the matrices at later timepoints could also be useful for promotion of angiogenesis. Alternatively, McFadden et al. (32) demonstrated a technique for in vitro pre-vascularization of a collagen-GAG matrix using human umbilical vein endothelial cells and MSCs. Additionally, a matrix could be transplanted subcutaneously for longer than 4 weeks without a cell population to establish vascularization in the desired site prior to the delivery of the graft. As this matrix crosslinks at 37°C, a liquid scaffold could be non-invasively injected to fill the subcutaneous space (17, 33). The cytotoxicity of higher crosslinker concentrations in this context requires further exploration. These could be promising approaches for engineering a matrix that can support NPI in a subcutaneous space that lacks sufficient initial vascularization to support the graft (10).

The natural extracellular matrix (ECM) of the pancreas is highly complex, and varies between species (16, 34). Although the specific roles of the various proteins on endocrine function are not well understood, it is well known that isolated islets suffer from anoikis, a form of apoptosis (35). The lack of TUNEL positive islet cells within the

matrix supports the rationale for including collagen, laminin, a GAG, and a polysaccharide such as chitosan to prevent this form of apoptosis by presenting a scaffold with similar components as the natural pancreatic ECM (16, 36). Continued research on the specific interactions between NPIs and these matrix components is warranted to further ameliorate NPI survival and function.

3.5. CONCLUSIONS

Overall, these data indicate that this collagen-based matrix with the incorporation of chitosan, chondroitin-6-sulfate, and laminin is reproducible, tunable, and may be used to create a vascularized site for cell delivery, specifically for but not limited to NPIs. This matrix offers the potential for creating a vascularized ectopic site that can be thoughtfully designed for diverse applications.

3.6. ACKNOWLEDGMENTS

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3.8. FIGURES

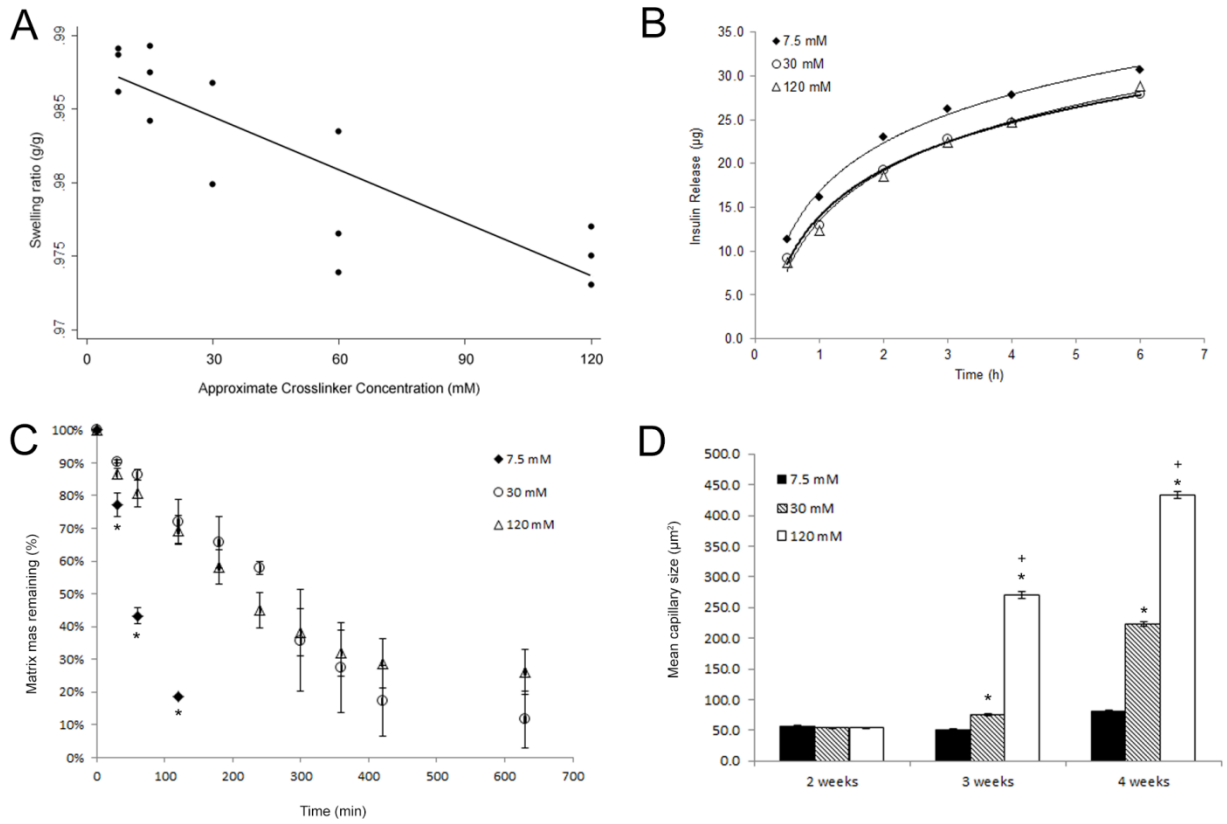

Figure 3.1. Effect of crosslinker concentration on swelling ratio, drug release, enzymatic degradation and vascularization.

(A) Matrices were weighed immediately after the crosslinking period and then dehydrated in increasingly concentrated ethanol solutions. The swelling ratio was calculated as the ratio of the difference between the crosslinked matrix mass and the dehydrated matrix mass to the crosslinked matrix mass. (B) One unit of insulin was added to matrices with 7.5, 30, or 120 mM crosslinker concentration, then PBS was added on top of the matrices. The insulin diffusion into the PBS was measured at 30 minutes, then 1, 2, 3, 4, and 6 hours. (C) Matrices were exposed to 25 U of type V collagenase. The masses of the matrices were measured at 30 minutes, then hourly up to 10 hours or until the matrices were completely degraded. (D) Matrices were

transplanted subcutaneously in naïve BALB/c mice then retrieved at 2, 3, or 4 weeks post transplantation. Matrices were paraffin embedded, then sections were stained for vWF and the capillary size was measured using Image/J.

* - $p<0.05$ vs. 7.5 mM crosslinking concentration; + - $p<0.05$ vs. 30 mM crosslinking concentration. All results are shown as mean±SEM.

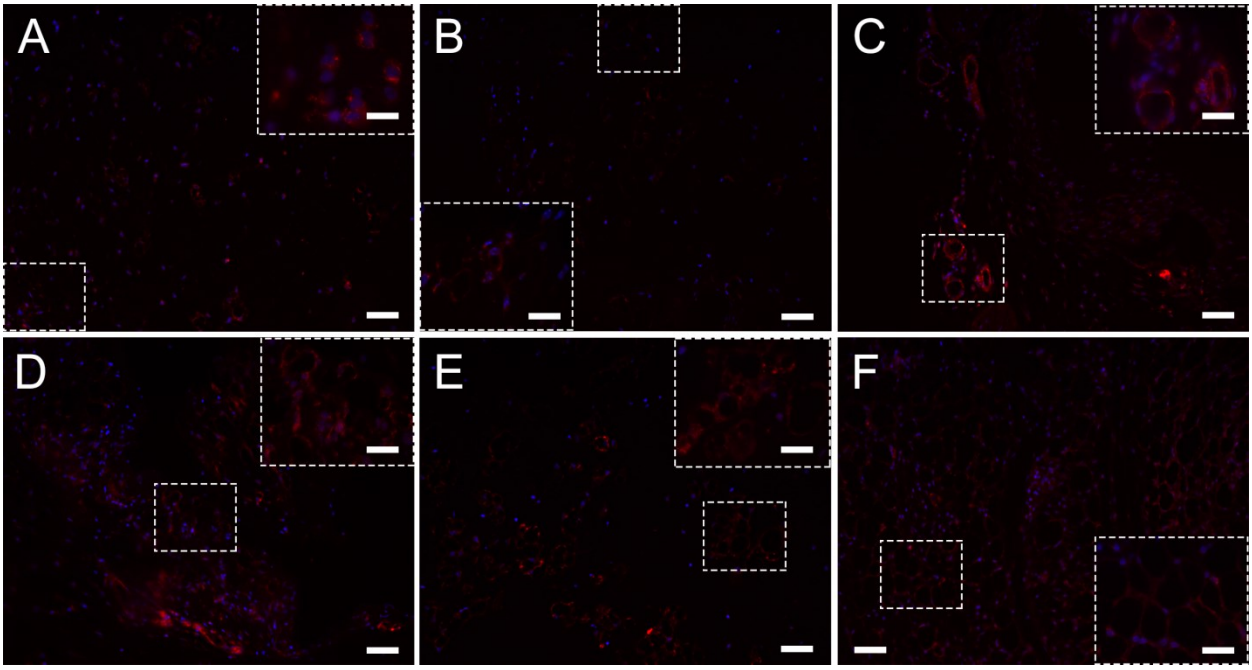

Figure 3.2. CD31 staining reveals vascularization increases over time and is crosslinker dependent.

Matrices were transplanted subcutaneously in naïve BALB/c mice then retrieved at 2 or 4 weeks post transplantation. Matrices were paraffin embedded; sections were stained for CD31 (red) and DAPI (blue). Vessels of matrices with 7.5 mM (A, D), 30 mM (B, E) and 120 mM (C, F) crosslinker concentration were visualized at 2 (A, B, C) and 4 (D, E, F) weeks post transplantation. Scale bars of A-F are 50 μm; scale bars of insets are 10 μm.

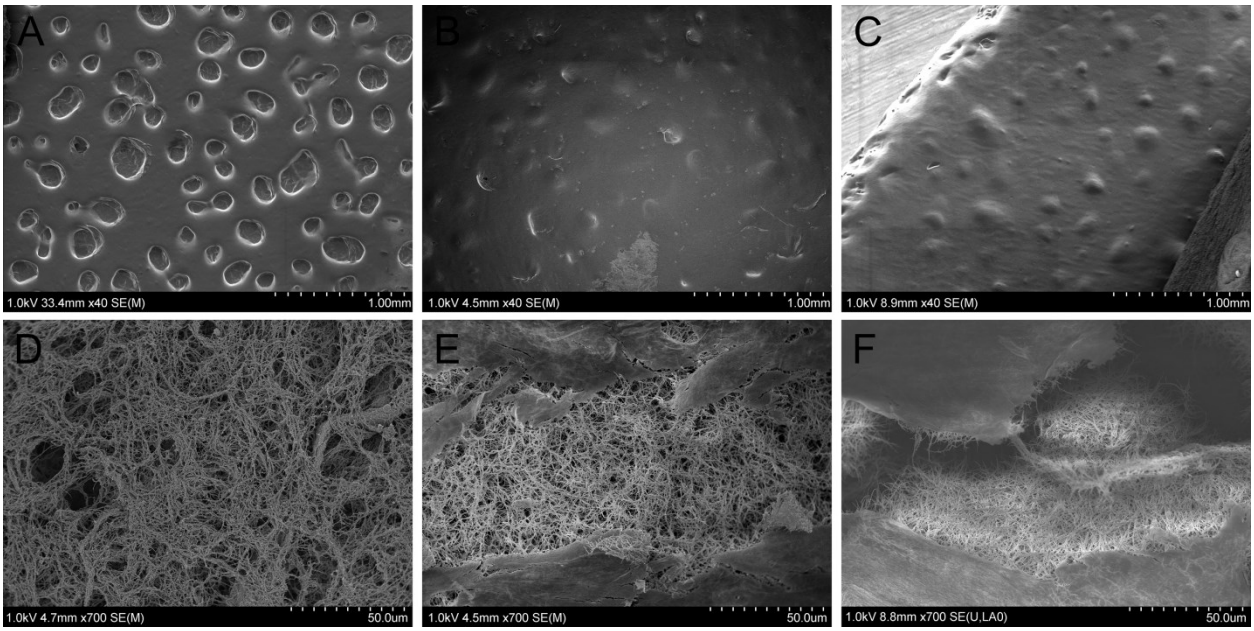

Figure 3.3. The microstructure and surface topography of the collagen-based matrices are crosslinker dependent, as revealed by SEM.

The microstructures of the matrices with 7.5 mM (A, D), 30 mM (B, E) and 120 mM (C, F) crosslinker concentration were examined using scanning electron microscopy (SEM). The surface topographies (A-C) and interior topographies (D-F) were examined.

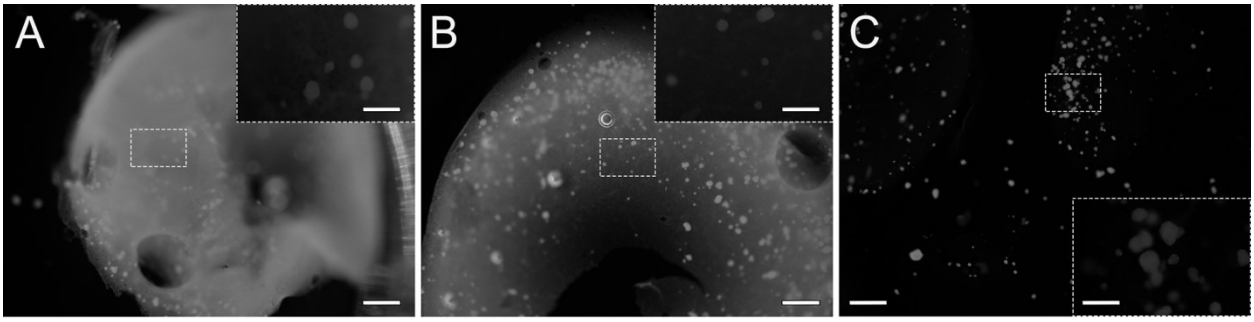

Figure 3.4. Effect of crosslinker concentration on matrix integrity with NPIs after 7 days in culture.

NPIs embedded in matrices with 7.5 mM (A), 30 mM (B), and 120 mM (C) crosslinking concentrations were cultured for 7 days then dark field images were taken of the matrices in a 6 well plate. Scale bars are 1.6 mm for A, B, and C; scale bars of insets are 400 μm.

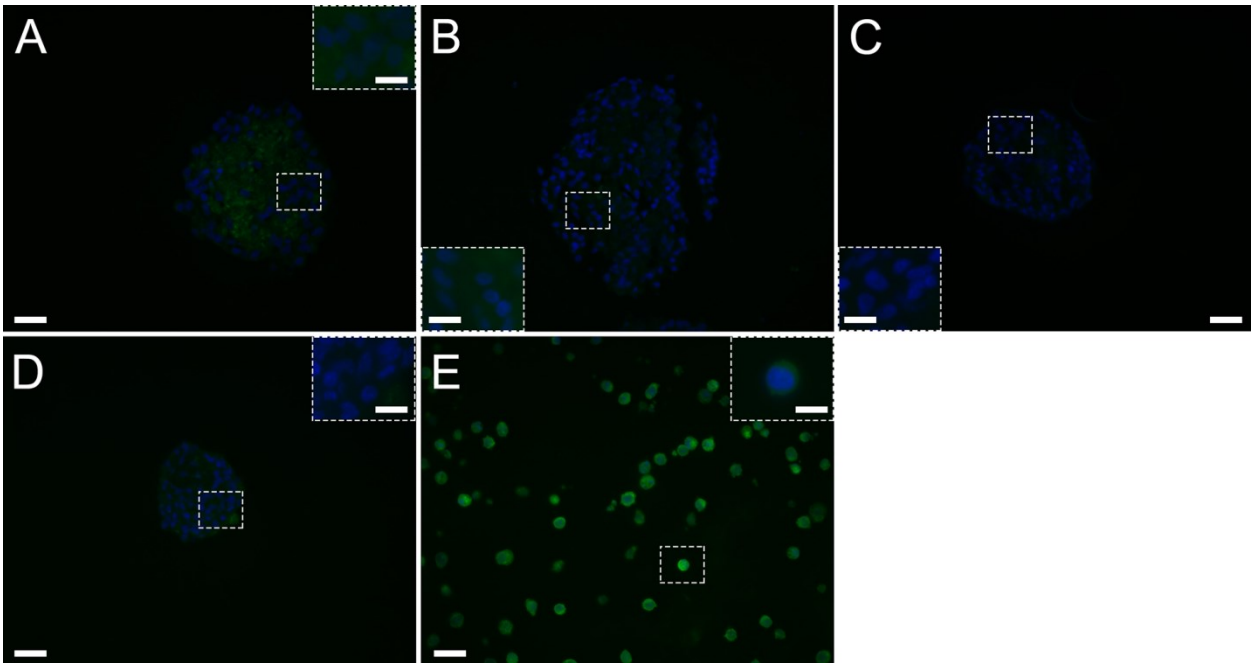

Figure 3.5. Effect of addition of copolymers on apoptosis of NPIs after 7 days in culture. NPIs embedded in matrices with 7.5 mM (B), 30 mM (C), and 120 mM (D) crosslinking concentrations and in standard Ham's F10 culture media as a control (A) were cultured for 7 days then fixed and paraffin-embedded; sections were stained for TUNEL and compared to TUNEL positive controls (E). Scale bars of A-E are 20 μm and scale bars of insets are 10 μm.

Chapter 4: Establishing a clinically scalable protocol for the isolation of neonatal porcine islets

4.1. ABSTRACT

One challenge that must be overcome to allow transplantation of neonatal porcine islets (NPIs) a clinical reality, include defining a reproducible and scalable protocol for the efficient preparation of therapeutic quantities of clinical grade NPIs. In our standard protocol we routinely isolate NPIs from maximally 4 pancreases, requiring tissue culture in 16 petri dishes (4 per pancreas) in Ham's F10 and bovine serum albumin (BSA). We have now developed a scalable and technically simpler protocol that allows us to isolate NPIs from minimally 12 pancreases at a time by employing automated tissue chopping, collagenase digestion in a single vessel and tissue culture/media changes in 75% fewer petri dishes. For culture, BSA is replaced with human serum albumin and supplemented with Z-VAD-FMK general caspase inhibitor and a protease inhibitor cocktail. The caspase inhibitor was added to the media for only the first 90 minutes of culture. NPIs isolated using the scalable protocol had significantly more cellular insulin recovered (56.9±1.4 μg) when compared to the standard protocol (15.0±0.5 μg; $p<0.05$). Compared to our standard protocol, recovery of β-cells ($6.0x10^6$ ±0.2 vs. $10.0x10^6$ ± 0.4; $p<0.05$) and islet equivalents (35,135±186 vs. 41,810±226; $p<0.05$) was significantly higher using the scalable protocol. During a static glucose stimulation assay the SI of islets isolated by the standard protocol were significantly lower than the scale-up protocol (4.3±0.2 vs. 5.5±0.1; $p<0.05$). Mice transplanted with NPIs from the scalable protocol had significantly lower glycemias than the mice that received NPIs from the standard protocol ($p<0.001$) and responded significantly better to a glucose tolerance test. Based on the above findings, this improved simpler scalable protocol is a significantly more efficient means for preparing therapeutic quantities of clinical grade NPIs.

4.2. INTRODUCTION

An attractive alternative to daily insulin injections is to transplant insulin-producing tissue to achieve a more physiological means for restoring glucose homeostasis, thereby potentially reversing the metabolic and neurovascular complications of diabetes. Seven patients transplanted in 2000 by the Islet Transplant Group in Edmonton attained insulin independence by receiving freshly isolated islets from multiple donors and steroid-free anti-rejection therapy - a procedure known as the Edmonton Protocol (1-3). Modifications of this protocol by other groups have also resulted in similar successes (4-9). To date, in Edmonton, approximately 300 patients have been transplanted; this is less than anticipated, in large part because of the limited supply of cadaveric pancreases (1). To allow islet transplantation to become a more wide spread form of therapy for more patients with type 1 diabetes, an unlimited source of islets must be identified.

There is a strong rationale to pursue the use of porcine donors as an unlimited source for clinical islet xenotransplantation. The principle challenges that must be met to make xenotransplantation of porcine islets a clinical reality include defining a reproducible strategy for the efficient preparation of large numbers of clinical grade islets; understanding and minimizing the risk of transmission of porcine pathogens; and overcoming the patient rejection barrier with clinically applicable immunosuppression and ultimately tolerance induction strategies. Our group reported a simple, inexpensive and reproducible method to isolate large numbers of NPI (10). These islets are comprised of differentiated endocrine and endocrine precursor cells that both *in vitro* and *in vivo* have the potential for proliferation and differentiation and have been shown to reverse hyperglycemia in immuno-deficient mice (10), allogeneic out-bred pigs (11), and moreover in non-human primates (12, 13). Furthermore, NPIs are appealing because of their resistance to hypoxia (14), human pro-inflammatory cytokines (15),

hyperglycemia (16) and toxicity of islet amyloid deposition (17) as well as their inherent ability to differentiate and proliferate (10) and achieve transplant tolerance induction in diabetic mice (18). The recent New Zealand Phase I/IIa clinical trial testing the safety and efficacy of alginate-encapsulated NPI showed low incidence of and quick resolution to reactions to the implantation, and no long term reactions; improved quality of life with a statistically significant reduction in the number of unaware hypoglycaemic events; decreased insulin use; and decreased HbA1c compared to baseline (19). Importantly, there was no evidence of xenogeneic infection of the recipients or those in close contact with the patient (19). Taken together, these observations clearly indicate that neonatal porcine islets are an ideal source of tissue for clinical islet xenotransplantation.

One principal challenge that must be met to make transplantation of neonatal porcine islets a clinical reality is defining a reproducible and scalable strategy for the efficient preparation of therapeutic quantities of clinical grade neonatal porcine islets. In our original publication (10) we routinely isolated islets from only four neonatal porcine pancreases, but the protocol is very laborious and at a high risk for potential contamination. A more scalable, technically simpler and clinically acceptable protocol is developed in this study that will facilitate and expedite clinical trials of neonatal porcine islets thereby allowing the treatment of many more patients with Type 1 diabetes.

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4.3. MATERIALS AND METHODS

4.3.1. Neonatal Porcine Islet Preparation

Donor pancreases were obtained from 1 to 3 day old Duroc neonatal piglets from the University of Alberta Swine Research Centre (1.5-2.0 kg body weight), and the islets

were isolated and cultured for 5-7 days as described previously (10) (Figure 4.1A). For our previously published standard protocol (Figure 4.1A), the retrieved pancreases (four per isolation) were cut into 1 to 3 mm tissue fragments in separate 50 mL conical tubes using scissors, collagenase digested (Type XI, 2.5 mg/mL, Sigma-Aldrich, Oakville, Canada), filtered through a 500 μm nylon screen, washed in Hank's Basic Salt Solution (HBSS) (Gibco, Burlington, Canada) supplemented with 0.25% bovine serum albumin (BSA) (fraction V; Sigma-Aldrich, Oakville, Canada), 10 mM HEPES (ICN Biomedicals, Inc., Costa Mesa, CA), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Lonza Walkersville, Inc., Walkersville, USA). The digest was then cultured in four non-tissue culture treated petri dishes per pancreas (Figure 4.1A; 16 petri dishes per isolation) in Ham's F10-BSA tissue culture media (Sigma-Adrich, Oakville, Canada) supplemented with 14.3 mM sodium bicarbonate (Fisher Scientific, Ottawa, Canada), 10 mM D-glucose (EM Sciences, Hartfield, PA), 2 mM L-glutamine (Sigma-Aldrich), 0.25% BSA (fraction V; Sigma-Aldrich), 50 μM isobutylmethylxanthine (IBMX) (Sigma-Aldrich), 10 mM nicotinamide (Sigma-Aldrich), 1.6 mM calcium chloride dihydrate (Sigma-Aldrich), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Lonza Walkersville, Inc., Walkersville, USA). The islets in this standard 4 plate protocol were cultured at 37°C for 5-7 days, with the medium changed the first, third and fifth days after isolation. A control group included pancreases processed as outlined above but the digest from each pancreas being cultured in 1 petri dish (standard 1 plate protocol) as opposed to 4 petri dishes per pancreas (standard 4 plate protocol).

For the experimental scalable protocol (Figure 4.1B), 12 pancreases per isolation were surgically removed and then cut into 1-2 mm^3 fragments in 160 mL HBSS without BSA using an automated tissue chopper (Retsch Grindomix GM 200, VERDER Group, Netherlands) at 2.0 rpm in two 5 second bursts (Figure 4.1B). All tissue from 12 pancreases was then collagenase digested in a single 225 mL conical tube, filtered through a 500 μm nylon mesh then each pancreas was cultured in a single petri dish (12

dishes for 12 pancreases) in Ham's F10-HSA-CI-PI described above but with no BSA and additionally supplemented with 0.5% human serum albumin (HSA) replacing the 0.25% BSA, 11.1 μM Z-VAD-FMK general caspase inhibitor (CI) (RD Systems, Cedarlane, Hornby, Canada), and a protease inhibitor (PI) cocktail (1:500) used for tissue culture (Sigma-Aldrich). The caspase inhibitor was added to the media for only the first 90 minutes then the islets were washed twice in HBSS then cultured in the same media but with no caspase inhibitor. The islets were cultured at 37°C for 5-7 days, with the medium changed the first, third and fifth days after isolation.

4.3.2. In Vitro Assessment of Islets

Following tissue culture, recovery of the NPI preparations was determined on the basis of cellular insulin and DNA contents as well as islet equivalents (10). All measurements were assessed from duplicate aliquots of the NPI suspensions. Cellular insulin content was measured after extraction in 2 mM acetic acid containing 0.25% BSA (10). Samples were sonicated in acetic acid, centrifuged at 800 g for 15 minutes, and then supernatants were collected and stored at -20°C until assayed for insulin content by ELISA (Boehringer Mannheim, Basel, Switzerland). For DNA content, representative 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 by Picogreen (Molecular Probes, Inc., Eugene, OR), a fluorescent nucleic acid stain for quantification of double-stranded DNA (10). Aliquots from each preparation were also counted and sized to determine the recovery of islet equivalents from each NPI preparation (10).

For assessment of *in vitro* viability, a static incubation assay (10) was used to determine glucose stimulated insulin secretion of NPIs prepared using each protocol. Representative NPIs from each condition were incubated in duplicate at 37°C for 2 hours in 1.5 mL RPMI supplemented with 2.0 mM L-glutamine, 0.5% w/v BSA and either 2.8 mM (low) or 20.0 mM (high) glucose (15). Culture supernatant was collected,

stored at minus 20°C and measured for insulin at a later time by a porcine insulin immunoassay (Meso Scale Discovery, Gaithersburg, MD). Stimulation indices were calculated by dividing the amount of insulin released at 20.0 mM glucose by that released at 2.8 mM glucose.

Immunohistochemistry was used to determine the cellular composition of the NPIs. The avidin-biotin complex (ABC) method was used with peroxidase and diaminobenzidine as the chromagen. NPIs were dissociated into single cells mechanically with a siliconized Pasteur pipette in a solution of 0.05% trypsin, 0.5 mM EDTA and PBS (15). The single cells were then fixed in formaldehyde on glass slides and stained to determine the proportion of insulin, glucagon and cytokeritin-7 (CK-7) positive ductal cells. Primary antibodies (Dako Corp., Carpinteria, CA) included guinea pig anti–porcine insulin (1:1000), rabbit anti-glucagon (1:100) and mouse anti-human CK-7 antibody (1:200); biotinylated secondary antibodies and the ABC-enzyme complexes were purchased from Vector Laboratories (Burlingame, CA). Primary antibodies were incubated for 30 minutes at room temperature, while secondary antibodies were applied for 20 minutes.

Since we are assessing the mean DNA content in each sample analyzed, and we have previously determined the DNA content of NPI cells to be approximately 7.1 pg/cell (10), we then determined the total number of NPI cells recovered in each preparation, and by using this value and the following equation we also calculated total number of cells staining positive:

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

Essentially, this methodology allows us to accurately calculate the number of insulin-glucagon- and CK-7 positive cells.

4.3.3. Transplantation and metabolic follow-up

Following culture, NPIs from either the clinically applicable or standard

laboratory protocol were transplanted under the left kidney capsule of Halothane-anesthetized diabetic B6.Rag$^{-/-}$ mice (15). Mice were rendered diabetic by intravenous injection of 185 mg/kg body weight streptozotocin (Sigma-Aldrich) 4–5 days before transplantation. All recipients entering this study exhibited blood glucose levels above 20 mmol/L. Blood samples were obtained from the tail vein for glucose assay (OneTouch glucose meter; LifeScan Canada Ltd., Burnaby, British Columbia, Canada). Animals were maintained in climatized rooms with free access to sterilized tap water and pelleted food. Aliquots consisting of 2000 NPI IEQs were aspirated into polyethylene tubing (PE-50), pelleted by centrifugation, and gently placed under the kidney capsule with the aid of a micromanipulator syringe. Once the tubing was removed, the capsulotomy was cauterized with a disposable high-temperature cautery pen (Aaron Medical Industries, St. Petersburg, FL).

Transplanted mice were monitored for blood glucose levels once a week between 8:00 and 11:00 a.m. The graft was deemed a success when the blood glucose level was ≤ 8.4 mmol/L. At post-transplantation week 12, an oral glucose tolerance test (OGTT) was performed on all NPI recipients with normalized basal glycemia (n=20 from each of the standard laboratory and clinically applicable protocols). After a 2 hour fast, D-glucose (3 mg/g body weight) was administered as a 50% solution intragastrically into nonanesthetized mice. Blood samples were obtained from the tail vein at 0, 15, 30, 60, and 120 minutes. Following the OGTT, survival nephrectomies were performed on the graft bearing kidneys and the mice were monitored until hyperglycemia returned to ensure the reversal of diabetes was attributed to the graft and not β cell regeneration.

4.3.4. Statistical Analysis

All statistics were performed using the one-way analysis of variance with the Bonferroni correction for multiple groups, or the Student's t-test for two groups; the level of significance was considered to be α=0.05. All statistical analyses were performed

with STATA 11 (StataCorp LP, College Station, TX). Results are presented as mean ± standard error of the mean (SEM).

4.4. RESULTS

4.4.1. NPI preparation

NPIs were isolated using our previously published (10) standard protocol and cultured in 4 petri dishes per pancreas in Ham's F10-BSA (Figure 4.1A; n=16 isolations of 4 pancreases per isolation), or a modified scalable protocol and one petri dish per pancreas in Ham's F10-HSA-CI-PI with BSA replaced with HSA and addition of caspase (CI) and protease (PI) inhibitors (Figure 4.1B; n=4 isolations of 12 pancreases per isolation). As a control, NPIs isolated according to the standard laboratory protocol were also cultured in Ham's F10-BSA at a density of one petri dish per pancreas (n=6 isolations of four pancreases per isolation). Using the scalable protocol and Ham's F10-HSA-CI-PI, significantly more cellular insulin content was recovered (56.9±1.4 μg) when compared to the standard laboratory protocol and culturing in either four petri dishes (15±0.5 μg) or one petri dish per pancreas (9.0±0.3 μg) ($p<0.05$) (Table 4.1). A significantly higher proportion ($p<0.05$) of insulin positive β cells were also recovered using the scalable protocol (31±1.5%) compared to the standard protocol with either four petri dishes (21±0.2%) or one petri dish (24±0.8%) per pancreas (Table 4.1). There were no differences in the proportion of glucagon positive α cells between the three conditions (Table 4.1); however, there were significantly fewer ($p<0.05$) CK7 positive ductal cells in the scalable protocol compared to the standard protocol and culture in Ham's F10-BSA with either four or one petri dishes (Table 4.1). Similarly, using the scalable protocol and Ham's F10-HSA-CI-PI the total number of β cells ($10\pm0.4x10^6$) and islet equivalents (41,810±226) recovered per pancreas were also significantly higher ($p<0.05$) compared to the standard protocol and Ham's F10-BSA when cultured using either four ($6\pm0.2x\ 10^6$, 35,135±186, respectively) or one ($7\pm0.2x\ 10^6$, 38,649±254,

respectively) petri dish (Table 4.1). In contrast, there were no differences between numbers of glucagon positive α-cells recovered per pancreas between the three protocols (Table 4.1). However, there were significantly fewer ($p<0.05$) CK7 positive cells recovered per pancreas using the scalable protocol and Ham's F10-HS-CI-PI ($4\pm0.1x10^6$) when compared to standard protocol and Ham's F10-BSA in either four ($8\pm0.2x10^6$) or one petri dish ($8\pm0.8x10^6$).

4.4.2. NPI insulin secretory responsiveness in vitro

During a static glucose stimulated insulin secretion assay significantly more insulin was secreted at high glucose (20.0 mM) compared to low glucose (2.8 mM) in all three experimental conditions (Table 4.2). I addition, no statistically significant differences were noticed amongst all experimental conditions in the amounts of insulin secreted at both low and high glucose (Table 4.2). When calculating the stimulation indices there were no significant differences between stimulation indices of NPIs cultured according to the standard protocol in either four (SI: 4.3±0.2) or one (SI: 4.4±0.2) petri dish per pancreas. However, when compared to both standard protocol conditions, those NPIs isolated and cultured using the scalable protocol exhibited a significantly higher ($p<0.05$) stimulation index (5.5±0.1; Table 4.2).

4.4.3. Transplantation of NPIs in diabetic mice

All mice transplanted with NPIs from either the standard protocol (n=20 mice) or the scalable protocol (n=20) achieved normoglycemia (Figure 4.2A). However, 100% of the mice transplanted with NPIs prepared from the scalable protocol became euglycemic within 8 weeks post-transplantation, while the mice implanted with NPIs using the standard protocol did not normalize until week 10 post-transplant. From week 2 until survival nephrectomies at week 12 post-transplant, mice grafted with islets from the scalable protocol had significantly lower glycemias than mice transplanted with NPIs prepared using the standard protocol ($p<0.001$). In both groups, removal of

the graft-bearing kidneys resulted in a rapid return to the diabetic state, indicating that the NPI grafts were responsible for normoglycemia.

Oral glucose tolerance tests performed at 12 weeks post-transplant demonstrated that mice transplanted with NPIs prepared using the scalable protocol had significantly lower blood glucose levels ($p<0.001$) at every time point during the OGTTs compared to mice implanted with NPIs using the standard protocol (Figure 4.2B). In both groups, the glycemia of each mouse 120 minutes after the bolus of glucose was not significantly different from the values at 0 minutes.

4.5. DISCUSSION

One challenge that must be overcome to allow transplantation of NPIs a clinical reality is defining a reproducible and scalable protocol for the efficient preparation of therapeutic quantities of clinical grade NPIs. In our previously published standard protocol (10) we isolated NPIs from maximally four neonatal porcine pancreases that required tissue culture in 16 petri dishes (four per pancreas) (Figure 4.1A). Furthermore, based our previously published non-human primate studies, at least 12 neonatal porcine pancreases will be required to reverse diabetes in human patients (19, 20).

We have now developed a more scalable and technically simpler protocol that allows us to isolate NPIs from minimally 12 pancreases at a time by employing automated tissue chopping, collagenase digestion in a single vessel and tissue culture/media changes in 75% fewer petri dishes (Figure 4.1B). In this new culture media BSA was replaced with the clinically acceptable human serum albumin (HSA) and the caspase and protease inhibitors were added since tissue from one pancreas was cultured in one not four petri dishes and is therefore significantly more concentrated. While the scalable protocol is faster, less laborious, more efficient, and has lower probability of contamination, it also significantly increased the recovery of total cellular insulin content, absolute number of β cells as well as islet equivalents recovered per

pancreas (Table 4.1). In addition, the scalable protocol also significantly improves the *in vitro* glucose stimulated insulin secretory response of the NPIs as indicated by higher stimulation indices after a static incubation (Table 4.2).

Moreover, the scalable protocol significantly ameliorates the restoration of glycemia and the glucose tolerance of transplanted mice compared to the standard protocol. This is likely due to the increased proportion of β-cells and insulin secretory capacity of NPIs isolated using the scalable protocol over the standard protocol. This enhanced function of NPIs isolated with the scalable protocol is potentially due to decreased β cell apoptosis as a result of the short term addition of the general caspase inhibitor (20-25). Pre-treatment of human (25) or adult porcine (24) islets with caspase-3 inhibitors for 24-48 hours by other groups show significant decreases in islet viability and function both *in vivo* and *in vitro*. The positive effect of 90 minute pre-treatment with Z-VAD-FMK in NPIs could be attributed to the fact that it is a general caspase inhibitor instead of only caspase-3 and exposure time was much shorter (21), as well as the inherent resistance of NPIs to apoptosis after hypoxia (14). Serine protease inhibitors have been demonstrated to have a protective effect on the membrane integrity of rat islets upon exposure to collagenase (26); this is likely another contributing factor to the improved viability and function of the islets.

Based on the above findings, this improved scalable protocol could be utilized to manufacture a clinical NPI product. This protocol could now be tested using clinically acceptable reagents such as replacing Ham's F10 tissue culture media and collagenase with Good Manufacturing Practice (GMP) certified products (27). GMP is a required regulatory approach recognized worldwide for ensuring the quality control of manufactured pharmaceuticals, blood products and medical devices. This designation guarantees that the cells and tissues will be consistently manufactured in strictly defined physical environments using high-quality systems and closely controlled procedures. The pancreatic chopper apparatus used in this study can be validated to

comply with these GMP standards, or replaced with an apparatus that has already received such certification (28). In addition to clinically approved reagents and protocols a GMP laboratory is required for producing live therapeutic cells and tissues for cell therapy treatments. FDA and Health Canada requires GMP certification for clinical grade cells and tissues produced for use in clinical trials. The protocol described in this study can thus be further adapted to comply with GMP regulations.

4.6. ACKNOWLEDGMENTS

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4.8. TABLES

Table 4.1. Comparison of neonatal porcine islets cultured in the standard culture protocol compared to a modified scaled-up protocol.

Condition	Content (μg)		Cellular Composition % (β /α / CK7)	Cell Number (10^6) (β /α / CK7 / Total)	IEQ
	Insulin	DNA			
A: Original Protocol 4 plates (n=16)	15±0.5*	208±6.8	21±0.2*/ 17±0.2 / 25±0.5	6±0.2*/ 5±0.2 / 8±0.2 / 31±1	35135±186*
B: Original Protocol 1 plate (n=6)	9±0.3*	227±10.6	24±0.8 / 21±0.4 / 25±0.8	7±0.2 / 6±0.4 / 8±0.8 / 30±1.6	38649±254*
C: Scalable protocol (n=4)	56.9±1.4	238±3	31±1.5 / 17±1.5 / 13±0.5	10±0.4 / 6±0.5 / 34±0.5	41810±226

Data are means±SEM. Islets were isolated and cultured using: A) our original protocol and 4 petri dishes per pancreas in standard Ham's F10 medium; B), our original protocol and 1 petri dish per pancreas in standard Ham's F10 medium; and C) our modified clinically scalable protocol and 1 petri dish per pancreas in modified Ham's F10 (BSA replaced with HSA and addition of caspase and protease inhibitors). *$p<0.05$ vs. modified protocol.

Table 4.2. Glucose stimulated insulin secretion of islets cultured in the standard culture protocol compared to a modified scaled-up protocol.

	% Cellular Insulin Content		
Condition	**2.8 mM**	**20 mM**	**SI**
A: Original Protocol 4 plates (n=16)	1.9±0.1	8.1±0.5	4.3±0.2
B: Original Protocol 1 plate (n=6)	1.8±0.1	7.9±0.3	4.4±0.2
C: Modified protocol (n=4)	1.5±0.04	8.2±0.05	5.48±0.1

Data are means±SEM. Islets were isolated and cultured using: A) our original protocol and 4 petri dishes per pancreas in standard Ham's F10 medium; B), our original protocol and 1 petri dish per pancreas in standard Ham's F10 medium; and C) our modified clinically scalable protocol and 1 petri dish per pancreas in modified Ham's F10 (BSA replaced with HSA and addition of caspase and protease inhibitors).

4.9. FIGURES

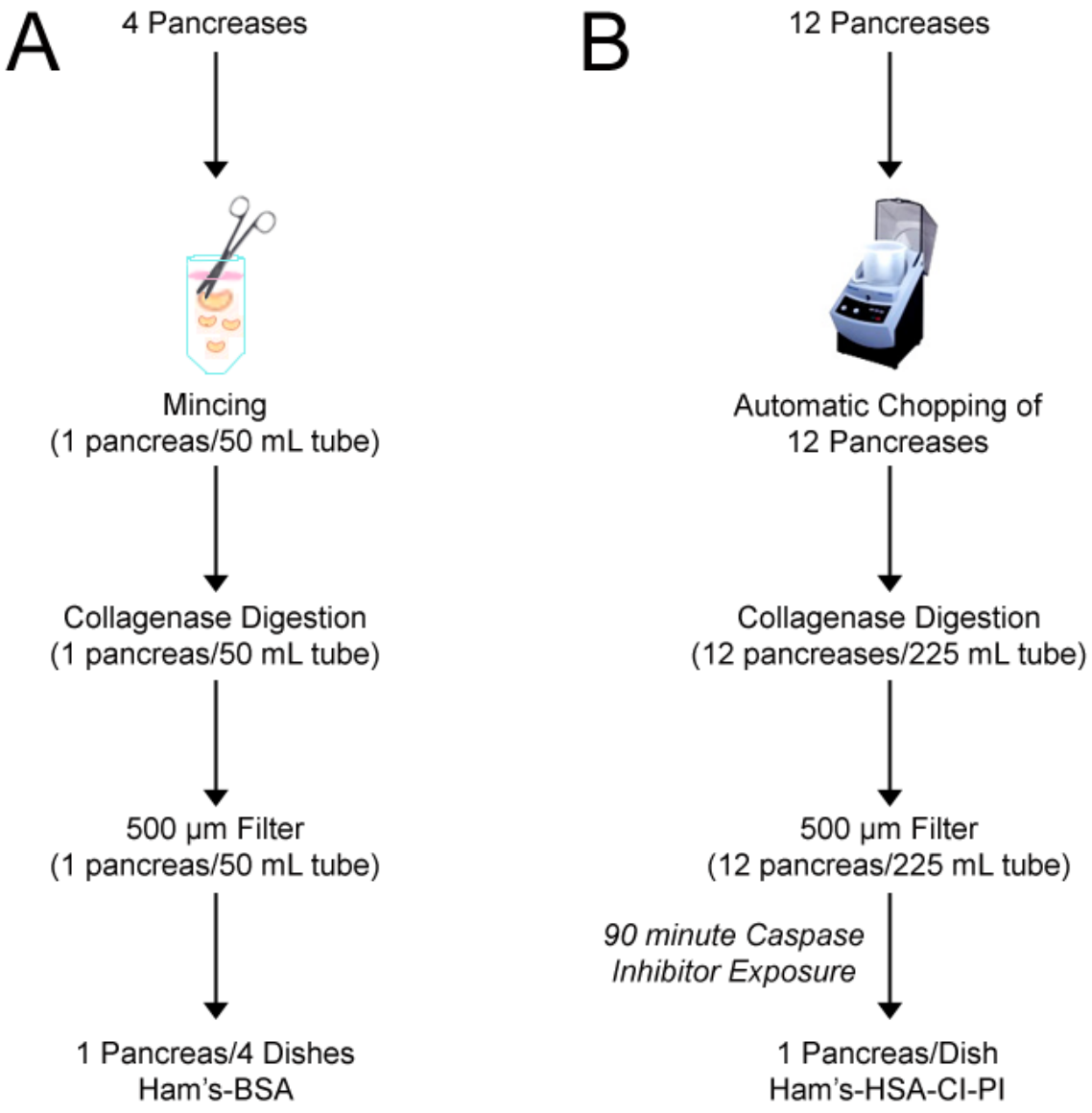

Figure 4.1. Schematic diagram of the isolation of neonatal porcine islets using our standard protocol (A) and cultured in Ham's-BSA (4 plates/pancreas) or a scalable protocol and cultured in Ham's-HSA-CI-PI (1 plate/pancreas (B).

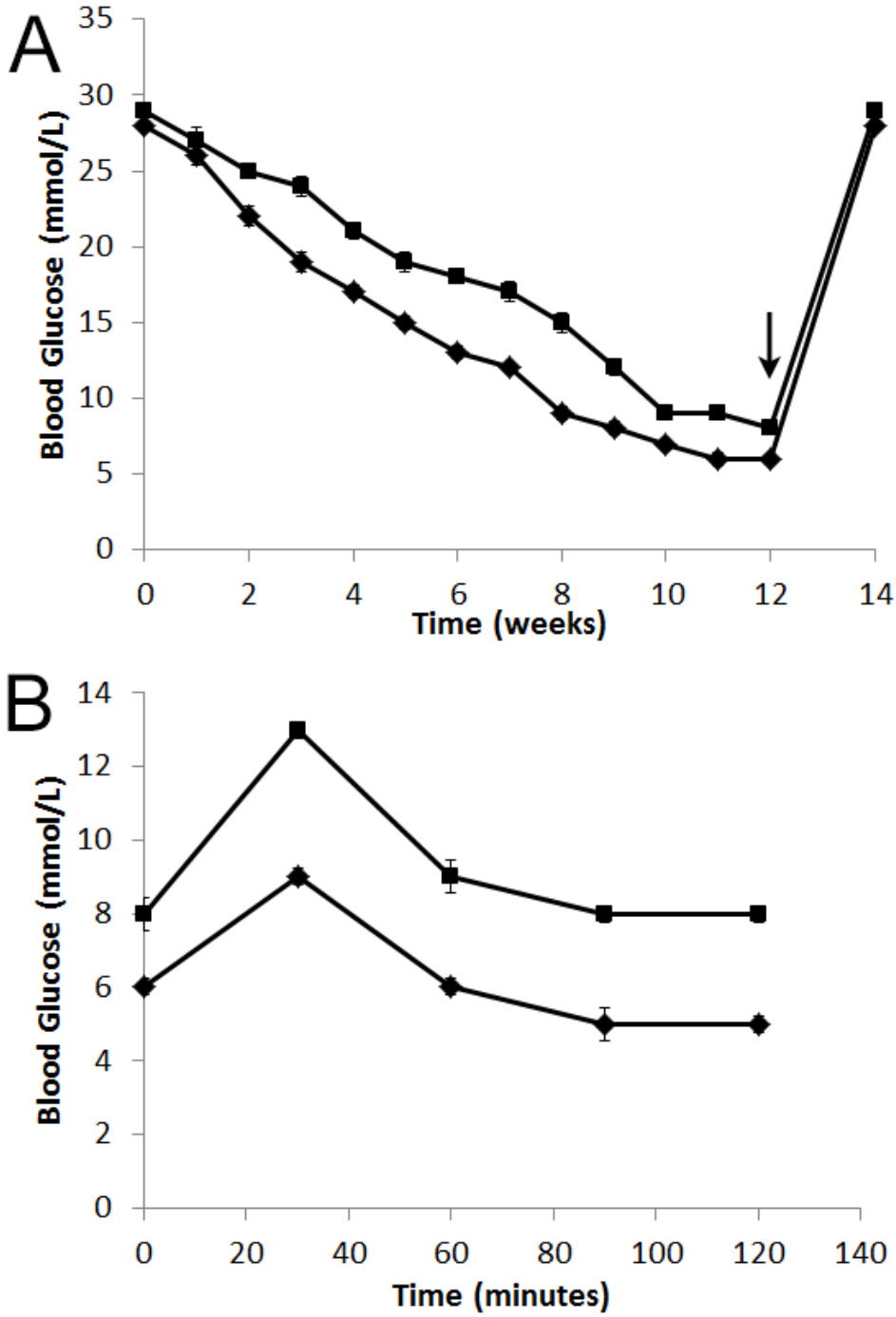

Figure 4.2. Blood glucose values measured weekly of (A) and during oral administration of glucose to (B, oral GTT) B6/Rag$^{-/-}$ mice transplanted with 2000 NPIs. Weekly blood glucose values of mice transplanted with neonatal porcine islets prepared from either our standard protocol (■; n=20) or clinically applicable protocol (◆; n=20). At week 12 post-transplant all mice underwent a nephrectomy of the graft bearing

kidney (arrow) to ensure a return to normoglycemia. (B) Blood glucose values during oral glucose tolerance tests in mice transplanted with neonatal porcine islets prepared from either our standard protocol (■; n=20) or clinically applicable protocol (◆; n=20). During weekly blood glucose measurements from 2 weeks to the survival nephrectomy (A) and at all time points during the OGTT blood glucose levels were significantly less in mice transplanted with NPIs prepared using the scalable protocol ($p<0.001$).

Chapter 5: Successful isolation of viable neonatal porcine islets from 24 hour cold-stored pancreases

5.1. ABSTRACT

In order to clinically transplant neonatal porcine islets (NPIs) the source pig will most likely need to be housed in a barrier facility that is Designated Pathogen Free (DPF). However, the transport of a DPF pig outside a barrier facility to a Good Manufacturing Practice (GMP) laboratory for NPI isolation while maintaining DPF status is considered complicated, not only with regard to infectious pathogen status but also with respect to regulatory compliance. Therefore, it is recommended that retrieving pancreases from these DPF pigs be conducted within the barrier facility and then shipped to a GMP facility for islet isolation. Routinely in human organ transplantation, organs are retrieved from donors and shipped to hospitals throughout a large region. In order to maintain viability of the transported organs they must be shipped on ice with specialized preservation solutions such as University of Wisconsin Solution. Therefore, we designed a study in which we collected pancreases from neonatal pigs and immediately isolated the islets whereas paired organs were stored at 4ºC in University of Wisconsin Solution for 24 hours (to mimic transportation) followed by islet isolation. The amount of cellular insulin recovered from islets isolated from cold stored neonatal porcine pancreases (17±2.0 μg) was comparable to NPIs isolated from those pancreases that were not cold stored (18±20.7 μg; $p>0.05$). Similarly, the total number of recovered β-cells ($6.0x10^{6}$±1.0 from cold stored vs. $7.0x10^{6}$±0.4 from non-cold stored pancreases; $p>0.05$) and islet equivalents (35,478±410 from cold stored vs. 36,149±621 from non-cold-stored pancreases; $p>0.05$) was also statistically comparable. When a glucose sensitive insulin secretion assay was used to measure islet function the calculated stimulation index (ratio of insulin secreted at 20 mM to insulin secreted at 2.8 mM after 2 hours) were also comparable at 4.9±0.4 for islets isolated from cold stored pancreases vs. 4.3±0.9 for islets isolated from non-cold stored pancreases ($p>0.05$). These data therefore

support the rationale for transporting pancreases from DPF porcine barrier facility to a GMP laboratory for the isolation of clinical grade neonatal porcine islets.

5.2. INTRODUCTION

Clinical islet transplantation has become increasingly successful since the development of the Edmonton protocol (1-3), with current results showing 3 year insulin independence rates approaching 50% (4) and at the very least improvements in quality of life, decreases in the burden of complications, and improved hemostatic and cerebral abnormalities (4, 5). However, challenges remain, including donor shortages, particularly due to the current requirement for multiple human donors (4). To address this supply issue, porcine islets are a strong contender as a potentially unlimited source. The possibility of genetically modifying the donor animals to decrease the required immunosuppression is an exciting advancement. Neonatal porcine islets are particularly well suited for clinical islet transplantation as they are resistant to hypoxia (6), human pro-inflamatory cytokines (7), hyperglycemia (8) and toxicity of islet amyloid deposition (9); additionally, because they are immature they can differentiate and proliferate (10).

As porcine islets are composed of live cells, they cannot be subjected to conventional disinfection used for medical devices prior to clinical transplantation. Therefore, the source pig must be depleted of infectious agents that can transmit to the recipient and cause disease. Among other requirements, regulatory guidance's specify that donor animals fulfill the Designated Pathogen-Free (DPF) status (11). Constructing and operating a closed pig herd with DPF status is extremely expensive; it is highly unlikely that many sites will have both a DPF facility and a current Good Manufacturing Practice (cGMP) laboratory connected such that the organ will remain inside a closed system. The transport of a DPF pig outside a barrier facility, while maintaining DPF status, is considered complicated, not only with regard to infectious pathogen status, but also with regard to regulatory compliance (12). Therefore, it is recommended that dissection of the pancreases from these DPF pigs be conducted

within the barrier facility, and then the organs shipped to a cGMP facility for development into a therapeutic product. Routinely in human organ transplantation, organs are retrieved from donors and shipped to hospitals throughout Canada. In order to maintain viability of the transported organs they must be shipped on ice with specialized preservation solutions such as University of Wisconsin (UW) Solution (13). This study examines the effects shipping would have on the viability and function of the NPI with the goal of developing a standardized protocol.

5.3. MATERIALS AND METHODS

5.3.1. Neonatal Porcine Islet Preparation

Donor pancreases were obtained from 1 to 3 day old Duroc neonatal piglets from the University of Alberta Swine Research Centre (1.5-2.0 kg body weight), and the islets were isolated and cultured for 5-7 days as described previously (10). Briefly, the retrieved pancreases were cut into 1 to 3 mm tissue fragments in separate 50 mL conical tubes, then exposed to 2.5 mg/mL collagenase, filtered through a 500 μm nylon screen, washed in Hank's Basic Salt Solution (HBSS) (Gibco, Burlington, Canada) supplemented with 0.25% bovine serum albumin (BSA) (fraction V; Sigma-Aldrich, Oakville, Canada), 10 mM HEPES (ICN Biomedicals, Inc., Costa Mesa, CA), 100 U/mL penicillin, and 0.1mg/mL streptomycin (Lonza Walkersville, Inc., Walkersville, USA). NPI were then cultured in non-tissue culture treated petri dishes containing Ham's F10 tissue culture media purchased from Sigma-Adrich supplemented with 14.3 mM sodium bicarbonate (Fisher Scientific, Ottawa, Canada), 10 mM D-glucose (EM Sciences, Hartfield, PA), 2 mM L-glutamine (Sigma-Aldrich), 0.25% BSA (fraction V; Sigma-Aldrich), 50 μM isobutylmethylxanthine (IBMX) (Sigma-Aldrich), 10 mM nicotinamide (Sigma-Aldrich), 1.6 mM calcium chloride dihydrate (Sigma-Aldrich), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Lonza Walkersville, Inc., Walkersville, USA). The islets were cultured at 37°C for 5-7 days, with the medium changed the first, third

and fifth days after isolation.

Pancreases from neonatal pigs were collected and divided into two paired groups; islets from the first group of pancreases were immediately isolated, whereas paired organs from the second group were stored at 4°C in University of Wisconsin Solution for 24 hours (to mimic transportation) followed by islet isolation as described above.

5.3.2. In Vitro Assessment of Islets

Following tissue culture, recovery of the NPI preparations was determined on the basis of cellular insulin and DNA contents as well as islet equivalents (10). All measurements were assessed from duplicate aliquots of the NPI suspensions. Cellular insulin content was measured after extraction in 2 mM acetic acid containing 0.25% BSA (10). Samples were sonicated in acetic acid, centrifuged at 800 g for 15 minutes, and then supernatants were collected and stored at -20°C until assayed for insulin content by ELISA (Boehringer Mannheim, Basel, Switzerland). For DNA content, representative 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 by Picogreen (Molecular Probes, Inc., Eugene, OR), a fluorescent nucleic acid stain for quantification of double-stranded DNA (10). Aliquots from each preparation were also counted and sized to determine the recovery of islet equivalents from each NPI preparation (10).

For assessment of *in vitro* viability, a static incubation assay (10) was used to determine glucose stimulated insulin secretion of NPIs prepared using each protocol. Representative NPIs from each condition were incubated in duplicate at 37°C for 2 hours in 1.5 mL RPMI supplemented with 2.0 mM L-glutamine, 0.5% w/v BSA and either 2.8 mM (low) or 20.0 mM (high) glucose (7). Culture supernatant was collected, stored at minus 20°C and measured for insulin at a later time by a porcine insulin immunoassay (Meso Scale Discovery, Gaithersburg, MD). Stimulation indices were

calculated by dividing the amount of insulin released at 20.0 mM glucose by that released at 2.8 mM glucose.

Immunohistochemistry was used to determine the cellular composition of the NPIs. The avidin-biotin complex (ABC) method was used with peroxidase and diaminobenzidine as the chromagen. NPIs were dissociated into single cells mechanically with a siliconized Pasteur pipette in a solution of 0.05% trypsin, 0.5 mM EDTA and PBS (7). The single cells were then fixed in formaldehyde on glass slides and stained to determine the proportion of insulin, glucagon and cytokeritin-7 (CK-7) positive ductal cells. Primary antibodies (Dako Corp., Carpinteria, CA) included guinea pig anti–porcine insulin (1:1000), rabbit anti-glucagon (1:100) and mouse anti-human CK-7 antibody (1:200); biotinylated secondary antibodies and the ABC-enzyme complexes were purchased from Vector Laboratories (Burlingame, CA). Primary antibodies were incubated for 30 minutes at room temperature, while secondary antibodies were applied for 20 minutes.

Since we are assessing the mean DNA content in each sample analyzed, and we have previously determined the DNA content of NPI cells to be approximately 7.1 pg/cell (10), we then determined the total number of NPI cells recovered in each preparation, and by using this value and the following equation we also calculated total number of cells staining positive:

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

Essentially, this methodology allows us to accurately calculate the number of insulin-glucagon- and CK-7 positive cells.

5.3.4. Statistical Analysis

All statistics were performed using the one-way analysis of variance with the Bonferroni correction for multiple groups, or the Student's t-test for two groups; the level of significance was considered to be α=0.05. All statistical analyses were performed

with STATA 11 (StataCorp LP, College Station, TX). Results are presented as mean±standard error of the mean (SEM).

5.5. RESULTS

Islets were isolated using our previously published original protocol (10) immediately following organ retrieval or after 24 hour storage at 4°C in University Wisconsin (UW) Solution. The amount of cellular insulin recovered from islets isolated from cold stored neonatal porcine pancreases (17±2.0 μg) was comparable to NPIs isolated from those pancreases that were not cold stored (18±20.7 μg; $p>0.05$) (Table 5.1). Similarly, the total number of recovered β cells ($6.0x10^{6}$±1.0 from cold stored vs. $7.0x10^{6}$±0.4 from non-cold stored pancreases; $p>0.05$) and islet equivalents (35,478±410 from cold stored vs. 36,149±621 from non-cold-stored pancreases; $p>0.05$) was also statistically comparable. When a glucose sensitive insulin secretion assay was used to measure islet function the calculated stimulation index (ratio of insulin secreted at 20 mM to insulin secreted at 2.8 mM after 2 hours) were also comparable at 4.9±0.4 for islets isolated from cold stored pancreases vs. 4.3±0.9 for islets isolated from non-cold stored pancreases ($p>0.05$) (Table 5.2).

5.6. DISCUSSION

Divers methods have been reported for the preservation of the porcine pancreas before islet isolation. Many of these methods focus on preserving the adult porcine pancreas, which is particularly sensitive to cold storage. These studies use techniques such as the two layer method or persufflation to attempt to achieve islet yields similar to a non-cold stored pancreas (14-16). Advantageously, our data show no difference between the cold-stored and non-cold-stored pancreases without these complex methods.

We did not perform an initial vascular flush with UW solution as is commonly done with human or adult porcine organ donors. Because the neonatal porcine pancreas

is much smaller than a juvenile or adult porcine pancreas, the initial exposure to 4°C UW solution after dissection was sufficient to keep the warm ischemic time less than three minutes as evidenced by the similarity of the islets isolated from the cold-stored pancreases to those isolated from non-cold stored pancreases. This will save resources including surgical time and costly cold storage solutions. Additionally, superior islets are isolated from cold-stored adult porcine pancreases when the pancreases are preloaded with collagenase (15); this is also not necessary with neonatal porcine islets, allowing dissection of the pancreas to be performed even more quickly as the pancreatic duct does not need to remain intact for cannulation. Although there are many strategies that could be adapted into the simple protocol described in our study, these are clearly not necessary for this neonatal porcine model as the results of cold storage do not differ from a freshly isolated pancreas and thus further improvement is unlikely.

While cold storage with UW solution, the current gold standard for organ transportation, is definitively supported by these data, the question remains of how to transport the organ from the biosecure DPF facility to a cGMP laboratory. The same standards for safety of human organs for human transplantation will likely apply to transported neonatal porcine pancreases, including appropriate packaging to maintain the organ integrity, temperature, and sterility; and labelling, including donor information such as DPF status, and the storage solution. While there is no gold standard for packaging for human organ transportation, the FDA approved LifePort® platform has shown promising results with juvenile porcine pancreases (15). However, as is the case with human organ transportation, it may be that inexpensive insulating materials are sufficient. As the porcine donors will have far less variability than human donors, it may be useful to standardize packaging materials; it will also be more feasible than for human donors as the organs will likely be released from only a small number of DPF source herds. Further work is required in this area to develop a definitive

standardized operating protocol for transporting neonatal porcine organs to a cGMP laboratory.

The cold storage protocol developed in this study could also be combined with the protocol developed in Chapter 4; it is possible that the superior islets obtained from the scalable protocol would be more sensitive to the cold storage protocol, and the combination of the two protocols would improve the clinical applicability of NPIs. It would also be valuable to transplant islets isolated after cold storage into diabetic recipients to ensure they restore normoglycemia. However, it is clear that the protocol developed in this study has the potential to allow the transport of pancreases between sites in a clinically applicable manner.

5.7. ACKNOWLEDGMENTS

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5.8. TABLES

Table 5.1. Effect of 24 hour UW cold storage on neonatal porcine islet cell recovery.

Condition	Content (μg)		Cellular Composition % (β /α / CK7)	Cell Number (10^6) (β /α / CK7 / Total)
	Insulin	DNA		
Control (n=4)	17±2	222±27	22±1 / 16±1 / 24±2	6±1 / 5±1 / 8±1 / 31±4
UW stored (n=4)	18±0.7	227±26	23±2 / 20±1 / 25±2	7±0.4 / 6±1 / 8±2 / 30±4

Table 5.2. Glucose stimulated insulin secretion of islets isolated from control and 24 hour UW cold stored neonatal porcine pancreases.

	% Cellular Insulin Content		
Condition	**2.8 mM**	**20 mM**	**SI**
Control (n=4)	1.8±0.2	7.8±2.1	4.3±0.9
UW stored (n=4)	1.6±0.2	8.0±0.7	4.9±0.4

Chapter 6: Justifying Clinical Trials for Porcine Islet Xenotransplantation

6.1. ABSTRACT

The development of the Edmonton Protocol encouraged a great deal of optimism that a cell-based cure for type 1 diabetes could be achieved. However, donor organ shortages prevent islet transplantation from being a widespread solution as the supply cannot possibly equal the demand. Porcine islet xenotransplantation has the potential to address these shortages, and recent pre-clinical and clinical trials show promising scientific support for xenotransplantation being a possible cure. It is therefore important to consider whether the current science meets the ethical requirements for moving towards clinical trials. Xenotransplantation is a therapy with great potential to benefit many people and improve global health equity. Despite the potential risks and the scientific unknowns that remain to be investigated, there is optimism regarding the xenotransplantation of some types of tissue, and enough evidence has been gathered to ethically justify clinical trials for the most safe and advanced area of research, porcine islet transplantation. Researchers must make a concerted effort to maintain a positive image for xenotransplantation, as a few well publicized failed trials could irrevocably damage the public perception of xenotransplantation, and because all of society carries the burden of risk it is important that the public be involved in the decision to proceed. As new information from pre-clinical and clinical trials develops, policy decisions should be frequently updated and if at any point evidence shows that xenotransplantation is unsafe, or alternatives are created that are superior, then clinical trials will no longer be justified and they should be halted. However, as of now the expected benefit of an unlimited supply of islets, combined with adequate informed consent, justifies clinical trials for islet xenotransplantation.

6.2. INTRODUCTION

The transplantation of human cells and tissues in patients with end-stage organ failure is a life-saving medical procedure that has become more effective with breakthroughs in immunosuppressive protocols and post-operative care. However, particularly because of this potential to transform the patient from being desperately ill to relatively healthy, difficult decisions must be made to determine which patients should get the limited supply of human organs and who will have to remain sick and perhaps die. Efforts have been made to address the organ donor shortage from many directions, including attempting to increase organ donor recruitment from the general population, but in 2012, 4612 Canadians were waiting for some kind of organ transplantation while only 1079 organs were donated (1). There is an unacceptably high rate of patient death while waiting for transplantation (2); in 2012, 230 Canadians died while waiting for an organ transplant (1).

The ideal treatment for many patients would be to bioengineer genetically matched tissues, but this technology is only beginning to be developed and will not be clinically available for many years. Another source must be found to address the immediate need for life-saving organs. A clinically relevant, unlimited supply could potentially be found in non-human organ donors, specifically pigs. The United States Food and Drug Administration (FDA) defined xenotransplantation in 2003 as "any procedure that involves the transplantation, implantation, or infusion into a human recipient of either (i) live cells, tissues, or organs from a nonhuman animal source or (ii) human body fluids, cells, tissues or organs that have had ex vivo contact with live non-human animal cells, tissues, or organs" (U.S. Food and Drug Admininstration, 2003), a widely accepted definition (Cozzi, et al., 2009). Perhaps because of the great potential to prevent death and improve quality of life for millions of patients, xenotransplantation is a highly controversial issue with many unique scientific, ethical, and legal issues.

From the late 1990s to the early 2000s there were discussions from many groups about the issues related to xenotransplantation, and some placed moratoriums on xenotransplantation (Cozzi, et al., 2009). In 2001, Health Canada and the Canadian Public Health Association utilized deliberative citizens fora modeled after a citizen's jury to pose the question, "should Canada proceed with clinical trials on xenotransplantation and if so, under what conditions?" The final recommendation of the project was that "Canada should not proceed with xenotransplantation involving humans at this time, as there are critical issues that first need to be resolved." According to the citizens involved in the discussion, the issues that required attention were exploring alternatives to xenotransplantation; the lack of pre-clinical trials and therefore knowledge on risk and efficacy of xenotransplantation; a need for clarified legislation and regulations for clinical trials; and the need for further education of Canadians on the complexities of xenotransplantation (3). Advances in pre-clinical trials in the past decade, and the development of regulations and recommendations by a variety of international groups warrant an updated discussion of Canada's xenotransplantation policy. Currently, no such national policy on clinical trials on xenotransplantation exists (4).

The development of the Edmonton Protocol in 2001 demonstrated the potential of islet cell transplantation to allow insulin independence in patients with type 1 diabetes (5). However, donor shortages remain a significant barrier to the widespread applicability of this therapy (6). Islet xenotransplantation is particularly relevant to the discussion of health policy as it is close to clinical trials in Canada; this therapy has already begun clinical trials in other parts of the world (7). An excellent recent discussion by Samy et al. explored the recent scientific progress of islet cell xenotransplantation as a therapy for type 1 diabetes (6). Samy et al. asked four questions, strikingly similar to those asked by the citizens' jury in 2000, about the scientific justifications for clinical trials; is there an applicable regimen? Is there an

established donor islet source? Are there eligible patients? and Are there other options with more favorable risk-benefit ratios? (6). The answers to these questions led the authors to conclude that the xenotransplantation community must be cautious and not allow enthusiasm to outweigh judgment as the immunosuppressive regimen has yet to be successfully defined and the specific genetic modifications of the source animals have not been perfected (6). However, the community must also not allow cautiousness to set overly stringent justifications for clinical trials. We do not require a perfect, fully developed model to move forward to treating the public; the minimum requirements for new medical technology are adequate informed consent and that its potential risks are outweighed by its potential benefits (8). In the case of xenotransplantation, the expected benefit to society is an unlimited source of islets for donation that could be used to treat the 39 million patients across the world suffering from type 1 diabetes (9).

6.3. EXPLORING ALTERNATIVES TO XENOTRANSPLANTATION

Islet transplantation is not the only biotechnology being explored as a treatment for type 1 diabetes. Samy et al. ask the same question as the citizens of Canada – what are the alternatives to xenotransplantation. Stem cells are a potential set of answers; since their famous 1963 discovery by Till and McCulloch (10), these cells have given rise to much optimism due to their ability to renew themselves and to become specialized cell types. There is a panoply of stem cell types that could potentially be utilized to treat diabetes, including endogenous adult stem cells, hematopoietic stem cells, and pluripotent stem cells.

A 2004 study first showed a clonal population of pancreatic precursor cells rising from the ductal cell population, possibly linked through a neural stem cell lineage (11, 12). The ability of human pancreatic duct cells to differentiate into β cells has been proven (13), but the protocol for β-cell engineering from this duct-derived population requires further development (14). It is also not clear that this precursor cell population

exists in diabetic patients, and if it does that it could be activated, or isolated and expanded ex vivo (15). Additionally, because auto-immune mediated destruction of β cells is characteristic of the disease, type 1 diabetic patients would still likely require immunosuppression to maintain the newly derived population. Alternatively, there is some evidence for replication of endogenous β cells; for example, a compensatory increase in β-cell mass is seen during periods of increased metabolic demand, such as obesity and pregnancy, due to a combination of replication, insulin secretion, and neogenesis (14). However, the mechanisms that control the β-cell cycle in the human pancreas are not yet fully elucidated, and immunomodulation to prevent destruction of newly formed β cells would still be required (14).

Hemopoietic progenitor cells, commonly isolated from bone marrow or umbilical cord blood, represent an easily accessible resource that could be used to modulate the initial autoimmune destruction of the β cells. A 2009 study showed improvement in C-peptide levels and insulin independence when autologous hematopoietic stem cells were transplanted in newly diagnosed type 1 diabetic patients (16). However, this therapy is likely only helpful in early diagnosis of diabetes and would not improve the health status of patients with long term disease.

Human embryonic (hESCs) or induced pluripotent stem cells (iPSCs) are a promising alternate strategy for cell therapy for Type 1 diabetes. Although momentous advances in differentiation protocols have been achieved this past year, the mass of cells produced using these protocols is low and certainly not yet an unlimited supply of stem cell derived beta cells (17, 18). Viacyte began in 2014 to recruit patients for a Phase I/IIa clinical trial utilizing the VC-01™ cell combination product, which is PEC-01™ stem-cell derived cells and the Encaptra® macroencapsulation and drug delivery system (19, 20). If this trial succeeds, it will begin to answer the many questions of safety and efficacy of using pluripotent stem cells in humans. A primary safety concern is the formation of malignant teratomas from undifferentiated cells in an

immunocompromised host; this concern is mitigated by macroencapsulation such as in the ViaCyte trial, and will continue to be decreased by further understanding of the differentiation protocol (17). It is also unclear whether β cells alone will be sufficient to cure diabetes in humans, or whether a complete islet is required (15). If human embryonic stem cells are utilized, ethical considerations on the destruction of a potential person must be considered; this has been discussed ad nauseum, particularly by politicians, but is still not yet fully resolved (21, 22). As mentioned above, bioengineering genetically matched tissue through the differentiation of iPSCs is an attractive and less ethically fraught alternative to hESCs transplantation. However, even if the β cells were genetically matched to the patient, the immune response when the cells are transplanted in patients with an autoimmune disease will likely mean that immunosuppression of some kind will still be required, with its associated toxicity.

Closed-loop insulin pumps, first developed in the 1980s, have the potential to reverse diabetes in a large patient population, but many challenges remain to be solved involving the insulin pump itself, glucose sensing, and control algorithms; these obstacles are thoroughly explored by Ricotti et al. (23). In November 2012, the FDA released a final artificial pancreas guidance document, based on recommendations by the Juvenile Diabetes Research Foundation (JDRF), demonstrating the commitment to and optimism for this technology. This guidance clearly details the FDA's expectations for human studies of artificial pancreas systems and established "time in range" as a potential endpoint measure of glycemic control. Subsequently, a 2013 randomized crossover controlled trial comparing dual-hormone artificial pancreases to conventional insulin pump therapy demonstrated significantly increased time in the target blood glucose range in the artificial pancreas system compared to the control group, and a decrease in the number of hypoglycemic events (24). That same year, a multicenter, multination, randomized crossover trial compared nocturnal glucose control in patients with either an artificial pancreas system or a sensor-augmented pump, showing

significantly fewer hypoglycemic events and less time below the target blood glucose range (25). The JDRF has funded the Artificial Pancreas Project (APP) since 2005, and has established a six step, three generation APP development pathway as a strategic funding plan and to define research priorities. These studies show promising first generation technologies, preventing unsafe high and low blood glucose levels and increasing time in range; however, a great deal of work is required before the third generation, fully automated multi-hormone closed loop artificial pancreas is feasible (26).

Despite some truly fascinating approaches to this biotechnology, including a sensorized insulin capsule that refills the implanted insulin reservoir after being swallowed (27), it is difficult to imagine developing a bioartificial pancreas that even begins to approach the complexity of the endocrine pancreas. As Ricotti et al. so aptly remind us, physiological glycemic control interprets data generated from substrates, hormones, paracrine compounds, and autonomic neural inputs and releases insulin with minimal delay (23). One of the most difficult aspects of bioartificial pancreas design is accurately sensing just one of the parameters, glucose. Perhaps these exciting biotechnologies can be combined with cell therapies to offer short term alternatives to islet transplantation, but it seems clear that the potential benefit of xenotransplantation is at the very least equal to these alternatives.

6.4. RISK AND EFFICACY OF XENOTRANSPLANTATION

One key risk for xenotransplantation is zoonosis, the cross-species transmission of a porcine disease to humans, either directly or through a third party. Therefore, microbial status of the source pigs is a major aspect of regulatory oversight (28). Sentinel animals can be tested regularly and frequently to ensure no infectious agents are present in the cells and tissues to be transplanted (29). With careful screening and designated pathogen free (DPF) breeding and housing, all potentially zoonotic pig

microorganisms should be removed, but there may also be unknown endogenous retroviruses that would not be identified until after transmission to the patient (30). The only known endogenous retrovirus is the porcine endogenous retrovirus (PERV), and studies have shown that subtypes of PERV can infect human cells (31). The AIDS pandemic has demonstrated the disastrous effects of zoonotic retroviruses, so it is extremely important that a similar situation be avoided for porcine xenotransplantation (32). There have been no reported transmission of PERVs in the past decade of porcine to human xenotransplantations, or in pre-clinical animal models (see (30) for a review), but because of the serious potential for harm to not only the patient receiving the transplantation but humanity as a whole, strategies are being developed to prevent the transmission of PERVs after xenotransplantation (29).

To further decrease the demonstrably low risk of zoonotic infection, careful handling of the source animals and tissue will be very important. In order to have DPF status, the herd must be free of a specified and comprehensive list of bacteria, fungi, protozoa, and viruses (33), and there must be meticulous documentation and standard operating procedures to maintain this status (34). If the animals are housed in a biosecure barrier facility, DPF status can be maintained and the donor organs and cells will be less likely to cause infectious complications than their allogeneic counterparts (28). Feed restrictions should also be incorporated into the animal husbandry procedures, as although prion disease is rare in pig species one suspected albeit unproven case has been documented (35). Thus, the feed should be free of mammalian material, as well as herbicides and pesticides, and the source animal should be on this certified feed for more than two generations before being considered as donors for xenotransplantation (34).

Once the donor tissues are procured from the source herd in a controlled, sterile operating room, the manufacturing of the xenotransplantation product must be carefully handled so that a therapeutic product can be made safely and reproducibly

(36). Manufacturing facilities must therefore comply with established current Good Manufacturing Practices (cGMP), and only organs that comply with stringent acceptance criteria should be considered (36). The tissues must be transported to the manufacturing facility or the clinical site under conditions such that the product remains sterile, viable, and potent. A sample of the final product should be tested for sterility before transplantation and PERV expression using *in vitro* co-culture with human cells or other techniques (37). Particularly for cells that require extra handling for isolation and subsequent culturing, such as pancreatic islets, the media and solutions used must have endotoxin levels below a safe, specified value (36). A sample of the final product should be characterized and tested for viability and potency, and samples should be stored against the possibility that future analysis is necessary (36). Although not all tests may be conducted prior to transplantation if the need for the xenograft is urgent, it is still important to maintain documentation that the product is well characterized and safe according to international and national regulations.

Although the worst case risk assessment of xenotransplantation is dire indeed, the current science demonstrated that the actual risk is quite low. Therefore, once safety is established, the efficacy of a porcine islet xenotransplantation must be considered. Porcine islet transplantation is by no means a new idea. In 1994, Groth et al. reported the transplantation of fetal porcine islet-like cell clusters (ICC) in ten insulin-dependent diabetic kidney-transplant patients (38). These ICC secreted detectable levels of porcine C-peptide in urine up to 400 days, and in one case insulin and glucagon staining was detectable in a renal-graft biopsy. In 2006, two groups published results demonstrating the survival of functional porcine islet grafts in diabetic non-human primates for longer than 3-6 months with immunosuppression (39, 40), and subsequent studies with transgenic porcine islets have shown islet survival for more than one year (41). Encapsulation of islets in an alginate matrix without immunosuppression has also demonstrated effectiveness in non-human primates (42) and humans (7). Neonatal

porcine islets are a focus as a source because they are easy to isolate and can proliferate after isolation, they are more resistant to injury than adult islets (43), and genetically engineered islet-source pigs could be utilized within a few days of birth, greatly decreasing housing costs in perhaps prohibitively expensive DPF facilities (44). Based on these pre-clinical and clinical trials, there is demonstrated scientific justification to expect a benefit from transplanting porcine islets into human diabetic subjects, particularly those with episodes of hypoglycemic unawareness who are currently eligible for allogeneic islet transplantation (45) as there is a limited supply of high quality human islets for transplantation in these patients. The therapy appears to be safe with no evidence of zoonotic infections, and because cells are being transplanted instead of whole organs there is likely to be a lesser immune response compared to whole tissues.

These pre-clinical data were considered enough evidence of benefit for the New Zealand Ministry of Health to approve a phase I/IIa clinical trial in 2009 for the transplantation of porcine islets in human subjects based on perceived safety as the source animals were DPF and housed in a biosecure barrier facility, the islets were produced in a GMP facility, and there is an accredited diagnostic laboratory for viral follow-up. The primary objectives for these clinical trials are to demonstrate safety and efficacy through an improvement in blood glucose levels. Although the New Zealand clinical trial is not yet completed, the most recent results show safety with an absence of PERV transmission, a reduction in the number of unaware hypoglycemic episodes, and an up to 30% reduction in exogenous insulin dosage (7).

If it is unlikely that close contacts or the general human population will be infected by PERV or a similar microorganism, and there is an expected benefit based on pre-clinical and clinical data, the risk of harm to the patient who receives the transplantation must be considered. Each case must be assessed individually to determine whether the expected benefit to the patient is greater than the potential risk, and again long term

monitoring will be vital to protecting the both patient and the general populace (2). The first potential patients for clinical trials should be chosen such that they are likely to benefit, the patient is not eligible for allotransplantation or the wait is untenable, and graft failure is unlikely to compromise the patient's ongoing management (6, 46); in these cases, the risks can be justified.

The well-described idea of a welfare baseline suggests that a person's normal welfare position is favorable, and a person who requires an organ transplantation is below their normal baseline state because of their illness (47). Xenotransplantation as an intervention would likely save their life and restore the patient to the baseline state, a decided benefit; if the organ was rejected, the patient would not be in a worse state than prior to the transplantation as they would not be sensitized to future allotransplantations, and therefore should remain entitled to receiving human organs to the same extent they were before the trial (48). The risk of zoonotic infection is small so they would not likely be made sicker and thus worse off than their baseline state prior to the intervention even if the intervention failed. Therefore, until more empirical information is discovered through pre-clinical and clinical trials, in select cases more harm may be done to the patient and the public by failing to perform a xenotransplantation than by involving the patient in a clinical trial. As clinical trials progress and more is understood about the long term immunological, physiological, and microbiological aspects of xenotransplantation, the benefits or harms to the patient and the public may be further elucidated. Although there are risks, some unknown, for moving forward in clinical trials for NPI xenotransplantation, these risks are justified if the benefits are expected to be comparable to human islet allotransplantation, with the advantage of immediate tissue availability, and the risks are minimized using regulations and surveillance, with careful and explicit informed consent.

6.5. LEGISLATION AND REGULATIONS FOR CLINICAL TRIALS

There are already some regulations and recommendations in place in many countries, including those for DPF source pig herds. The most comprehensive regulatory frameworks have been developed in the US, Europe, and New Zealand, and the WHO sponsored a process to develop the Changsha Communiqué as an internationally applicable model (for a more detailed review than presented here, see (49)).

In the United States, five agencies within the US Department of Health and Human Services (HHS) have been involved with the establishment of regulations, including the FDA, the Centers for Disease Control and Prevention, and the National Institute of Health. The resulting guidelines demonstrate the commitment of the US to creating a robust, empirical regulatory framework. The 1996 "US Public Health Service draft guideline on infectious disease issues in xenotransplantation" first made clear the regulations that would minimize the risk of xenogeneic infections, and this document was followed by two public meetings in 1997 and 1998 (49). In 2001 the FDA released an updated guideline on infectious disease issues (50), and in 2003 a more thorough document entitled the "Guidance for industry: source animal, product, preclinical, and clinical issues concerning the use of xenotransplantation products in humans" (34). Considerations for source animal regulations from this guidance statement include animal welfare and husbandry issues, herd and source animal origin, DPF status of the herd, and qualifications for the facilities and personnel.

The US document also recommends regulations for harvesting the xenotransplantation products, transporting these products and the source animals, archiving of samples, herd records, and restrictions of the fate of by-products from the source animals (49). The US regulatory framework also stipulates guidelines for clinical issues, including an exemplary peer-reviewed national regulatory template for clinical trial protocols and sites(34). Review criteria include patient selection; patient and close

contact education, counseling, and informed consent; an individualized risk/benefit assessment; careful screening for infectious microorganisms; long term patient follow-up; and archiving of patient samples for up to 50 years to allow epidemiologic and public health risk analysis (34).

The 2008 Changsha Communiqué that resulted from the First World Health Organization (WHO) Global Consultation on Regulatory Requirements for Xenotransplantation Clinical Trials began the process of creating uniform international regulations as guidelines for national policies, focusing on safety in source animals, adequate pre-clinical data, careful selection of trial participants, and long-term patient follow-up and sample archiving (described in(49)). The Communiqué included a call for action for the establishment of consistent, effective regulation of clinical xenotransplantation trials in all member states (49). The document encourages the WHO to offer resources and support to member states developing regulations, to help educate the publics of member nations about the risks and benefits of xenotransplantation, and to maintain a registry of xenotransplantation trials; it also encourages member states to take immediate steps to begin regulating xenotransplantation practices within their jurisdictions and to ban unregulated trials(51). A coordinated international effort for uniform ethical and regulatory standard for xenotransplantation clinical trials is integral to minimizing the risks to society(49).

6.6. EDUCATING CANADIANS

Because xenotransplantation can potentially affect more than just the recipient, and because of its controversial nature, it is important for the public to be involved in the decision to move forward in clinical trials. Deliberative democracy is a form of democracy that can be used to make legitimate decisions on controversial issues that affect the public (52). It has been argued that technology decisions should be made more democratically (53), and xenotransplantation is certainly a new biotechnology.

With xenotransplantation, deliberative democracy is a form of informed consent for the public as it involves citizens from a range of social and ethical values and educates them thoroughly on the scientific background required to make a decision (54), and because of the risk to the public from clinical trials for xenotransplantation it is important to obtain this third party consent (55). The public fora in Canada in 2000 previously mentioned were a deliberative approach to deciding whether xenotransplantation was ready for clinical trials (3). The citizen's jury model utilized for the deliberative process was comprised of six fora of 15 to 18 lay citizens from a balanced demographic held at six locations across Canada to ensure a balanced geographic profile. First, the participants met for an orientation session where they viewed a 50 minute video on xenotransplantation. The following day, a meeting was held wherein experts gave an eight minute presentation followed by questions from the panelists and the general public, who were invited to attend the meeting. A two hour discussion between the citizen participants and the experts followed the public session. The third days of the fora were spent in small groups and in plenary where the citizens deliberated until final positions on the policy question were recorded (3). The overall conclusion was that there was not enough evidence at the time to justify clinical trials, alternatives should be explored, Canadians should be further educated on the issues, further pre-clinical research needed to be conducted, and more stringent legislation and regulations needed to be developed.

Fourteen years later, there have been advances in all areas of xenotransplantation and there is cause to ask for new input from the public. As Sobbrio and Jorqui discuss, it is important to be clear about who is included as the "public" (56). In New Zealand, following an application for approval for clinical trials from Living Cell Technologies (LCT) for their encapsulated porcine islets, the National Health Committee requested public opinion on the application. Unlike in Canada, this was not done using a citizen's model, but rather through submissions from both individuals and organizations,

including LCT, Diabetes New Zealand, New Zealand Society for the Study of Diabetes, Diabetes Youth New Zealand, and various groups representing the Maori population (57). The NHC report noted that while there are no requirements mandating public involvement in decisions regarding trials that involve risk to the public, it is nonetheless important that such involvement is advantageous to promote transparency (57). Stakeholders, such as industry and patient advocacy groups, clearly have much invested in advancing technologies that directly improve their situations; however, the risk is shared by all citizens, albeit not equally. For this reason, any public consultation must engage a public that represents all the citizens of Canada, with information and education provided by patients, health professionals, scientists, industry, and other stakeholders such that the citizens can give informed consent on behalf of the country.

Critics of the Health Canada and Canadian Public Health Association citizen's jury state that the question posed to the public was too vague, and that each organ should be treated differently; because of Canada's large geography, the quality of experts varied broadly between regions, which led to a varied response from the public; and the public were not given sufficient information to make meaningful comment on the issue (58). Because of its demonstrated low risk and high likelihood of benefit, and because safe and ethical clinical trials are already occurring in other countries, phase I/IIa islet xenotransplantation trials could progress prior to conducting a time-consuming public consultation; the results of these early clinical trials would be very helpful for the citizens participating in the consultation. As it is not practical to simultaneously consult the citizens of every nation, Canadian citizens should be consulted on whether or not xenotransplantation trials should occur in Canada in national deliberative democratic fora. Advancements in communications technology in the past decade, such as online video conferencing, would allow the same group of experts to speak to all fora located across Canada so that the citizens all receive the same quality of information on which to base their decisions. The same general principles apply for the xenotransplantation of

all types of tissues, but because the biotechnology is at different stages for different organs and cells the citizens should be asked to recognize the variety in risk profiles in their recommendations. The initial public consultation, as well as public consultations in Australia, the UK, and the US, should be used a basis for a second deliberatively democratic public consultation in Canada (4, 59).

6.7. SHOULD CLINICAL TRIALS IN XENOTRANSPLANTATION PROCEED?

There still remains the important question that Health Canada and the Canadian Public Health Association asked in 2001 – should clinical trials in xenotransplantation proceed, and if so, under what circumstances? In 2003, the International Xenotransplantation Association (IXA) released the "Position Paper of the Ethics Committee of the International Xenotransplantation Association" on the ethics of xenotransplantation. This position paper considers the ethical considerations relevant to xenotransplantation, including beneficence and risk/benefit analysis; respect for persons and informed consent; xenotourism; and the use of animals (2). With advances in the biotechnology, these issues remain relevant and can be reconsidered.

Samy et al. rightly suggest that the current science does not answer all possible questions regarding NPI xenotransplantation (6). It is difficult to make a decision based on the 'harm principle' as the potential harms are not yet known, and it is equally difficult to discuss the benefits (or the harms from not transplanting) because they have not been fully proven. Judgment must instead be made balancing the likelihood and gravity of the potential harms and benefits, and there must be significant expectation of benefit for xenotransplantation to move forward (2). Much is unknown about the long term effects of xenotransplantation, and unless clinical trials move forward much will remain unknown, but based on the current knowledge the potential benefits are convincing for NPI xenotransplantation and the risks seem unlikely to be realized.

The body of research for pre-clinical and clinical xenotransplantation trials is increasing, and with more information it appears that although the potential harm of an epidemic caused by PERV transmission is very grave, the likelihood of this occurring appears to be low (6, 7). It is important that the risks of zoonotic infections be predicted by scientific evidence and not fantastical fears (60). Many mitigating measures can be taken to further diminish these risks, including regulating GMP at all stages of animal breeding, housing, and organ or cell preparation; long-term monitoring of patients and close contacts; and maintaining preserved samples of the donor animals' tissues for study in the future if required. Extensive monitoring of xenotransplantation recipients in accordance with regulatory guidelines will be necessary and a variety of effective strategies have been developed for screening both donor animals and human patients (37).

6.8. CONCLUSIONS

Besides benefiting recipients and society, xenotransplantation will also decrease health disparities, a stated goal for many public health agencies including the Nuffield Council on Bioethics (48) and the World Health Organization (61). Health has long been considered an important aspect of justice as ill health prevents the pursuit of the goals an individual may want to pursue (62). Alan Williams' fair innings approach to health equity suggests that each member of society has the right to a 'certain level of achievement in the game of life' (63) such that they are free to live how they wish (64); the paucity of organs for transplantation prevents many members of society from that level of achievement, and is a definite health disparity that prevents every individual who requires an organ from having positive freedom. An unlimited supply of safe, functional organs would mean that every patient who requires a transplant can receive the intervention they need, and patients who are not eligible for transplantation based

on the current, restrictive criteria could have xenotransplantation as an option before they are as sick (2).

Xenotransplantation is a therapy with great potential to benefit many people and improve global health equity. Despite the potential risks and the scientific unknowns that remain to be investigated, there is optimism regarding the xenotransplantation of some types of tissue, and enough evidence has been gathered to ethically justify clinical trials for the most safe and advanced area of research, porcine islet transplantation. Porcine islet xenotransplantation could be utilized as a case study to explore long terms effects, and as the technology progresses for other organs that framework could be adapted and updated as evidence develops; thus, the benefits could be seen more quickly. Researchers must make a concerted effort to maintain a positive image for xenotransplantation, as a few well publicized failed trials could irrevocably damage the public perception of xenotransplantation, and because all of society carries the burden of risk it is important that the public be involved in the decision to proceed. As new information from pre-clinical and clinical trials develops, policy decisions should be frequently updated and if at any point evidence shows that xenotransplantation is unsafe, or alternatives are created that are superior, then clinical trials will no longer be justified and they should be halted. However, as of now the expected benefit of an unlimited supply of islets, combined with adequate informed consent, justifies clinical trials for islet xenotransplantation.

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Chapter 7: Conclusions

7.1. SUMMARY OF MY THESIS

In this thesis I performed studies focused on translating NPI cell therapy to the clinic as an alternate source to cadaveric human donor islets using the Edmonton protocol. This thesis addresses two issues related to this translation, namely the development of an alternate site with the potential for retrieval and of a clinically applicable protocol for transport, isolation, and culture of neonatal porcine islets. Using a collagen-based matrix designed using knowledge of the interactions between islets and ECM proteins, we detailed the ability to design such a site subcutaneously in Chapter 2. Specifically, this matrix contains chitosan, chondroitin-6-sulphate, and laminin, three copolymers chosen for their theorized ability to promote islet cell survival or vascularization. Chitosan is a natural polymer that has been shown to accelerate wound healing and vascularization (1), as well as increasing the resistance of the collagen-based matrix to enzymatic degradation. Chitosan also improves cell adhesion and antimicrobial properties of the matrix (1). Collagen-chitosan constructs are successful cell delivery vehicles that promote neovascularization (1). Chondroitin-6-sulfate is a sulfated GAG that, as described in Chapter 1, may bind and sequester growth factors for sustained release post transplantation (2). Chitosan may also regulate matrix elasticity and by creating ionic bonds between its numerous amine groups and the carboxyl groups of collagen, stabilizing the overall matrix structure (3). Laminin, an important basement membrane protein for islets, may aid in the retention of transplanted islets *in vivo* in addition to acting as a guidance molecule for vascularization and islet survival (4, 5). This matrix was crosslinked with EDC and NHS as opposed to other chemical crosslinkers such as genipin or gluteraldehyde; although these other crosslinkers were tested in early experiments, the approximately 30 mM EDC/NHS combination resulted in the best balance between matrix integrity, resistance to enzymatic degradation and islet survival (data not shown).

In Chapter 3, we continued exploring the properties of this collagen-based matrix by examining the effect of altering the crosslinking concentration on the matrix microstructure and other material properties, including *in vivo* vascularization after subcutaneous transplantation. We utilized three different concentrations of the EDC/NHS combination and observed definite effects on drug release, matrix integrity, nanotopography, resistance to enzymatic degradation and capacity for vascularization. The higher crosslinker concentrations demonstrated improved capacity for vascularization *in vivo* when transplanted subcutaneously in a murine model while having no negative effects on NPI survival. Combined with the results of Chapter 2, it is apparent that this collagen-based matrix may be tuned for various purposes, including islet delivery and prevascularization of an ectopic site.

The second objective of this thesis focused on developing a clinically applicable protocol for the isolation, culture, and transport of NPIs. Chapter 4 explored a clinically applicable and scalable modified protocol for the isolation and culture of NPIs. Replacing time consuming and laborious hand mincing with automated chopper and adding a general caspase inhibitor for 90 minutes plus a protease inhibitor cocktail during culture in one petri dish instead of four resulted in islets with improved insulin content, β cell mass, and glucose responsiveness both *in vitro* and *in vivo* in a murine model. This modified protocol allows for the isolation of islets from minimally 12 pancreases by employing automated tissue chopping, collagenase digestion in a single vessel and tissue culture and media changes in 75% fewer petri dishes. This protocol is therefore more cGMP compliant than the original protocol, with less chance of contamination as well as superior islets. Then, Chapter 4 detailed a cold storage protocol allowing the transport of pancreases from a DPF facility to a cGMP facility for isolation. We showed with no differences between islets isolated from cold stored pancreases and those isolated from pancreases immediately post-surgery. These two

protocols may be further modified, replacing reagents such as Ham's F10 with cGMP certified reagents (see section 7.2.3.)

Finally, in Chapter 6 we examined utilizing deliberative democracy to gain the informed consent of the public to begin clinical trials for xenotransplantation in Canada. If xenotransplantation is not first proven to be an effective therapy, it is clearly difficult if not impossible to justify the risks involved in clinical trials. The three major scientific barriers to efficacious xenotransplantation that must be addressed in pre-clinical trials are immunological, physiological, and microbiological. Immunological concerns include humoral and cellular rejection, both of which may be avoided or ameliorated by using genetically modified pigs, immunosuppression, or an encapsulation with small enough pores to protect the tissue from the immune response (6). With respect to the hyperacute rejection caused largely by the humoral response, genetically modifying porcine sources to knock out the specific galactose (Galα1,3Gal) antigen to which the natural primate antibodies bind has resulted in prolonged heart and kidney graft survival in non-human primates (7-9). Pigs without the galactose antigen are known as α1,3-galactosyltransferase gene-knockout (GTKO). Genetically modified (GM) pigs have also been developed that express human complement-regulatory proteins and therefore mediate the humoral response, extending porcine heart and kidney graft survival in non-immunosuppressed non-human primates (10, 11). These two approaches could be combined to overcome the initial antibody-mediated complement response (12).

The cellular immune response involves the activation of macrophages, natural killer cells, and antigen specific T-cells, as well as the release of various cytokines, small cell-signaling protein molecules. Again, GTKO donor cells incite a weaker cellular immune response in primates compared to wild-type, non-genetically modified cells; in fact, the response is similar to the immune reaction to human donor cells (13). Similar to the allotransplantation of human organs, the cellular immune response can be

controlled with the use of immunosuppressive drugs that are already clinically approved, but the ability to genetically modify the source animals provides an alternative approach. The pigs may be engineered to express an immunosuppressive gene, inducing local immunosuppression around the graft and reducing the need for exogenous immunosuppressive therapy (14). Specifically, genetically modifying porcine cells to express cytotoxic T-lymphocyte antigen 4 significantly reduced the primate cellular response to porcine cells (15).

With the advances in genetic modification, relatively long term xenograft survival has been achieved, and both hyperacute and acute rejection due to the humoral response are not usually seen, and the cellular immune response is not the primary cause of long term failure (6). The final immunological barrier to whole organ xenotransplantation is chronic graft failure associated with coagulation dysfunction between the porcine donor and the primate recipient (6). Coagulation dysfunction is any disorder related to the inability of the blood to clot, and can lead to consumptive coagulopathy wherein small clots form within blood vessels throughout the body (16, 17). This dysfunction can be corrected following transplantation of porcine hearts and kidneys by surgically removing the donor organ (18); therefore, organs such as porcine livers that have been shown to function adequately in the short term could be used as a bridge to allotransplantation for some patients to temporarily sustain the patient (17, 19, 20). Ideally, however, once the complex coagulation dysregulation response is better understood, the source pigs could be genetically modified with human anticoagulant or antithrombotic genes in order to completely remove this barrier (12). A secondary advantage to utilizing porcine organs as long term replacements or as bridges for allotransplantation for patients with fulminant organ failure is that recipients who have already received a human organ and are therefore allosensitized are no more likely to reject the porcine organ than humans without a xenograft; if the xenograft fails or, if the porcine organ is being utilized as a bridge, upon availability of a human organ the

patient will not be sensitized and thus he or she remains eligible for an allotransplantation (21, 22).

As the understanding of the immune response is improved and porcine to non-human primate xenografts can be studied in the longer term, the physiological barrier may be addressed. As humans and pigs are clearly different animals with different sizes, postures, and life-spans, there are differences between human and porcine organs that are not yet fully understood. However, some promising research showing physiological functionality in primates is being conducted at the pre-clinical stage. GTKO and hCRP porcine hearts have been shown to survive heterotopically, in conjunction with the primate heart, for 6-8 months in baboons (7, 8, 23), and other groups have shown that orthotopic porcine hearts can maintain the life of baboons as substitutes for the native heart for up to two months, with primary graft failure due to postoperative complications and not cardiac function the limiting factor (11). GTKO kidney transplants have functioned at an adequate level for up to 3 months (9, 24), however proteinuria (the excess secretion of serum proteins in urine, a sign of kidney damage) has been observed in every case after pig kidney xenotransplantation; this could be due to the immune response or a physiological incompatibility. Porcine livers have also been transplanted in non-human primate models, and although the recipient survival is approximately one week, an orthotopically transplanted porcine liver functions adequately during that time by producing porcine proteins that perform satisfactorily in the primate, at least in the short term (16, 19, 25-27). Porcine lung xenografts are severely damaged by rapid coagulation dysfunction despite efforts to extend survival through depletion of von Willebrand factor and pulmonary macrophages (28). Some whole organ xenografts have shown physiological efficacy at least in the short term, but it is not necessarily clear that porcine organs will function adequately in human recipients even once the coagulation dysfunction barrier is addressed.

Better experimental results demonstrating physiological efficacies have been observed in cell-based therapies wherein cells are transplanted instead of whole organs. This strategy could be advantageous as it may avoid the acute humoral rejection of the xenograft because of the lack of antibody deposition on vascular endothelial cells (Ekser, Gridelli, Verhoux, & Cooper, 2011). Porcine hepatocytes transplanted in the portal vein of cynomolgus monkeys survived for 243 days with acceptable graft function (29). Another potential target for xenotransplantation is porcine corneas. There is not currently a shortage of human cornea for the clinical demand in developed countries, but in less developed countries the supply is far outweighed by the demand (30). Corneas from wild-type pig donors survived in monkeys with only topical steroid treatment to the eye for several months (31), and corneas from genetically modified porcine donors promise to survive longer as they are protected from the humoral response (30). Finally, the greatest success in xenotransplantation has been seen with porcine pancreatic islet transplantation (32, 33), as discussed in Chapter 6.

If at least some types of xenografts are both viable and functional for at least some amount of time, there is justification for considering xenotransplantation as a therapy. However, it is extremely important to consider the microbiological barrier as it is the source of the most controversy and concern. Human viruses, including cytomegalovirus and Epstein-Barr virus, Hepatitis A, B, and C, and other microorganisms, including West Nile fever, rabies, toxoplasma and HIV, are currently transplanted with allogeneic organs and can cause complications in recipients (6). With proper handling of the source pig herd, these would not be transferred to the recipient because they would be eradicated from that herd; the microbial status of the source pigs is a major aspect of regulatory oversight (34). Sentinel animals can be tested regularly and frequently to ensure no infectious agents are present in the cells and tissues to be transplanted (6), and to maintain DPF status (35), as discussed in Chapter 6. There is a particular concern for zoonosis, the cross-species transmission of a porcine disease to humans, either

directly or through a third party. With careful screening and DPF breeding and housing, all potentially zoonotic pig microorganisms should be removed, but there may also be unknown endogenous retroviruses that would not be identified until after transmission to the patient (34). The only known endogenous retrovirus is the porcine endogenous retrovirus PERV, and studies have shown that subtypes of PERV can infect human cells (36). However, as discussed in Chapter 6, the actual risks for PERV transmission are demonstrated to be low (6, 37), and may be mediated using cGMP protocols.

Based on the potential for xenotransplantation to address the paucity of human organs for transplantation, and because NPIs are already being utilized for clinical trials in other countries, we believe that clinical trials for NPI xenotransplantation should proceed. The data generated by these necessary studies could then be provided to the public in a citizen's jury model, similar to the one held by Health Canada in 2001. Because technology has advanced significantly since 2001, it would be a trivial matter to broadcast expert testimony to many centers across Canada to ensure that each site receives the same quality of evidence.

Collectively, these studies demonstrate that NPIs have the potential to be utilized as a clinical cell therapy for type 1 diabetes, isolated and cultured following a clinically applicable protocol and delivered in a collagen-based matrix that can be further modified as required. This matrix could be readily retrieved if necessary. Furthermore, sharing of expertise and expenses across centers is feasible with a successful cold storage protocol, further improving the translational relevance of NPIs.

7.2. FUTURE DIRECTIONS

An obvious limitation of this thesis is that the ability of this collagen-based matrix with NPIs was not demonstrated to reverse diabetes in mice. Although NPIs were demonstrated to survive for up to 28 days, survival longer than this time point was not

examined. Based on previous studies of the subcutaneous space, however, it is likely that the NPIs would have superior viability and function if they were transplanted in a site with existing vasculature. Additionally, it would like be useful to protect the NPIs with an immunological barrier device such as alginate microencapsulation or in a device similar to the Viacyte Encaptra® macroencapsulation device; however, while the exploration of these technologies is highly important it is beyond the scope of this thesis.

7.2.1 Prevascularization

These data clearly demonstrate that the collagen-based matrices can become highly vascularized, allowing for NPI survival. Although islets can be supported in a collagen-based matrix as it becomes vascularized over time, it would be ideal to transplant the islets into an environment with an already established vascular system. As described, the negatively charged GAG side chains of chondroitin-6-sulfate are known to bind and sequester growth factors, so the matrix developed in this thesis may readily be modified in this manner. This approach has successfully been used in other bioengineered devices, with both natural and synthetic polymers, with prevascularization periods ranging from 14 days to 60 days (38-43). Growth factors such as fibroblast growth factor (FGF) (38, 39, 41) and vascular endothelial growth factor (VEGF) (40) as well as stromal cell-derived factor-1 (SDF-1) have demonstrated efficacy for presvascularizing transplanted biomaterials (44). VEGF has previously been shown to increase vascularization in collagen-based matrices, and it is known to bind to chondroitin-6-sulfate (45). SDF-1 is an alternative growth factor that has also been shown to improve recruitment of angiogenic cells in a collagen-based matrix *in vivo* (44). Delivering bFGF in gelatin microspheres intermuscularly in diabetic rats demonstrated prevascularization within 10 days (39). A stainless steel mesh with PTFE stoppers coated with aFGF was also successfully prevascularized (38). As it would be

technically very simple to add these or other growth factors to the matrix, this is a clear future direction that should be explored; insulin diffused more quickly out of matrices with lower crosslinking concentration, supporting the rationale to adjust crosslinker concentration as a technique for controlling drug release. Prevascularizing the matrix could also be achieved by transplanting a stem cell population to promote angiogenesis.

7.2.2. Co-transplantation with mesenchymal stem cells

As an alternative to growth factors, MSCs have been shown to promote vascularization in various models, including in a collagen-glycosaminoglycan scaffold wherein the mesenchymal stem cells were observed to adopt an endothelial phenotype, thereby enhancing vascularization (40). Moreover, MSCs have been also shown to have beneficial effects when co-transplanted with islets, such as promoting graft revascularization and prevention of islet allograft rejection (46-49). In particular, the immunomodulatory and anti-inflammatory cytoprotective factors secreted by MSCs have been shown to protect islets from pro-inflammatory cytokines (46). The properties of the collagen-based matrix could then be tuned to support MSC viability and function; investigations into the best collagen-based matrix for MSCs are therefore warranted. MSCs may also modulate the autoimmune destruction of β cells and therefore preserve existing β cell function (50); however, the immunological interactions with β cells or with the NPIs are explored elsewhere are and beyond the scope of this thesis (33, 51-54).

7.2.3. Improving clinical applicability of NPI isolation and culture protocol

The protocol developed in Chapters 4 and 5, although significantly improved over the standard laboratory protocol, nevertheless may be further improved by using clinically acceptable reagents such as replacing Ham's F10 tissue culture media and the standard Sigma collagenase with Prodo Islet Media (PIM) (PRODO Laboratories Inc, Irvine, CA, USA) and other cGMP certified reagents. In preliminary data (see Appendix) we demonstrate significantly higher IEQ recovered from islets cultured in

PIM compared to Ham's F10 controls (41062±1250 vs. 35229±1926, $p<0.05$) with no significant differences in DNA recovery, GSIS, or cellular composition following the standard laboratory protocol. These reagents remain to be tested using the clinically applicable protocol.

Additionally, other reagents may be added to further improve islet viability and culture. For example, rho-associated protein kinases (ROCKs) are a family of serine-threonine kinases that are effectors of the RhoA, a small GTP-binding protein that stimulates actin-myosin contractility, which in turn results in the bundling of actin filaments to generate stress fibers and clustering of integrins and associated proteins to form focal adhesions (55). Focal adhesions, specialized sites of cell attachment, allow cell attachment to the ECM as well as carrying mechanotransduction signals related to cell growth, survival, and function (55). ROCKs modulate the activity of RhoA through effects on the phosphorylation of myosin light chain; RhoA activity, through focal adhesion formation, inhibits cell spreading and exocytosis (56). The inhibition of the Rho-ROCK pathway through ROCK inhibitors has been demonstrated to improve GSIS during both phases of insulin secretion, likely through interactions with integrins and ECM (56). This effect is acute and rapidly reversible, suggesting that ROCK inhibitors affect actin cytoskeleton remodeling; the first phase of insulin secretion is known to involve actin cytoskeleton depolymerization leading to exocytosis of granules already at the plasma membrane, whereas the second phase (longer than 10 minutes post-stimulus) involves the movement of granules from the cell interior by the actomyosin cytoskeleton (57, 58). Attachment to ECM also initially inhibits RhoA activity through integrin signaling (55); culturing rat β cells on laminin through integrin-based interactions with focal adhesions (56). Because the islet ECM is lost during isolation, the addition of ROCK inhibitors to culture media could preserve islet function in through RhoA inhibition until the islets are transplanted in the prevascularized collagen-based matrix.

7.2.4. Characterizing NPI integrin expression

To date, no study has yet examined integrin expression in NPIs. It is known that integrin expression is modified during development, and also following isolation. In the adult pig, the structural integrity of the porcine islet depends almost entirely on cell-cell adhesion at the endocrine/exocrine interface rather than cell-ECM interactions (59); it is currently unknown if the immature NPI requires the same interactions. Integrin expression is indicative of the type of ECM or BM component that the cells require for normal function. Therefore, it would be highly useful to determine which integrins are expressed in islets in the context of both whole pancreas and isolated islets. Although the studies in this thesis explore the functional outcomes of the interactions between NPIs and the collagen-based matrix, through viability and glucose responsiveness, identifying NPI integrins would provide insight into specific modifications that can be made to the collagen-based matrix to improve support for long term survival and function in the transplant recipient. For example, the αv integrin subunit is found in human islets, while the β1 subunit is found in both adult porcine and adult human islets (59); as $\alpha v\beta 1$ is known to interact with vitronectin and fibronectin, this could indicate that a matrix with those glycoproteins would be beneficial for human but not porcine islets. If developing NPIs were found to express αv, the collagen-based matrix described above could readily be modified with either glycoprotein. The integrin expression of NPIs could be determined by using immunohistochemistry on serial paraffin embedded sections for both whole pancreas and isolated islets.

7.2.5. Alternate sources to NPIs

NPIs, although an excellent alternate source to human cadaveric donor islets, are not the only area of research being explored to address tissue shortages. The two other primary spheres of research are stem cells and bioartificial pancreases, as explored in section 6.3. Briefly, a pancreatic precursor cell population (60), hemopoietic progenitor

cells (61), and hESCs or iPSCs (62-65) are being examined as potential sources. Alternatively, there is some evidence for replication of endogenous β cells (60). However, as previously discussed these therapies have many remaining challenges and are unlikely to be clinically relevant before NPIs.

Closed-loop insulin pumps, first developed in the 1980s, have the potential to reverse diabetes in a large patient population, but many challenges remain to be solved involving the insulin pump itself, glucose sensing, and control algorithms; these obstacles are thoroughly explored by Ricotti et al. (66). However, it is difficult to imagine developing a bioartificial pancreas that even begins to approach the complexity of the endocrine pancreas. As Ricotti et al. so aptly remind us, physiological glycemic control interprets data generated from substrates, hormones, paracrine compounds, and autonomic neural inputs and releases insulin with minimal delay (66). Perhaps these exciting biotechnologies can be combined with cell therapies to offer short term alternatives to islet transplantation, but it seems clear that the potential benefit of xenotransplantation is at the very least equal to these alternatives.

7.3. REFERENCES

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Appendix: Use of Prodo Islet Media for NPI Culture

A1. METHODS

A1.1. Neonatal Porcine Islet Preparation

Donor pancreases were obtained from 1 to 3 day old Duroc neonatal piglets from the University of Alberta Swine Research Centre (1.5-2.0 kg body weight), and the islets were isolated and cultured for 5-7 days as described previously (114). Briefly, the retrieved pancreases were cut into 1 to 3 mm tissue fragments in separate 50 mL conical tubes, then exposed to 2.5 mg/mL collagenase, filtered through a 500 μm nylon screen, washed in Hank's Basic Salt Solution (HBSS) (Gibco, Burlington, Canada) supplemented with 0.25% bovine serum albumin (BSA) (fraction V; Sigma-Aldrich, Oakville, Canada), 10 mM HEPES (ICN Biomedicals, Inc., Costa Mesa, CA), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Lonza Walkersville, Inc., Walkersville, USA). Control NPIs were then cultured in non-tissue culture treated petri dishes containing Ham's F10 tissue culture media purchased from Sigma-Adrich (Oakville, Canada), and supplemented with sodium bicarbonate (Fisher), calcium D-glucose (EM Sciences,), L-glutamine (Sigma-Aldrich, Oakville, Canada), 0.25% BSA (fraction V; Sigma-Aldrich, Oakville, Canada), isobutylmethylxanthine (IBMX) (Sigma-Aldrich, Oakville, Canada), nicotinamide (Sigma-Aldrich, Oakville, Canada), calcium chloride dihydrate (Sigma-Aldrich, Oakville, Canada), and 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Lonza Walkersville, Inc., Walkersville, USA). The islets were cultured at 37°C for 5-7 days, with the medium changed the first, third and fifth days after isolation.

Experimental NPIs were isolated and washed following the same protocol as the control islets, but then cultured in Prodo Labs PIM(R)® islet recovery media for at 37°C for 48 hours according to the supplier protocol. This recovery media contains 5% Human AB Serum and a glucose concentration of 5.8 mM. After 48 hours, the medium was replaced with PIM(S)®, which also contains 5% Human AB Serum and a glucose concentration of 5.8 mM. These media were designed for culture of human islets, and

the effect on NPIs was previously not explored.

A1.2. In Vitro Assessment of Islets

A static incubation assay (114) was used to determine glucose responsiveness in islets prepared using each protocol. Each condition was incubated in duplicate at 37°C for 2 hours in 1.5 mL RPMI supplemented with 2.0 mM L-glutamine, 0.5% w/v BSA and either 2.8 mM (low) or 20.0 mM (high) glucose. Stimulation indices were calculated by dividing the amount of insulin released at 20.0 mM glucose by that released at 2.8 mM glucose. Hormone content was measured after extraction in 2 mM acetic acid containing 0.25% BSA. Samples were sonicated in acetic acid, centrifuged at 800 g for 15 minutes, and then supernatants were collected and stored at -20°C until assayed for insulin content by ELISA (Boehringer Mannheim). Total insulin content was measured after extraction in 2 mM acetic acid containing 0.25% BSA. Samples were sonicated in acetic acid, centrifuged at 800 g for 15 minutes, and then supernatants were collected and stored at -20°C until assayed for insulin content by ELISA (Boehringer Mannheim, Basel, Switzerland). For DNA content, representative 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 by Picogreen (Molecular Probes, Inc., Eugene, OR), a fluorescent nucleic acid stain for quantification of double-stranded DNA.

Immunohistochemistry was used to determine cellular composition of the NPI. The avidin-biotin complex (ABC) method was used with peroxidase and diaminobenzidine as the chromagen. NPI were dissociated into single cells mechanically with a siliconized Pasteur pipette in a solution of 0.05% trypsin, 0.5 mM EDTA and PBS. The single cells were then fixed in formaldehyde on glass slides and stained for insulin or glucagon. Primary antibodies (Dako Corp., Carpinteria, CA) included guinea pig anti–porcine insulin (1:1000) and rabbit anti-glucagon (1:100); biotinylated secondary antibodies and the ABC-enzyme complexes were purchased

from Vector Laboratories (Burlingame, CA). Primary antibodies were incubated for 30 minutes (room temperature), while secondary antibodies were applied for 20 minutes. A minimum of 500 cells were randomly selected and counted.

A1.3. Statistical Analysis

All statistics were performed using the Student's paired t-test; the level of significance was considered to be α=0.05. All statistical analyses were performed with STATA 11 (StataCorp LP, College Station, TX). Results are presented as mean±standard error of the mean (SEM).

A2. RESULTS

There were no significant differences between islets cultured using the PIM (4.5±0.54 μg) compared to the standard Ham's F10 (3.7±0.77 μg) (Table A1). There were also no differences between the percentage of β cells (12.4±0.91% with Ham's F10 culture media compared to 9.2±1.7% using PIM) (Table A1). There were no differences in the proportion of glucagon positive α cells or CK7 positive ductal cells between the two conditions (Table A1). There were significantly more IEQ recovered from NPIs cultured using PIM (41062±1250) ($p<0.02$) compared to the Ham's F10 (35229±1925) (Table A1); however there was no difference in DNA recovered per pancreas (200±22 μg DNA from NPIs cultured using Ham's vs. 227±21 μg DNA from NPIs cultured using PIM). There were also no difference in total insulin recovered per pancreas from NPIs cultured in Ham's (3.7±0.78 μg) or PIM (4.5±0.54 μg).

During a static glucose stimulated insulin secretion assay there were no differences between insulin secreted at significantly more insulin was secreted at high glucose (20.0 mM) compared to low glucose (2.8 mM) in both experimental conditions (Table A1). No statistically significant differences were noticed amongst all experimental conditions in the amounts of insulin secreted at low or high glucose. When calculating the stimulation indices there was no significant difference between

stimulation indices of NPIs cultured with either Ham's (SI: 1.75±0.08) or PIM (SI: 1.94±0.18).

A3. DISCUSSION

These data demonstrate that the PIM, although designed for human islet culture, result in islets of similar quality to the Ham's F10 culture media specifically designed for NPI culture. This supports the rationale to replace Ham's with a PIM or a similar cGMP certified islet culture media. Further studies remain to be performed on NPI cultured in these media, including cell viability studies using TUNEL staining and transplantation studies in a murine model.

A4. TABLES

Table A1. Comparison of neonatal porcine islets cultured in the standard culture media compared PIM.

Condition	Content (μg)		Cellular Composition	IEQ
	Insulin	DNA		
Ham's F10 Culture Media (n=11)	3.7±0.77	200±21.8	12±0.91 / 16±3.3 / 10±1.0	35229±1925*
PIM (n=11)	4.5±0.54	227±21.4	9.2±1.7 / 13±2.1 / 12±1.4	41062±1250

Data are means±SEM. Islets were isolated and cultured using standard Ham's F10 medium or PIM(R)® for 48 hours then PIM(S)® for 3-5 more days. *$p<0.02$ vs. PIM.