THE ROLE OF ISLET AMYLOID AND CHOP IN ISLET GRAFT DYSFUNCTION AND FAILURE

by

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ABSTRACT
Islet transplantation has great promise as a treatment for patients with insulin-dependent diabetes but its long-term success is limited by progressive graft dysfunction. Many measures of β cell dysfunction in transplantation are similar to those observed in the type 2 diabetic β cell. We focused upon two particular genes underlying pathology of the latter, namely islet amyloid polypeptide (IAPP) and C/EBP homologous protein (CHOP). We hypothesized that CHOP and islet amyloid composed of IAPP played distinct roles in progressive dysfunction and loss of the transplanted β cell.

Human islets and murine islets expressing the IAPP transgene developed islet amyloid following transplantation. Amyloid deposition correlated with loss of glycemic control and was exacerbated by transplantation of a marginal islet mass. Further, pre-existing amyloid in human islets prior to transplantation correlated with graft dysfunction at one year following islet transplantation into human allograft recipients. We tested several strategies to protect against islet amyloid toxicity in a pre-clinical model of human islet culture. Human IAPP deposition and toxicity was abrogated by siRNA against IAPP and by peptide inhibitors of IAPP aggregation. As an alternate strategy, transplantation of porcine islets expressing minimally amyloidogenic forms of IAPP yields excellent long-term outcomes.

Islet amyloid deposition and toxicity is particularly rapid in the transplanted islet. This phenomenon may relate to factors in the transplant environment. We demonstrated that heparin, used in clinical islet transplantation, potentiates amyloid deposition in human islets. In addition, it exacerbates IAPP toxicity to cultured cells and accelerates graft failure in marginal mass human islet grafts.

CHOP is activated by prolonged endoplasmic reticulum or oxidative stress. We demonstrated that CHOP immunostaining is increased in marginal mass islet grafts. Transplantation of islets in which CHOP has been deleted or silenced by RNA significantly improves glycemic normalization in marginal mass grafts and reduces apoptosis. These data suggest that CHOP plays a detrimental role in islet graft dysfunction.
These studies demonstrate a role for two independent non-immune factors in mediating islet graft dysfunction and for which therapeutic modulation may improve β cell function and survival in both islet transplantation and type 2 diabetes.
PREFACE

Ethics approval was obtained for the use of human samples from the University of British Columbia / Children’s and Women’s Health Centre of British Columbia Research Ethics Board (Ethics Certificates #H06-03112, #H0370453). Animal studies were reviewed and approved by the University of British Columbia Committee on Animal Care (Protocols # A10-0294, #A05-1003-A006).

In Chapter 3, K. Potter monitored mice, performed i.p. glucose tolerance tests, retrieved and fixed grafts for histological processing, and performed graft histology and analysis for all murine islet grafts. Technical assistance in islet isolation and transplantation was provided by D. Dai and G. Soukhatcheva. A. Klimek performed proinsulin and insulin ELISAs and determined the proinsulin:insulin ratios; these data and thioflavin S analysis of human islets grafted into NOD.scid mice performed by K. Potter are also presented in A. Klimek’s Ph.D. thesis (1). For human islet grafts, A. Klimek and S. Butterworth contributed human islet graft samples and corresponding blood glucose data. Islet allograft donor pancreas histological sections and clinical data were provided by Dr. G. Korbutt (University of Alberta). Islet autograft donor pancreas histological sections and clinical data were provided by Dr. H. Rilo (University of Cincinnati). K. Potter performed all histological staining and analysis for human autograft and allograft donor pancreas and islets. Lipid nano-particles containing one of the short-interfering RNAs (siRNA) for human IAPP (si-hIAPP4) were generated by Dr. C. Tam in the laboratory of Dr. P. Cullis (University of British Columbia). All cultured studies using these liposomes and subsequent data analysis was performed by K. Potter. Hexa-peptides used for studies presented in Figure 14 were synthesized by Dr. P. Fraser (University of Toronto). Islet culture studies in Figures 14 and 15A were performed by Dr. L. Marzban; all immunostaining and histological analysis presented in this these figures was performed by K. Potter. Figures appearing in Figure 14 and 15A have been published in Potter et al. *Biochim Biophys Acta* 1790(6):566-74, 2009 (2) and Marzban et al. *Diabetes*. 57(11):3045-55, 2008 (3), respectively. Islet culture studies for Figure 15 B-D were performed by K. Potter.

In Chapter 4, all studies except data presented in Figure 17 were performed by K. Potter. Transmission electron microscopy in Figure 17 was performed by Y. Chen in the laboratory of Dr. P. Fraser (University of Toronto). J. Cheng aided in amyloid quantification of cultured human islets and in the development of the insoluble fraction IAPP assay. Dr. Fraser suggested the idea for the insoluble fraction IAPP assay. Technical assistance with islet transplantation was provided by Derek Dai.

A version of Chapter 5 contributed to a manuscript published in the Proceedings of the National Academy of Sciences (USA) (Potter et al. *Proc Natl Acad Sci* 107:4305-10, 2010). Islet lysis, western blot, porcine IAPP sequencing, thioflavin T kinetics studies, cell culture studies, immunohistochemistry and related analysis were performed by K. Potter. Dr. R. Baker aided in the design of constructs to sequence porcine IAPP. Circular dichroism and electron microscopy presented in this thesis were performed by Drs. A. Abedini and P. Marek in the laboratory of Dr. D. Raleigh (Stony Brook University, NY). Neonatal porcine islets, histological sections from neonatal porcine islet grafts, and blood glucose data from the recipients of these grafts were provided by Dr. G. Korbutt. Histological sections from adult porcine islet grafts, and blood
glucose from the recipients of these grafts were provided by Dr. S. Bertera and Dr. M. Trucco (University of Pittsburgh).

In Chapter 6, K. Potter performed all studies. C. Zhang aided in studies of glucose-stimulated insulin secretion from isolated islets and several histological analyses. Lipid nano-particles were synthesized by Dr. C. Tam in the laboratory of Dr. P. Cullis. Technical assistance with islet isolation and transplantation was provided by D. Dai, G. Soukhatcheva, and M. Obach.
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ABBREVIATIONS

4-PBA  4-phenyl butyric acid  
AA  Amyloid A  
AFU  Arbitrary fluorescence units  
AL  Amyloid light chain  
AMP  Adenosine monophosphate  
ANOVA  Analysis of variance  
APC  Antigen presenting cell  
ATF  Activating transcription factor  
ATP  Adenosine triphosphate  
ATTR  Transthyretin  
BB  Biobreeding  
BCA  Bicinchoninic acid protein assay  
BiP  Binding immunoglobulin protein  
Bl/6  C57BL/6 mice  
BMDMS  Bone marrow-derived mesenchymal stem cells  
BMI  Body mass index  
bZIP  Basic leucine zipper domain  
CCAC  Canadian Council on Animal Care  
CCL  Chemokine (C-C motif) ligand  
CD  Cluster of differentiation: Circular dichroism (Chapter 5 only)  
C/EBP  CCAAT/enhancer-binding protein  
CFRI  Child and Family Research Institute  
CHOP  C/EBP homologous protein (same as DDIT3)  
CHOP<sup>−/−</sup>  B6.129S-Ddit3<sup>tmIDron</sup>/J mice  
CIC3  Cleaved caspase-3  
CPE  Carboxypeptidase E  
CXCL1  Chemokine (C-X-C motif) ligand 1  
DCCT  Diabetes control and complications trial  
DDit3  DNA damage-inducible transcript 3 (same as CHOP)  
DMSO  Dimethyl sulfoxide  
DPP-IV  Dipeptidyl peptidase 4  
EDTA  Ethylenediaminetetraacetic acid  
EGFR  Epidermal growth factor receptor  
ELISA  Enzyme-linked immunosorbent assay  
eIF  Eukaryotic initiation factor  
EST  Expressed sequence tag  
ER  Endoplasmic reticulum  
ERAD  ER-associated degradation  
ESRD  End-stage renal disease  
FBS  Fetal bovine serum  
FKBP  FK506 binding protein  
FTIR  Fourier transform infrared spectroscopy  
FVIIa  activated factor VII  
GAD65  Glutamate decarboxylase 65
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>NP-40</td>
<td>Nonyl phenoxypolyethoxylethanol, or Igepal CA-630</td>
</tr>
<tr>
<td>PAM</td>
<td>Peptidyl-glycine α-amidating monooxygenase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>PC1/3</td>
<td>Prohormone convertase 1/3</td>
</tr>
<tr>
<td>PC2</td>
<td>Prohormone convertase 2</td>
</tr>
<tr>
<td>Pdx-1</td>
<td>Pancreatic and duodenal homeobox 1</td>
</tr>
<tr>
<td>PDI</td>
<td>Protein disulfide isomerase</td>
</tr>
<tr>
<td>PERK</td>
<td>Protein kinase RNA-like endoplasmic reticulum kinase</td>
</tr>
<tr>
<td>PERV</td>
<td>Porcine endogenous retrovirus</td>
</tr>
<tr>
<td>PI</td>
<td>Proinsulin</td>
</tr>
<tr>
<td>PI/PI+I</td>
<td>Proinsulin:insulin ratio</td>
</tr>
<tr>
<td>Pme-1</td>
<td>Protein phosphatase methylesterase-1</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethanesulfonylfluoride</td>
</tr>
<tr>
<td>PNDM</td>
<td>Permanent neonatal diabetes</td>
</tr>
<tr>
<td>proIAPP</td>
<td>pro-islet amyloid polypeptide</td>
</tr>
<tr>
<td>PTDM</td>
<td>Post-transplantation diabetes mellitus</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RAMP</td>
<td>Receptor modifying protein</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>si-hIAPP</td>
<td>Small interfering RNA targeted against the human IAPP gene</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SRP</td>
<td>Signal recognition particle</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline with tween</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>Tg</td>
<td>Thapsigargin</td>
</tr>
<tr>
<td>Tg-hIAPP</td>
<td>Human IAPP transgene</td>
</tr>
<tr>
<td>T_h</td>
<td>T-helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNDM</td>
<td>Transient neonatal diabetes</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TUDCA</td>
<td>Tauroursodeoxycholic acid</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Transferase-mediated dUTP nick-end labeling</td>
</tr>
<tr>
<td>UKPDS</td>
<td>United Kingdom prospective diabetes study</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type mouse</td>
</tr>
<tr>
<td>ZnT8</td>
<td>Zinc transporter 8</td>
</tr>
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</table>
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DEDICATION

To my parents, Dean and Judith Potter
Chapter 1. Introduction

1.1 Amyloid

Amyloid was first identified by Rudolph Virchow in 1854 in his studies of macroscopic pathology in diseased cerebral cortex (4). He discovered that areas of pathology stained blue when treated with iodine. He coined the term “amyloid” to describe these and similar deposits also found in liver and spleen, based upon his assumption that iodine bound to starch. Although these deposits were shown to be proteinaceous in nature in 1859 (4), the term amyloid held fast.

1.1.1 Current definition of amyloid

Today, amyloid refers to deposits of insoluble protein with characteristic β-sheet quaternary structure (5). It should be noted that the β sheets characteristic of amyloid differ biochemically from β sheets found within globular proteins, as determined by differing Fourier transform infrared (FTIR) spectroscopy peaks and differing antibody-binding epitopes (5). Many proteins may adopt this conformation and can be subdivided into two classes. Prions and the mammalian protein Huntingtin have primary sequences rich in glutamine, which stabilizes intramolecular hydrogen bonding (6). The second class of pro-amyloidogenic proteins have regions containing a high proportion of hydrophobic amino acid residues and sequence containing alternating polar and non-polar residues (7). Amyloid fibrils are unbranched and 5 to 25 nm in diameter. They are long polymers of constituent monomers assembled through intermolecular hydrogen bonding between cross-β sheets that are oriented perpendicular to the long axis of the amyloid fibril (5).

In biophysical studies of amyloid, fibrils are identified by several methods. Amyloid fibrils may be visualized by transmission electron microscopy and atomic force microscopy (5). β sheet confirmation is confirmed by circular dichroism (5). Additional biophysical techniques used to study amyloid fibrils include infrared spectroscopy, X-ray diffraction, and electron diffraction (5). In histological sections, several dyes, such as thioflavin S, thioflavin T, and Congo red, are known to bind to amyloid fibrils (8). Thioflavin dye fluorescence is dramatically enhanced upon binding to amyloid fibrils (9). Thioflavin T interaction with amyloid fibrils is used to monitor amyloid fibril formation in vitro by fluorescence spectroscopy. In histological sections, the binding of Congo red and thioflavin S is used to identify amyloid deposits. Congo red emits a
weakly fluorescent signal in the red spectrum upon amyloid binding and is strongly birefringent under polarized light when bound to amyloid. Thioflavin S strongly fluoresces in the blue spectrum upon binding to amyloid.

1.1.2 Amyloid formation
Amyloid fibrils are insoluble polymers that form by hydrogen bonding between β-sheets of misfolded peptides. These polymers are highly resistant to degradation, although synthetic fibrils may be solubilized in formic acid (10) and enzymes such as neprilysin (11) may degrade fibrils in vivo (11). Amyloid fibril formation is classically monitored by thioflavin T fluorescence (9). The kinetics of amyloid fibril formation comprise three phases: 1) a lag phase; 2) a phase of rapid fibril growth; and 3) a steady-state plateau (12). Rapid fibril growth begins following formation of a nucleus which seeds fibril growth. Seeding of monomers with pre-formed fibrils greatly accelerates progression to the second phase (13). In the third phase, visualization of amyloid fibrils by transmission electron microscopy reveals a dense meshwork of long, unbranched fibrils (5) and circular dichroism indicates complete conversion to β sheet structure. Thioflavin T kinetics do not characterize prefibrillar assembly well. Thioflavin dyes bind to the cross β-sheet structure of amyloid fibrils (9), but may also bind to prefibrillar species which have distinct β-sheet conformation (12, 14-19) and exist in multimeric assemblies, ranging from dimers to small oligomers to large multimeric assemblies (12). Prefibrillar species form during the lag phase of thioflavin-monitored kinetics.

1.1.3 Functional amyloid
Although typically known as a pathology, many proteins form amyloid to fulfill physiological functions. Bacterial biofilms, essential for adherence to surfaces, are composed of proteins that have adopted amyloid-like structure. For example, the TasA protein (14) secreted by Bacillus subtilis and the Curli protein (15) secreted by Escherichia coli form amyloid fibrils which are principal components of biofilm. The malarial coat protein adopts an amyloid conformation to protect Plasmodium falciparum (16). The major silk protein, spidroin, adopts an amyloid conformation (17). Amyloid formation by yeast prions promotes replication, phenotypic diversity, and growth in nitrogen-poor environments (15, 18). In mammals, the melanosome protein Pme17 forms functional amyloid that serves as a template for melanin polymerization.
Prohormones adopt amyloid-like conformations within secretory granules of mammalian peptide-hormone producing cells (15). The concept of functional amyloid has been well characterized amongst bacterial and simple eukaryotes, but is novel in the vertebrate world. It is highly likely that additional roles for functional amyloid will be identified in vertebrates in the years to come.

1.4 Pathological amyloid
Over 40 amyloid-forming proteins have been implicated in disease pathology (4, 19, 20). Although the traditional definition of amyloid encompassed proteins forming β-sheet structure due to hydrophobic residues in their primary structure, this definition has recently been expanded to include cross-β sheet structure formed by glutamine rich proteins, such as huntingtin (21-23), and proteins localized to pathological inclusions with cross-β sheet properties, such as α-synuclein (22, 24-26). Amyloid diseases may be divided into two classes, namely systemic and localized amyloidoses. In systemic amyloidosis, such as amyloid light chain (AL), amyloid A (AA), transthyretin (ATTR), and β-2 microglobulin amyloidosis, amyloid deposits may be found throughout multiple tissues (27). Other amyloidoses are localized to a particular tissue, such as amyloid β in the cerebral cortex in Alzheimer’s disease and islet amyloid in the pancreatic islet.

1.5 Amyloid toxicity
In recent literature, it has become apparent that amyloid fibrils are less cytotoxic than pre-fibrillar forms, which are the predominant cytotoxic species (12, 28). The toxicity of aged fibrils is minimal and several amyloids have been characterized to have early toxicity that diminishes over time. Protein engineering of amyloid β to stabilize an oligomeric state and prevent formation of mature amyloid fibrils yielded highly toxic species (29). The term “pre-fibrillar species” refers to a heterogenous ensemble of entities with β sheet structure that are on-pathway to amyloid formation. Another vague term, oligomer, is commonly used to describe β-sheet containing, non-fibrillar toxic species which may be on- or off-pathway to fibril formation (12). Prefibrillar species have been proposed to exert toxicity through many mechanisms for both intra- and extracellular toxicity. Intracellular mechanisms of toxicity include endoplasmic reticulum (ER) stress-mediated apoptosis following aggregation of misfolded protein in the ER (30), perforation of organelle membranes and subsequent calcium toxicity (28), oxidative stress
perturbation of mitochondrial function (33, 34), and disruption of autophagy and lysosomal degradation (35). Extracellular mechanisms include membrane perforation or channel formation leading to cytosolic calcium overload (28, 36) and death receptor activation (37). Apoptosis is potentiated by activation of CHOP (30), caspase-3 (38), and calpain signaling (39). Although amyloid fibrils are considered minimally toxic, their presence within tissues likely limits metabolite clearance and nutrient delivery and disrupts organ architecture. The characteristic β sheet structure of amyloid species may also stimulate pattern recognition receptors in the innate immune system (40), leading to local inflammation, cytokine production (40), and release of reactive oxygen and nitrogen species.

1.1.6 Public health burden of amyloid diseases
Amyloid underlies several notable diseases for which there is no cure, and for many of which treatments are minimally effective. Many neurodegenerative diseases have underlying amyloid pathology, such as Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and spongiform encephalopathy. Alzheimer’s disease may be moderately improved in the early stages by anti-cholinesterases. Its course, however, is unrelenting, leading to severe dementia. Patients may persist for years with dementia. The global economic burden of Alzheimer’s disease is staggering. Between 10 and 15% of adults over the age of 65 may have dementia (41). In the Canadian Outcomes Study in Dementia, 83% of enrolled dementia patients had Alzheimer’s disease (42). Canadian studies place the yearly economic burden of mild dementia at approximately $13,000 per patient and at $40,000 per patient for severe dementia (43).

Parkinson’s disease may be treated early in its course with pharmaceuticals that enhance the lifetime of dopamine in the substantia nigra or with deep brain stimulation. Similar to Alzheimer’s disease, therapeutics do not alter the course of the disease, which ultimately leads to severe impairment of mobility, fine motor function, speech, and swallowing; a subset of Parkinson’s patient also develop dementia. Parkinson’s disease had a prevalence of 12% in the Canadian Study on Health and Aging (44).

There is no available treatment for Huntington’s disease or spongiform encephalopathy. The cost of care for affected individuals is extensive (45). Spongiform encephalopathy, also known as Creutzfeldt-Jakob disease, is unique among the amyloidoses in that it is infectious. Disease
control to minimize exposure to the underlying infectious prions must be exercised at hospitals treating affected human patients, at farms in which infected animals are identified, and at meat processing facilities which may have processed an infected carcass. Millions of dollars are lost sterilizing hospital equipment and destroying entire herds of animals in which a single case of Creutzfeldt-Jakob disease is encountered (46-48). Amyloidoses affecting the central nervous system are a considerable economic burden.

Amyloidoses affecting organ systems other than the central nervous system also engender significant cost. Amyloid deposition in the renal glomeruli precipitates renal dysfunction requiring dialysis. The estimated cost of dialysis for a single patient is $60,000 per annum, according to the Canadian Institute for Health Information. Type 2 diabetes is gaining growing recognition as an amyloid disease in which islet amyloid accelerates the course of the disease and may contribute to diabetic renovascular pathology (49). The annual cost to treat an individual with diabetes mellitus in Canada exceeds $3000 (50), not including the cost of care of complications such as dialysis, blindness, and limb amputation. Though this cost is not extreme on an individual level, it will become considerable as type 2 diabetes prevalence is expected to double by 2030.

There is essentially no treatment available to target amyloid pathogenesis for any of the 20 or more known amyloid diseases. Even in the face of cardiac, renal, and hepatic transplantation, amyloid disease recurrence has been documented (51-58) and graft survival and function may be limited in recipients previously diagnosed with amyloid diseases (55, 56, 59). Indeed, *de novo* amyloid deposition has also been documented in transplant recipients not diagnosed with amyloidosis prior to transplantation (60). The development of anti-amyloid strategies is critical to reduce worldwide disease burden of several of the most prominent incurable diseases of the 21st century.

**1.2. Diabetes mellitus**

Diabetes mellitus encompasses an ensemble of metabolic disorders with the common features of hyperglycemia and serum insulin levels insufficient for the metabolic needs of the peripheral tissues. This results in the classic diagnostic triad of polyuria, polydipsia, and polyphagia. A clinical diagnosis of diabetes mellitus may be established upon evidence of persistent
hyperglycemia, including fasting plasma glucose > 7.0 mM, plasma glucose > 11.0 mM two hours following a 75 g oral glucose challenge, or glycated hemoglobin above 6.5%. An individual is considered “pre-diabetic” if their fasting glucose exceeds 6.1 mM or their plasma glucose exceeds 7.8 mM at 2 hours post-glucose challenge. According to the World Health Organization, the worldwide diabetes prevalence is 171 million individuals (2010). Type 1 diabetes affects approximately 10% of these individuals, while type 2 diabetes accounts for the majority of cases. Increasing prevalence of type 2 diabetes is predicted to increase diabetes prevalence to 366 million worldwide by 2030 (61).

1.2.1 Type 1 diabetes
Type 1 diabetes results from insufficient insulin secretion due to autoimmune destruction of pancreatic β cells. β cells are selectively destroyed by autoreactive CD8+ T cells that recognize β cell antigens (62, 63). CD4+ T cells and macrophages also infiltrate the pancreatic islets (64). CD4+ T-cells are critical to disease pathogenesis and promote the activation and maturation of CD8+ T cells (64). Much of our understanding of type 1 diabetes is based upon the development of diabetes in the NOD mouse and the BB rat. In these rodents, the disease process is preceded by inflammatory infiltration of the islet (63). Disease susceptibility in the NOD mouse lies in the Idd5 (65) and Idd9 (66) loci that govern T cell stimulation and survival, in MHC II genes, such as the Idd1 locus (67-69), and in additional susceptibility loci, such as Idd3 (68), Idd12 (69), and Idd18 (69). Upon defective thymic negative selection, islet antigen specific T cells are activated in pancreatic lymph nodes followed by subsequent infiltration of the islet. β cells are destroyed by CD8+ T cells; β cell mass, however, may not be immediately depleted. A honeymoon period has been described in some patients who may become experience remission and insulin independence for up to a year following a diabetes diagnosis and initial insulin therapy (70).
Type 1 diabetes tends to be diagnosed in children above 4 years of age, with a bimodal distribution of onset peaking at 5 and 11 years of age (71). The pathological process, however, begins much earlier as evidenced by the appearance of autoantibodies against known β cell antigens, including preproinsulin, glutamate decarboxylase 65 (GAD65), islet autoantigen 2 (IA-2), and zinc transporter 8 (ZnT8) (72) in human patients prior to metabolic dysfunction. The onset of overt type 1 diabetes is thought to occur upon loss of >80-95% of pancreatic β cell mass (73, 74), although recent studies suggest that diabetes may occur upon loss of as little as 40% of pancreatic β cell mass (75) or upon loss of insulin-positive immunostaining in 70% of islets (74).
Type 1 diabetes results from a combination of genetic susceptibility and environmental triggers. Genetic susceptibility is illustrated by the 27-54% (70, 76) concordance rates of type 1 diabetes in monozygotic twin studies (70) and the development of impaired glucose tolerance in non-symptomatic first-degree relatives of type 1 diabetic patients (77). Strong genetic susceptibility is associated with DR3 (DRB1*0301 DQA1*0501–DQB1*0201) and DR4 (*0401,0402, 0404 and 0405) haplotypes of human leukocyte antigen class II (76). Additional genes that confer risk include the insulin (INS) gene, the CTLA4 gene, and possibly PTPN22 genes (76).

Environmental causes, such as Group B Coxsackie viral infection (78), are thought to play a role in the development of type 1 diabetes in genetically susceptible individuals. Viral infection of β cells may lead to the presentation of β cell antigens to autoreactive T-cells, resulting in autoimmune attack (79).

1.2.2 Type 2 diabetes

Type 2 diabetes results from a combination of inadequate insulin secretion and insufficient insulin sensitivity. Inadequate insulin secretion by the pancreatic β cell results from deficits in both β cell function and mass. The type 2 diabetic β cell is characterized by multiple defects in insulin secretion and gradual cell loss by apoptosis. Defective first-phase glucose-stimulated insulin secretion (GSIS) is detectable in patients with impaired fasting glucose or impaired glucose tolerance (80). Islets from individuals with type 2 diabetes have absent first phase GSIS, reduced second-phase GSIS, and reduced insulin secretion in response to non-glucose secretagogues (81-83). Peripheral serum insulin concentration normally oscillates due to pulsatile insulin release, caused by coordinated bursts of insulin granule release (84). In type 2 diabetic patients, both diurnal and ultradian oscillations are decreased early in the disease process due to impaired rapid pulsatile insulin release (85). Processing of the insulin precursor, proinsulin, is also impaired in type 2 diabetes. Basal and stimulated proinsulin levels and proinsulin:insulin ratios are significantly elevated in type 2 diabetic patients in whom rates of clearance of proinsulin and insulin do not differ from healthy controls (86, 87). Type 2 diabetic β cells also show evidence of de-differentiation (88, 89) and exhibit markers of oxidative (90) and ER stress (91).
Progressive loss of β cell mass is critical to disease pathogenesis. In autopsy studies, type 2 diabetic patients have greatly reduced β cell mass as compared to controls matched for the degree of obesity (73). Prior to diabetes onset, fractional β cell volume is reduced by approximately half in individuals with impaired fasting glucose (73). Loss of β cell mass in type 2 diabetes is due to apoptosis (92) thought to result in part from prolonged glucotoxicity (93). Prolonged ER or oxidative stress, for which there is evidence in human type 2 diabetic β cells (90, 91), may also lead to apoptosis. Islet amyloid and inflammation may also contribute to apoptotic loss of β cells. Islet amyloid is a characteristic pathology of the type 2 diabetic islet (94): amyloid fibrils (95) and prefibrillar (96) intermediates are preferentially toxic to β cells and likely contribute to the progressive loss of β cell mass (97, 98). Type 2 diabetic islets have elevated numbers of macrophages (99) and increased production of pro-inflammatory cytokines, such as IL-1β (100). A local pro-inflammatory milieu may promote β cell dysfunction and loss. Thus, loss of β cell mass in diabetes is progressive and exacerbated by β cell dysfunction, islet amyloid deposition, and inflammation, all of which will be described in more detail in later sections.

The progressive decline in β cell mass and function in type 2 diabetes is exacerbated by peripheral insulin resistance. In adipose, skeletal and hepatic tissue, glucose uptake into cells is dependent upon insulin-receptor-mediated translocation of GLUT4 to the plasma membrane. In an insulin resistant cell, the concentration of insulin required to sufficiently activate the insulin receptor is much greater than in a normal individual. This results from a combination of fewer insulin receptors and reduced capacity of the insulin receptor to activate cytosolic tyrosine kinases (101). In the face of insulin resistance, the β cell must compensate by increasing insulin secretion. β cell dysfunction worsens under chronically elevated secretory demand. Insulin resistance may be increased in the β cell itself, thus decreasing first-phase glucose-stimulated insulin secretion and impairing glucose tolerance (102). A primary β cell defect becomes evident as first-phase insulin secretion in response to glucose declines. Weir et al developed a five-stage model of progression of β cells from compensation to decompensation (103). Early in the disease, the β cell is able to compensate for insulin resistance. In the early stages of β cell exhaustion, corresponding to impaired glucose tolerance in an individual, first phase insulin secretion diminishes and there is evidence of β cell de-differentiation. This will progress to frank
diabetes, in which glycemic control is lost and β cell de-differentiation is more severe, and β cell mass is profoundly reduced. While a β cell defect is intrinsic to the progression of diabetes, this defect is exacerbated by insulin resistance.

1.2.3 Other causes of diabetes mellitus

A subset of diabetes diagnoses (1-3% of diagnoses in children) is directly attributable to single gene mutations that affect transcription factors critical to β cell development, cause severe β cell dysfunction, or are critical to mature β cell function. Diabetes may present as permanent neonatal diabetes (PNDM), transient neonatal diabetes (TNDM), or maturity-onset diabetes of the young (MODY). PNDM and TNDM are diagnosed within the first 3 months of life and have a prevalence of 1/400,000 births. Diabetes does not resolve in PNDM, whereas in TNDM, hyperglycemia resolves but 50% of individuals later relapse. TNDM is most commonly associated with paternal isodisomy of chromosome 6. Gene mutations underlying PNDM are commonly found in the insulin gene; in glucokinase, which serves as the β cell glucose sensor; in FOXP3; in EIF2AK3 and in genes for subunits of the β cell K$_{ATP}$ channel, ABCC8 or KCNJ11. MODY, in which diabetes develops prior to 25 years of age, has a prevalence of 2% of diabetes diagnoses. In MODY2, mutations in glucokinase dampen responsiveness of insulin secretion in response to elevation in plasma glucose. Other causes of MODY involve mutations in genes critical to insulin gene transcription, IPF-1 (MODY4) and Ngn1 (MODY6), or other transcription factors critical to β cell mass and function, HNF4α (MODY1), HNF1α (MODY3), and HNF1β (MODY5).

Gestational diabetes occurs in 2-5% of pregnancies and resolves following pregnancy. Development of gestational diabetes is a strong risk factor for type 2 diabetes. Placental hormones and pregnancy-induced increases in cortisol, leptin, and TNFα increase insulin resistance in pregnant women. Diabetes may also be caused by medications, endocrinopathies, and defects of the exocrine pancreas. Drugs known to cause diabetes include glucocorticoids and immunosuppressive agents such as tacrolimus. Endocrinopathies in which diabetes may develop include Cushing syndrome, acromegaly, hyperthyroidism, and pheochromocytoma. Diabetes may result from damage to the pancreas such as in chronic
pancreatitis, cystic fibrosis, pancreatic neoplasm, and hemochromatosis, or following pancreatectomy.

1.2.4 Complications of diabetes
The long-term complications of diabetes mellitus are common between disease subtypes. Chronic hyperglycemia leads to both macro- and micro-vascular complications. The macrovascular complications of diabetes are cardiovascular, cerebrovascular, and peripheral vascular (109, 110). Diabetes is one of the strongest predictive risk factors for stroke and myocardial infarction (109). Chronic hyperglycemia and inflammation associated with diabetes increases the risk of atherosclerotic plaque formation, hypercoagulability, and platelet adhesion, and decreases fibrinolysis (111). Microvascular complications of diabetes include retinopathy, nephropathy, and neuropathy (109); these may lead to blindness, renal failure, and progressive limb amputation, respectively. The most likely mechanisms underlying microvascular complications are oxidative damage, advanced glycosylation end-products, and intracellular polyol accumulation leading to osmotic stress (112). Rigid glycemic control has been shown to slow the progression of complications in type 1 diabetes in the Diabetes Control and Complications Trial (DCCT) (113) and to minimize the development of complications in type 2 diabetes in the United Kingdom Prospective Diabetes Study (UKPDS) study (114). As detailed in a subsequent section, islet transplantation may be superior to rigid glycemic control in slowing, at least temporarily, the progression of complications in type 1 diabetes (115).

1.2.5 Treatment of diabetes mellitus
Type 1 diabetes is treated with insulin replacement therapy, frequent glycemic monitoring, and dietary management. Insulin replacement therapy may be given by multiple daily insulin injections or by insulin pump. β cell replacement, by pancreas or islet transplantation, may be considered to slow the progression of diabetes. Treatment of type 2 diabetes depends upon the stage of the disease. Initial modifications in diet and lifestyle dramatically slow diabetes pathology. In the early years of the disease, while there is adequate remaining β cell mass and function, β cell function may be potentiated to promote adequate insulin secretion. Sulfonylureas close the ATP-dependent potassium channel of the β cell, thus promoting insulin secretion by β cell depolarization. Incretin analogues such as exendin-4 potentiate insulin release by receptor-
mediated increases in cytosolic AMP. An alternate strategy to potentiate the effects of endogenous incretins is to administer dipeptidyl peptidase (DPP-IV) inhibitors. Insulin resistance may also be reduced using thiazolidinediones or biguanides to reduce secretory pressure on the β cell. Once an individual has progressed to overt β cell failure, treatment more closely resembles that of type 1 diabetes, by insulin injection and rigid glycemic monitoring.

1.3. β cell replacement

β cell replacement remains a “holy grail” of diabetes research, with the potential to dramatically improve quality of life and minimize complications of diabetes. Insulin therapy only estimates insulin requirements and cannot tune plasma insulin concentration to transient fluxes in plasma glucose. Transplantation of β cells with a glucose sensor is thus theoretically superior to the best medical therapy in minimizing glycemic fluxes. Many strategies have been proposed to replace β cells, from stem cells to whole organ transplantation.

1.3.1 Stem cell therapy

An effective β stem cell therapy would require generation of a cell able to produce and store large quantities of insulin and to effectively secrete sufficient insulin in response to a stimulus such as glucose. Strategies to generate glucose-responsive, insulin-producing cells have been attempted with both embryonic and adult stem cells. Established protocols transform human embryonic stem cells (hESC) into cell colonies capable of synthesizing multiple islet hormones (116). By current protocols, 2-8% of cells are C-peptide positive and have similar insulin content per cell as an adult human β cell (117). Insufficient glucose-stimulated insulin secretion in vitro remains a considerable challenge of the hESC approach, although these cells achieve improved GSIS several months following transplantation. To circumvent ethical issues of hESC origin, induced pluripotent stem (iPS) cells may be derived from adult human somatic cells or umbilical vein cord blood. While the iPS strategy is promising, it has not yet been possible to differentiate sufficient numbers of functional, mature β cells in vitro (118). Bone marrow-derived mesenchymal stromal cells may promote formation of insulin-positive cells or may differentiate into insulin-producing cells. Although evidence remains preliminary, transplantation of bone marrow-derived mesenchymal stem cells (BMDMS) promotes insulin secretion and glycemic
control in diabetic rodents (119). While stem cell-derived β cell replacement therapies show promise, all strategies remain pre-clinical.

1.3.2 Pancreas and islet transplantation

Whole pancreas and islet transplantation are clinically available therapies for β cell replacement. In whole pancreas transplantation, the pancreas is grafted such that the arterial and venous circulation of the recipient are connected to the iliac artery and vein, respectively, and the pancreatic duct is connected to the recipient duodenum. Pancreas transplantation is currently restricted to type 1 diabetic individuals with end-stage renal disease (ESRD), typically in conjunction with or following renal transplantation. Islet transplantation offers a less-invasive alternative to pancreas transplantation with fewer potential complications for patients not undergoing renal transplantation. Islet transplantation involves separation of islets from pancreatic acinar tissue by collagenase digestion and purification; a suspension of isolated islets is infused into the portal vein of the recipient.

Success in pancreas and islet transplantation was attained only after decades of research. Early unsuccessful attempts at transplantation of pancreatic tissue included subcutaneous xenotransplantation of sheep pancreatic tissue in 1893 (120) and autologous pancreatic tissue grafts in canines (121). In 1924, Pybus et al described the transplantation of cadaveric human pancreas to ameliorate glycemic control (121); the graft was lost due to lack of immunosuppression. In 1966, pancreas transplantation performed using azathioprine immunosuppression achieved long-standing insulin independence in an adult type 1 diabetic recipient (122). Subsequent success rates remained as low as 10%. Advances in surgical technique and immunosuppression over the last three decades greatly improved outcomes and reduced technical surgical complications from 25% (1980) to 8% (123). To date, 30,000 pancreas transplants have been performed worldwide (124).

1.3.2.1 Pancreas transplantation

Pancreas transplantation is recommended by the American Diabetes Association as an acceptable therapeutic alternative in diabetic patients with end-stage renal disease. Current estimates of pancreas graft survival are 95% and 90% at 1 and 3 years’ post-transplantation, respectively
Long-term metabolic control is maintained in pancreas transplantation. Recipients have robust insulin secretory responses to glucose and arginine (125), although basal and stimulated insulin levels are 2-3 fold greater than in normal adults. Kendall et al reported maintenance of glycemic control in 21 patients for >5 years and in 3 patients for > 10 years (122). Multiple centres have reported glycemic control for 5 years, while some have reports of 10-20 years of glycemic control in individuals (126). Hypoglycemic events and hypoglycemic unawareness are uncommon and vascular complications of diabetes improve or stabilize (124). Despite considerable success in graft survival and metabolic control, pancreas transplantation presents many potential complications. Peri-transplant and early post-surgical complications include graft thrombosis, deep wound infections, leakage, hemorrhage, graft pancreatitis, and graft non-function or delayed function (123). Other common complications are cardiovascular and cerebrovascular (124).

1.3.2.2 Islet transplantation
Islet isolation was first attempted by Paul Lacy in 1967 and Moskalewski in 1969 (121). The technique was optimized and automated in 1988 (121). Lacy reversed diabetes in rats made diabetic by treatment with the β cell toxin streptozotocin (STZ) by syngeneic islet grafting in 1972 (120). He achieved similar results in diabetic primates (124). Human islet autografts were first attempted in 1980 in patients undergoing pancreatectomy to treat pain associated with chronic pancreatitis; insulin independence was achieved in 3 of 10 patients for 3-38 months (120). The first successful allogeneic islet transplantation was performed in 1980 and insulin independence was maintained for 9 months (120). The first successful trials of human islet allo-transplantation were performed at the University of Pittsburgh in 1990 (127) and at the University of Alberta in 1992 (128). Overall success, however, remained low, with insulin independence rates at 1 year post-transplantation not exceeding 10% (120, 124). The Edmonton Protocol, introduced in 2000, revolutionized islet transplantation by eliminating xenoproteins from islet media, eliminating overnight islet culture, transplanting an excess of 11,000 islet equivalents/kg, and eliminating glucocorticoids (121). In the International Trial of the Edmonton Protocol for Islet Transplantation (129), 44% of graft recipients achieved insulin independence at 1 year post-transplantation and 28% achieved partial graft function. At two years post-transplantation, 31% were insulin-independent.
Islet transplantation offers a less-invasive alternative to pancreas transplantation with fewer potential complications. Nevertheless, islet graft survival is limited. Successful transplantation appears limited to centres with extensive experience. A rate of 90% insulin-independence was achieved in three well-established transplant centres (120), while success rates at other sites hovered around 23%. Long-term success of islet transplantation is also limited. In the International Trial of the Edmonton Protocol, 75% of recipients required exogenous insulin at two years following achievement of insulin independence. Similar findings were reported by the University of Miami and the Collaborative Islet Transplant Registry (121). To restore insulin independence, graft recipients require additional islet infusions (130), with some receiving as many as four total infusions (124). Even multiple infusions do not preserve long-term normoglycemia (124). Despite lack of long-term insulin independence in recipients, islet transplantation is successful in slowing the progression of vascular complications (115) and reducing hypoglycemic events (121) in patients that maintain detectable levels of C-peptide. Glycemic control was improved in patients back on insulin but who remained C-peptide positive as compared to C-peptide negative (120). When graft success is evaluated in terms of C-peptide secretion, 83% of patients demonstrate islet function at 5 years following transplantation (131). Reported complications of islet transplantation are primarily complications of immunosuppressive treatment or immunosuppressive drug toxicity (121). Risks of immunosuppression include impaired renal function, hypertension, and hypercholesterolemia, among others (124). Additional complications include elevated liver function tests, procedural hemorrhage, and abdominal pain (130).

Pancreas and islet transplantation are strategies for β cell replacement that have undergone clinical trials in humans and that are currently available. Pancreas transplantation is a clinically established procedure accepted for the treatment of patients with type 1 diabetes and ERSD or diabetes not adequately controlled by insulin therapy (132). Over 30,000 cadaveric pancreas transplants have been performed worldwide with one-year graft success rates of between 76-95% (124, 133-135), 3 year survival of ~60-90% (124, 133), and 5 year survival of 40-70% (135-137). The majority of these procedures are performed in tandem with (72%) or following (17%) a kidney transplant, and only 7% of recipients receive a pancreas alone (124). By several reports, pancreas transplantation maintains long-term normoglycemia over years (122, 124, 126), decreases the occurrence of hypoglycemic events (124), and slows progression of diabetic vascular complications (124). Reported rates of insulin independence range from 80-98% (137-
1 year following transplantation, 65% (135) after 2 years, 24-41% (136) after 5 years, and 28% after 10 years (142). Comparisons of islet function in transplanted pancreas and isolated transplanted islets are limited, although β cells in transplanted pancreas secrete greater amounts of C-peptide than those of transplanted islets (143). When procedures were performed at the same institution, three-fold more pancreas transplant recipients were insulin independent 1 year following the procedure (141), although a comparison of the functional outcomes of the procedures is biased in favour of pancreas transplantation as it receives higher quality organs from younger and healthier donors (144-147). Pancreas transplantation is limited by surgical complications and morbidity (123, 124, 141), particularly in type 1 diabetic recipients with long-standing disease and complications of diabetes. Islet transplantation is an experimental therapy (132) offers a non-surgical approach with proven benefit in improving glycemic control and slowing progression of microvascular complications in long-standing diabetic patients for whom invasive surgery has high risks of peri- and post-operative morbidity. Nevertheless, improvements in long-term graft function and survival would increase support for islet transplantation.

1.4 Graft dysfunction in islet transplantation
At 2 years following islet transplantation, 75% of initially successful islet grafts can no longer maintain insulin independence (129), such that graft recipients require partial supplementation or full medical therapy with exogenous insulin. Both immune and non-immune mechanisms may contribute to islet graft loss and dysfunction.

1.4.1 Immune-mediated graft destruction

1.4.1.1 Alloimmunity
Graft tissue is at risk of rejection in allotransplantation. Rejection may be hyperacute (immediately following transplantation), acute (in the first week following transplantation), or chronic (occurring months to years following transplantation). Hyperacute rejection occurs within minutes to hours of engraftment. It may be mediated by both inflammatory and humoral mechanisms. Most commonly, tissue factor (TF) and macrophage chemoattractant protein (MCP-1) exposed on the surface of isolated islets promotes destruction of up to 70% of transplanted islets by the instant blood mediated inflammatory reaction (IBMIR) (148, 149).
IBMIR is characterized by activation of the coagulation and complement systems, rapid activation and consumption of platelets (150, 151), and subsequent inflammation and leukocyte infiltration (152). Heparin is administered peri-operatively and up to 7 days post-operatively to inhibit the clotting cascade (152, 153) to promote graft survival. Alternate strategies to reduce TF production, block exposed tissue factor, or interfere with the complement and clotting cascades show promise in minimizing IBMIR (148, 149, 152, 154-156).

Acute and chronic rejection are primarily lymphocyte mediated. Rejection is initiated by presentation of donor antigens to T cells in regional lymph nodes. Donor antigens are presented on major histocompatibility complexes (MHC II) on the surface of antigen presenting cells (APCs). APCs may be derived from the donor or recipient. Presentation by donor or recipient APCs is termed direct or indirect presentation, respectively. Direct presentation is of particular importance in acute rejection, while chronic rejection is mediated by indirect presentation (157). CD4\(^+\) lymphocytes are activated if a second signal is generated by binding interaction of the APC cell surface co-stimulatory molecular B7 (CD80, 86) with the T-cell CD28 receptor (158). Activated CD4\(^+\) lymphocytes undergo IL-2 driven proliferation and release cytokines and other ligands to activate CD8\(^+\) T-cells, macrophages, natural killer cells and B cells. CD8\(^+\) T cells kill graft cells by granulysin and perforin-induced cell lysis, by granzyme activation of caspases, and by activation of Fas receptors on target cells (157). CD4\(^+\)-mediated immunity has a critical role in mediating islet allograft rejection (159-161). Recent studies have also established a role for CD8\(^+\) T cells in allograft rejection (162-164). CD8\(^+\) cytotoxic T cells may be activated independently of CD4\(^+\) lymphocytes (165), by Th\(_1\) cytokines such as IFN\(_\gamma\) (162).

The humoral, or antibody-mediated response, may contribute to acute and chronic rejection. It typically plays a role in acute rejection if a patient has been previously sensitized, by means of previous transplants (166), blood transfusions (167), and pregnancy (168). In chronic rejection, alloantibodies develop to the graft over time (169). Antibody binding to graft antigens activates the complement cascade or phagocytosis of graft cells via Fc or C3 receptors.

Immunosuppression in islet transplantation was revolutionized by the Edmonton Protocol. Introduction of the Edmonton protocol dramatically improved 1-year islet transplant outcomes, most significantly by omission of glucocorticoids (129, 170). Prior to the Edmonton Protocol,
the insulin independence rate at 1 year post-transplantation was less than 10% (152). In this steroid-free protocol, immunosuppression was achieved using daclizumab for induction and low-dose sirolimus, tacrolimus, and mycophenylate mofetil for maintenance therapy (129). Each of these therapies interferes with lymphocyte activation and proliferation. Daclizumab, sirolimus, and tacrolimus inhibit IL-2 driven proliferation of activated T cells (158, 171). Daclizumab is a humanized monoclonal antibody that binds to the T-cell IL-2 receptor to inhibit binding of interleukin-2 (158). Sirolimus forms a complex with FKBP12, which binds to mTORC1 and inhibits mTOR signaling (158, 172). Tacrolimus inhibits calcineurin signaling and subsequent IL-2 transcription by binding to FKBP12-FK506 (158). Mycophenylate inhibits purine synthesis, which is critical to T cell proliferation (158). Rituximab has been proposed as an adjuvant therapy to inhibit humoral responses (173). Rituximab is a monoclonal antibody that binds CD20 on B cells (158). Immunosuppression protocols continue to be refined using combinations of these available immunosuppressive agents.

1.4.1.2 Autoimmunity
Autoimmune destruction of pancreatic β cells is intrinsic to the pathophysiology of type 1 diabetes. In type 1 diabetes, up to 90% of β cells are destroyed by T-cell mediated processes, although β cells and residual insulin production are still detectable years after the onset of overt disease (174). Differentiation of T cells involved in initial β cell destruction into memory cells allows the persistence of a population of T cells that are activated by β cell antigens. Presentation of such antigens following islet transplantation may re-activate these cells, leading to destruction of transplanted β cells. Activation of memory CD8+ T-cells has been associated with rapid destruction of β cells in twin-to-twin pancreas transplantation (175), although CD4+ memory T cells have also been implicated in recurrent autoimmunity (176). In some cases of islet transplantation, rapid graft loss has been associated with the re-appearance of GAD-65 and IA-2 autoantigens (152). Depletion using a CD8-specific monoclonal antibody (177) or with anti-CD4 antibody (178) has proven effective in protecting islet grafts in BB rats and NOD mice, respectively, from recurrent autoimmune destruction of β cells.
1.4.1.3 Innate immunity

The innate immune system recognizes pathogens in a pattern-specific manner. While traditional immunosuppressive therapies target the adaptive immune response, blockade of innate immunity also promotes islet allograft and xenograft survival. Many factors in the pre- and peri-transplant periods can activate the innate immune system. Islet isolation leads to cytokine and chemokine production by human islets (179). In the peri-transplant period, multiple factors promote activation of the innate immune system in the recipient. Surgical procedures and anesthesia promote systemic cytokine production (180). The infusion of isolated islets triggers IBMIR (149), which activates the coagulation and complement cascades, promotes cytokines and free radical production, and recruits neutrophils and macrophages to the graft. Further inflammation may result upon reperfusion of islets that were ischemic during pre-transplantation islet culture (181).

Activation of the innate immune system in transplanted islets promotes pro-inflammatory signaling and recruitment of natural killer cells, macrophages, neutrophils, and dendritic cells (182). These cell types are recruited by cytokines and complement activation (182). Elevated levels of cytokines are expressed in transplanted islets and contribute to graft dysfunction and loss. Syngeneic murine islet grafts express high levels of cytokines and chemokines (183). Within the graft, pro-inflammatory mediators are released by intra-islet macrophages (184) and damaged β-cells (185). When islets are transplanted into the portal vein, hepatic Kupffer cells also secrete pro-inflammatory cytokines (186). Secreted cytokines include IL-1β, TNF-α, IFN-γ, IL-2 and IL-6 (187-191) and known chemokines include IL-8, MCP-1, and macrophage inflammatory protein-1β (MIP-1β) (184, 185). Cytokines may induce β cell apoptosis. Release of IL-1β, TNF-α, and IFN-γ within the islet contributes significantly to early β-cell loss (187-191). The direct and indirect detrimental effects of released cytokines on transplanted islets are demonstrated by cytokine blockade and macrophage depletion. Cytokine blockade with neutralizing antibodies preserves β cell mass and decreases the number of islets required to achieve normoglycemia in mice (192). Systemic depletion of macrophages prolongs allograft survival (182). Toll-like receptor (TLR) signaling is an additional means by which the innate immune system may be activated in islet transplantation. TLR activation may play a role in the early function of both allografts and xenografts (193-197). TLR signaling is also important in mediating inflammation in islets. While TLR signalling in donor islets is not essential to long-
term rejection of allografts or xenografts (198, 199), TLRs on immune cells of the graft recipient are involved in the innate immune response to syngeneic islet transplantation (200) and may inhibit T regulatory cell generation (201). Innate immunity may be activated by multiple mechanisms in islet transplantation and its activation may be detrimental to graft survival and function. Additionally, innate activation of dendritic cells may also promote adaptive immune responses (182) which mediate allorejection.

1.4.2 Non-immune causes of graft failure

Much focus on islet loss in transplantation has centered around immune-mediated mechanisms. Accumulating evidence from animal studies and clinical data suggests that non-immune factors also contribute to early and long-term graft failure.

Early graft failure encompasses both primary non-function of engrafted tissue and rapid loss of graft function and survival in the early post-transplantation period. Up to 60% of graft mass is lost in the first 7 days following transplantation in a syngeneic rodent model not subject to auto-or alloimmunity (202).

Long-term graft failure refers to declining graft function following an extended period of graft function. Islet grafts fail slowly and progressively over time, despite aggressive immunosuppressive therapy. Whereas T-cell mediated destruction of target cells is rapid even in chronic rejection, human islet transplant recipients maintain C-peptide secretion despite loss of insulin independence (129, 203, 204). Failing grafts in humans and primates commonly demonstrate little evidence of rejection. In three autopsies performed on allogeneic islet transplant recipients with marginally functioning grafts, islet mass was reduced to 10-15% of the infused islet mass yet there was no evidence of inflammation or infiltration (205). Toso et al failed to detect significant peri-islet inflammation in biopsies from 16 human islet allograft recipients (206). In those biopsies in which a minimal immune cell infiltrate surrounded some islets, there was no correlation with graft function even for 2 patients with worsening glycemic control (206). Slowly progressive loss of islet function is observed in cynomolgus monkey islet isografts and allografts despite adequate immunosuppression and no evidence of rejection (207). Further, human and other large mammal islet autografts demonstrate a slowly progressive loss of insulin independence despite absence of allo- and auto-immunity (205, 208, 209). Biopsies in
beagle autotransplants prior to graft failure showed that 84% of islets contained well-granulated β cells, while this decreased to 5% following graft failure (209). Of human patients achieving insulin independence following autotransplantation, 20% return to requiring insulin between 1-1.5 years following transplantation and of patients with partial graft function, 50% returned to requiring insulin several months post-transplant (210). Webb et al reported that only 26% of islet autotransplant patients experienced periods of insulin independence, with 11% remaining stably independent, and the median time of insulin independence being 16.5 months (211). Insulin dose increased with each year following islet auto transplantation (211, 212). Loss of β cell mass and a gradual progressive β cell dysfunction in islet autografts suggest that non-immune factors are important contributors to loss of insulin independence in graft recipients.

1.4.2.1 Causes related to early graft dysfunction

1.4.2.1.1 Pre-existing conditions within donor tissue

Characteristics of donor tissue may impact islet engraftment and function following transplantation. Donor organs are scarce (213), with the highest quality organs being utilized for pancreas transplantation (144, 145). Ideally, an organ used for islet transplantation should fall within selection criteria for solid organ transplants, with a donor age range between 18 and 45 years, a body weight between 40 and 100 kg, and without a history of diabetes mellitus or pancreatitis (214).

Organs allocated to islet transplantation are more likely to come from aged donors with higher body mass index (BMI) (146, 147) and tend to have longer cold ischemic time (144). A higher BMI is preferential in islet isolation as the digestion procedure is more efficient (214-217) and the islet yield is greater (218). Donors for islet transplantation have been as old as 70 years (219). Islets from younger donors have improved function in vitro and in vivo (214, 220). Once donor age exceeds 45 years, there is increased risk of poor glycemic control and early graft dysfunction in whole pancreas transplant (221). Some studies have shown lower insulin content and responsiveness in islets of older donors (218, 222). High-BMI donor pancreata tend to be fatty (216), more susceptible to reperfusion injury (216), and have significantly lower survival and long-term maintenance of insulin independence following pancreas transplantation (217). Although type 2 diabetic pancreata are not used in islet transplantation, high BMI donors have a
high probability of being pre-diabetic. It is not known whether β cell dysfunction is reversible in islets from pre-diabetic donors. There is, however, evidence that islets of type 2 diabetic donors function poorly in transplantation. Hyperglycemia in a donor has been shown to have poor outcomes following pancreas transplantation (145, 223). Islet yields from human type 2 diabetic pancreata are reduced (205). Islets from type 2 diabetic humans are smaller, have an increased α:β ratio, have abnormal GSIS and do not reverse hyperglycemia in transplanted mice (205). In spite of correlations of elevated donor age and BMI with reduced islet quality, each of these characteristics improve islet yield following isolation in all but one report (214, 216, 224-227). Such donors continue to be used for islet transplantation due to the high islet yield and the allocation of better quality pancreata for whole pancreas transplantation.

1.4.2.1.2 Isolation procedure and islet culture
Islets may sustain considerable damage during the isolation procedure. Many factors influence final islet yield and quality. Islets are subject to warm ischemic damage during collagenase digestion of the pancreas (228). Furthermore, islet isolation damages islets by osmotic, chemical, ischemic, enzymatic and mechanical insults (229-231). Isolation-induced damage to islet ultrastructure includes plasma membrane breakage (232), loss of peripheral cells (232), and disrupted islet capillary integrity and islet extracellular matrix. Disruption of the islet matrix during isolation leads to reduced islet viability and subsequent anoikis (233) or apoptosis (234). Cold ischemic time following collagenase digestion also affects islet yield and quality (218, 224). Minimization of cold ischemic time improves outcomes (228). Warm and cold ischemia subject β cells, which express particularly low levels of anti-oxidants (235), to ischemic damage. Following islet isolation, pro-apoptotic factors are elevated in isolated islets (229, 236). Islet isolation decreases insulin mRNA by 40% and upregulates stress genes (237). Islet isolation also leads to induction of proinflammatory cytokines and chemokines (179, 230, 238-240). An additional important consideration in islet isolation is the purity of the islet preparation. One might predict that impure preparations would be less effective. Surprisingly, islet preparations containing more ductal epithelial cells, thought to be islet progenitor cells, are more likely to maintain long-term metabolic success (222).

The necessity of islet culture prior to transplantation is debatable. Although the Edmonton Protocol recommended transplantation of islets within 2 hours of isolation, the majority of islet
transplant centres culture islets between 48-72 hours to give the transplant team and recipient more flexibility (241). Pre-transplant culture also allows quality assessment of islets and may improve islet metabolic activity as compared to islet function immediately following the stress of isolation (242). Islet culture may reduce immunogenicity and the culture medium CMRL reduces MCP-1 and TF expression (179, 243). Deng et al reported that improved outcomes in islet transplantation may have resulted from a shift to pretransplant culture (205). Islets are cultured at 22°C pre-transplant to promote apoptotic loss of remaining acinar tissue, decrease β cell metabolism, and slow production of pro-apoptotic molecules prior to transplantation (244). Reports on the effects of islet culture on viability and function are variable (243, 245-247). Islet morphology and function have been reported to be maintained between 1 and 2 weeks (241, 243, 248). Kin et al reported that islet mass is reduced in culture despite improvements in morphology (249). Established negative consequences of islet culture may result from microbial contamination (250) and hypoxia (235). Culture may promote β cell de-differentiation and replication (229, 250) as well as islet amyloid deposition (2, 3). Finally, large islets tend to undergo central necrosis and have reduced secretion and survival (244, 251). While overnight pre-transplantation has both practical and some functional advantages, additional strategies employed in pre-transplant culture might improve outcomes following transplantation. Additionally, overnight culture of islets allows a window for ex-vivo treatment with compounds to promote islet survival following transplantation.

1.4.2.1.3 Site of transplantation

In clinical islet transplantation, islets are infused into the portal vein following percutaneous catheter insertion under radiological guidance (252). The portal vein has been the site used in the majority of human islet transplants. Complications of portal vein infusion are rare but do include thrombosis (253), areas of local focal necrosis following islet infusion (254), modest increases in portal venous pressure with each subsequent islet infusion (253), hemorrhage, portal vein thrombosis, hemothorax, pneumothorax, hemobilia, and puncture of adjacent organs (252). The liver as a transplant site has both advantages and disadvantages. A critical advantage in humans is that this site may be accessed non-surgically under alternating ultrasound and fluoroscopic guidance (255). This is of particular value for type 1 diabetic patients for whom invasive surgery poses increased risk of morbidity, infection and complications relative to the general population (256). Transplantation into the portal vein leads to insulin independence given a sufficient islet
mass (129) and islets achieve pulsatile insulin secretion in response to hyperglycemia (257, 258). Over the long-term, however, this site is not ideal for islet function and survival. Insulin content, glucose-stimulated insulin release, insulin biosynthesis, glucose oxidation rate, and glucagon responsiveness to hypoglycemia were markedly decreased in islets transplanted to the liver relative to native islets (259-261). Islets transplanted into the portal vein showed downregulation of critical β cell proteins including Pdx-1, GLUT-2, glucokinase, mitochondrial glycerol-phosphate dehydrogenase, and pyruvate carboxylase (261, 262). Islet survival is limited in the portal vein. Survival rates of rat islets transplanted into the portal vein declined from 89% at day 1 to 43% at day 7 (202). There are many reasons the portal vein may not be the optimal transplantation site. Oxygen tension within the portal vein may be inadequate to support the high metabolic demand of pancreatic islets (263). Further, vascular density within portal vein transplanted islets is much lower than in native islets (264). Local insulin release by intrahepatic islets stimulated triglyceride accumulation and upregulation of lipogenic enzymes in adjacent hepatocytes, which may promote β cell dysfunction and death by lipotoxicity (265), evidenced by a reduction in islet β cell area. The local hepatic milieu may generate a proinflammatory milieu for islets, as its reticuloendothelial system has a large population of macrophages which may produce ROS, NO and cytokines detrimental to islet function (263). Finally, the portal vein has elevated concentrations of glucose and metabolites. Immunosuppressive drug concentrations in the portal vein are 2-3 fold higher than in the peripheral circulation (171, 172, 266, 267). Although the portal vein is easily accessible for transplantation, it is not an ideal heterologous transplantation site for islets.

Other sites have been considered for islet transplantation. Different organs may have different paracrine activity, local metabolite levels, oxygen tensions, inflammatory responses, and revascularization capacity (268). Other sites in which islet transplantation has achieved glycemic control in animal models include the spleen (269-271), omentum (262, 271, 272), renal subcapsular space (272, 273), gastrointestinal wall (273), pancreas (273, 274), thymus (275), bone marrow (273, 276), celiac artery (277), skeletal muscle (262, 273), and subcutaneous space (278, 279). Due to practical reasons and associated risks, the renal subcapsular space, spleen, and pancreas are limited in their suitability for clinical application (273, 280). These and other proposed sites remain experimental and further research is required to validate their utility in clinical islet transplantation.
1.4.2.1.4 Hypoxia following transplantation

Islets have a high oxygen tension in the native pancreas and their metabolic activity is highly dependent upon sufficient oxygen availability. Islets have substantially reduced oxygen tension in culture. Furthermore, islet endothelial cells die rapidly in culture (229) and islets are avascular following transplantation (281). Islets are not revascularized until 7-14 days post-transplantation (152, 282). Final graft vascular density is half that of native islets and oxygen tension is reduced five-fold regardless of site of implantation (281-283). In the portal site, capillaries may fail to penetrate islets (244). Islets may be lost by apoptosis due to hypoxia (284). Numerous areas of local focal necrosis are found in the liver following islet infusion and up to 2 weeks post-transplantation (254, 285). Reperfusion injury may also occur following re-establishment of the vasculature, in which re-introduction of oxygen may lead to production of reactive oxygen species; β cells have very limited antioxidant defense and are highly susceptible to ROS injury (244). An avascular period in early transplantation, altered vascular phenotype and lower islet oxygen tension may partly account for differences in long-term function between transplanted isolated islets and islets transplanted as part of a whole pancreas.

1.4.2.1.5 Hyperglycemia

Islets are transplanted into diabetic individuals, who by definition are subject to chronic hyperglycemia. In the early post-transplantation period, individuals may remain hyperglycemic until islets are revascularized and islet stress due to isolation and transplantation has subsided. Surgical procedures promote insulin resistance and temporary impairment of glucose-stimulated insulin secretion (286). Elevated portal vein glucose levels likely contribute to islet loss prior to engraftment and in the first few weeks following transplantation. To illustrate the effect of recipient hyperglycemia on islet function, primary non-function was observed in transplants into severely diabetic recipients (287). Recipient hyperglycemia at time of transplant greatly affected graft survival (288, 289). Hyperglycemia in the immediate post-transplantation period may stimulate β cell oxygen consumption and promotes apoptosis (290). Furthermore, hyperglycemia aggravates monocyte chemokine and cytokine expression, activation and adhesion to endothelium (291). Chronic exposure to high levels of extracellular glucose negatively affects β cell function. Prolonged exposure of islets to high glucose concentrations impaired glucose-
stimulated insulin secretion and induced apoptosis in β cells (85, 292-297) and decreased insulin content (294). Hyperglycemia in transplanted murine islets led to degranulation and swelling of ER and mitochondria (298). In rat islet transplant recipients that remained hyperglycemic for extended periods of time, glucose-stimulated insulin secretion was reduced or absent and grafts had 10% lower insulin content (295). Grafts which subsequently normalized after 2 weeks retained defects in glucose-stimulated insulin secretion (295).

β cells adapt to hyperglycemia by expansion of β cell mass and by metabolic changes. Glucose is a critical determinant of β cell mass (299) and hyperglycemia promotes neogenesis and β cell replication in a transplantation model by activation of Akt/mTOR signaling (289, 300). This effect was reported by Donath et al following acute glucose stimulation while chronic exposure to glucose impairs proliferation (293). Metabolic adaptation includes downregulation of glucose sensing and secretion mechanisms, increased glycolysis, and switching to futile cycles and production of uncoupling proteins (301); these effects are reversible in the short-term (299) but may not be in the long-term. β cell de-differentiation, apparent by increased LDH expression and reduced levels of transcription factors and genes critical to β cell function, may occur in transplanted islets and partially pancreatectomized animals due to prolonged hyperglycemia (237, 302-304). Additional changes noted in β cells following exposure to hyperglycemia include intracellular glycogen deposition (305, 306) and hypertrophy (303). Prolonged hyperglycemia promotes β cell exhaustion and glucotoxicity. β cell exhaustion occurs when insulin production cannot keep up with demand for secretion (301) and is likely linked to prolonged ER stress. Glucotoxicity negatively impacts insulin gene transcription, content, and secretion and insulin receptor expression and signaling in the β cell and promotes β cell apoptosis (294, 300, 307). Glucotoxicity may also impair β cell function by altering metabolic pathways, increasing intracellular calcium concentration, promoting ER stress from increased demand for insulin production, oxidative stress (246) and mitochondrial dysfunction, and stimulation of IL-1β production (300, 308). IL-1β suppresses β cell function and promotes Fas-mediated apoptosis (293).

Reducing demand on β cells slows β cell metabolism and reduces insulin secretion and ER and oxidative stress (309). Insulin and K_{ATP} channel activators have induced β cell rest in animal models and clinical studies (301). Short-term insulin therapy in new-onset diabetic patients with
severe hyperglycemia is highly effective in achieving adequate glycemic control and improving insulin secretion after 6 months as compared to oral hypoglycemic agents (299, 310). Peri-transplant insulin treatment promotes superior graft outcomes (311). Treatment of partially functioning autografts with insulin for the first 3 months post-transplantation allowed 50% of grafts to achieve periods of insulin independence (211). Insulin treatment for 14 days promoted function in marginal autografts (312). Supplemental insulin treatment preserved and promoted the function of second allograft islet infusions in human recipients with marginally functioning grafts despite infusion of low numbers of islet equivalents (313). Restoration of euglycemia using insulin promotes β cell regeneration from islet precursors (314). In marginal mass transplants, insulin treatment beginning 7 days prior to transplantation promoted graft function after ceasing insulin therapy (289). Islets treated with insulin during the peri-transplant period restored normoglycemia while the same mass of islets without insulin treatment did not (315).
Marginal mass grafts initially treated with insulin had a greater β cell mass, increased β cell replication, and a lower rate of apoptotic β cells at 1 month post-transplantation than mice that remained hyperglycemic (289).

1.4.2.1.6 Transplanted islet mass
In humans, an adequate graft mass in islet allotransplantation is considered to be above 10,000 islet equivalents (IE)/kg (316). Insulin independence in islet autotransplantation was more likely to be achieved if greater than 2,000 (317) or 6,000 IE/kg (210, 317, 318) were transplanted. Beyond a critical threshold of islets, canine autografts maintained normoglycemia (209). Patients receiving fewer islets are less likely to become insulin-independent and allograft recipients with single donor islet infusions have poorer long-term outcomes (319). This is particularly surprising given that >90% of pancreas mass must be removed in rodents or 50-60% in humans to induce glucose intolerance or hyperglycemia (87, 303, 320, 321). These findings suggest that a strong majority of transplanted isolated islets fail to engraft; thus, infusion of an optimal number of islets does not guarantee an optimal mass graft. β cell replication in transplanted human islets is particularly low (322), as has also been demonstrated in islet culture (323, 324). Substantial apoptosis occurs in the first days following transplantation in human and murine islets (236, 325-328) and the rate of apoptosis is particularly high in human islets following isolation (329). Furthermore, a continuous decline in β cell mass was observed over weeks in marginal mass murine grafts (330). Marginal mass grafts also exhibit metabolic dysfunction (326, 331),
increased apoptosis (331), and lower resistance to secretory stress (287) and insulin resistance (332) in animal models.

1.4.2.1.7 Instant blood mediated inflammatory reaction
The instant blood-mediated inflammatory reaction (IBMIR) is stimulated by TF (149) exposed on the islet surface following islet isolation. Human islets exposed to blood are rapidly destroyed (333). In the peri-transplant period, IBMIR activates the coagulation and complement cascades (150, 151). The extent of IBMIR in an islet graft recipient may depend upon the quality of an islet preparation (150) and may be monitored indirectly by thrombin-antithrombin (TAT) levels (149). The signaling cascades activated by IBMIR lead to consumption of platelets, generation of cytokines and free radicals at the site of islet engraftment, and recruitment of leukocytes and macrophages (152). Each of these processes contribute to islet destruction by the innate immune system. In rats, it is estimated that only one third to half of the transplanted islet mass engrafts (202); IBMIR likely plays a role. Numerous areas of local focal necrosis, which likely correspond to sites at which IBMIR has been initiated, are found in the liver following islet infusion (254) or 2 days later (285), but not at 4 weeks following transplantation (285). Islet cells adjacent to these focal necrotic regions of hepatic tissue demonstrate markers of apoptosis (254).

Multiple solutions have been proposed to protect islets against IBMIR. One approach is to minimize the exposure of TF on the islet surface by reducing its production with nicotinamide (152) or pre- and perioperative treatment of recipients with an anti-TF antibody (148). The latter solution was effective in a primate marginal islet allograft model (148). Alternative solutions have attempted to minimize the activity of the complement cascade with soluble complement receptor and the coagulation cascade with activated protein C, the thrombin inhibitor Melagtran, TF inhibitor active site-inactivated FVIIa, heparin, dextran sulfate, or thrombomodulin (149, 152-156, 334). In clinical islet transplantation, heparin is administered peri-operatively and for up to 7 days post-operatively to inhibit the clotting cascade (152, 153) and promote graft survival. The efficacy of soluble heparin, however, is questionable (149). Islet surface heparinization has been demonstrated to be more effective at protecting islets of multiple species against IBMIR (149).
1.4.2.1.8 Inflammation

Inflammation affects islets immediately following engraftment (192) and long-term following transplantation. Islet isolation and culture promote the production of inflammatory mediators. Islet isolation induces hypoxic and mechanical injury leading to activation of stress signalling pathways (335), including c-Jun N-terminal kinase (JNK) and p38 (336). Islet culture promotes cytokine and chemokine secretion in human or murine islets and the static environment promotes prolonged exposure of islets. Cytokines and chemokines, such as MCP-1, CCL2, CCL25 and CCL28 are produced by human and mouse islets in culture (230, 239, 337). Markers of inflammation are elevated in islets following transplantation. IL-1β and iNOS are substantially upregulated in the first week following syngeneic islet transplantation and localized to CD68-positive macrophages (191). Cytokine expression within the graft indicates the presence of inflammation. IL-1β, IFN-γ and TNF-α are elevated in islets following portal vein islet transplantation (186). In a study of the effects of adenoviral expression on post-transplant islet inflammation, control non-transduced grafts were not infiltrated but expressed comparable levels of IFN-γ, IL-4 and CCR7 and greater levels of CCR5 than did adenovirally transduced syngeneic grafts (338); this demonstrates that grafted islets express cytokines and chemokines. Pro-inflammatory cytokines have a detrimental effect on β cell survival and function (339-343). Local chemokine and cytokine release attracts host macrophages to the graft, which further propagate local inflammation.

Many established causes of inflammation in the type 2 diabetic islet may similarly promote inflammation in transplanted β cells. Long-term allograft survival may be impaired by inflammation associated with metabolic stress, including chronic hyperglycemia and dyslipidemia (289, 344). Hyperglycemia promotes β cell production of IL-1β and subsequent induction of the Fas receptor (293). One month duration of hyperglycemia in the Cohen rat promoted macrophage appearance in the islets (345). Anakinra, an IL-1 receptor antagonist, has been shown to improve β cell secretory function in type 2 diabetic patients (346) and to reduce hyperglycemia in the GK rat (345). Overexpression of IL-1R antagonist in murine islets improves syngeneic transplantation outcomes (347). Long chain fatty acids may also promote islet production of IL-1β, IL-6, IL-8 and CXCL1, potentially via stimulation of TLRs (293).
1.4.2.1.9 Immunosuppressive drug toxicity

Although progressive graft failure occurs in islet autograft recipients, it occurs at a faster rate in islet allograft recipients in many (129, 210, 312, 317, 348, 349), though not all (210, 211, 317), human clinical studies. The disparity between success of islet allografts and autografts may result in part from the absence of toxic immunosuppressive agents in autotransplantation. Immunosuppressive agents currently employed to prevent islet allograft rejection, including tacrolimus, sirolimus, and mycophenolate, have all been shown to have direct adverse effects on β cells. Tacrolimus is the most diabetogenic agent employed in the immunosuppressive cocktail. In whole organ transplant recipients, tacrolimus causes post-transplant diabetes mellitus (PTDM) in up to one third of patients (238, 350-353). Decreased PTDM correlates with reduced doses of tacrolimus (354). Tacrolimus directly affects the β cell, causing a reversible insulin secretion defect (152, 355-360), decreased insulin mRNA transcription (266, 357), loss of normoglycemia in murine (266, 361) and canine (362) islet graft recipients, and decreased β cell survival (356). Portal vein levels of tacrolimus exceed those of the peripheral circulation by three-fold, further potentiating its dose-dependent toxicity (266).

Reports are conflicting as to whether sirolimus negatively impacts β cell survival and function. Although sirolimus is not to be diabetogenic (238, 363), by some reports it may promote PTDM (353). In many animal transplant models, sirolimus had no adverse effects on β cell function (171, 364-367), while other studies in normal or transplanted animals have shown sirolimus-induced hyperglycemia, impaired islet graft function and β cell apoptosis (171, 364, 368, 369). In transformed β cell lines, sirolimus promoted a concentration- and time-dependent reduction in viability, Pdx1 and GLUT expression, and GSIS and increased apoptosis (171, 172, 360, 370).

Diabetogenic effects of sirolimus on the β cell may only be evident at supra-therapeutic doses (171, 172), although human islets transplanted into the portal vein are exposed to 2-3 fold higher concentrations of sirolimus than in the peripheral circulation (171, 172, 266). Additional deleterious effects of sirolimus are anti-proliferative and anti-angiogenic. Sirolimus inhibits the proliferation-promoting effects of IGF-1 on β cells (172, 371). It also suppresses the expansion of human ductal cells at therapeutic concentrations and in pregnant mice (171). In a transgenic model of conditional β cell destruction, treatment with a combination of sirolimus and tacrolimus
prevented β cell regeneration (372). Sirolimus inhibits angiogenesis and VEGF-mediated revascularization (373, 374), which is critical to graft revascularization.

Mycophenylate, by most reports (358, 366, 375), has no deleterious effects on the β cell (358, 366). Mycophenylate did suppress insulin secretion in transformed β cell lines and rat islets (360) and promote apoptosis in human islets (369) but had no effect upon maintenance of normoglycemia in transplanted human islets (361). As it is necessary to continue use of immunosuppressive agents in islet allograft recipients, further development of immunosuppressants with less β cell toxicity is required.

1.4.2.2 Intrinsic features of the β cell that may promote graft failure

1.4.2.2.1 Metabolic stress on the graft

The transplanted β cell is typically under a high degree of metabolic stress. As described previously, a limited β cell mass engrafts, estimated to be ~30% of the β cell mass in a native pancreas. Secretory demand per β cell is high and exacerbated by factors such as recipient hyperglycemia and elevated insulin resistance in transplant recipients (376). Many factors in the transplantation environment, such as in immunosuppressive drugs, inflammation, hypoxia, and elevated free fatty acid concentration the vicinity of transplanted islets, have a detrimental effect on β cell viability and secretory function. Transplanted islets may be derived from obese donors likely to have metabolic syndrome. The β cell’s capacity to adapt to chronic secretory stress is finite. In order to successfully adapt, the β cell must maintain homeostatic regulation of physiological endoplasmic reticulum (ER) and oxidative stress responses and its pro-hormone processing capacity. In contrast, β cell dysfunction is apparent by impaired GSIS, impaired processing of proinsulin, and amyloid deposition.

1.4.2.2.1.1 Endoplasmic reticulum stress responses in β cell physiology

β cells, as professional secretory cells, have a highly developed ER (236, 377). The ER is fundamentally important in many cellular processes. It is involved in phospholipid synthesis (378), free fatty acid esterification (379), and steroid production and storage (378). The ER is an important calcium buffer and modulates cellular calcium signaling via inositol-3-phosphate and
ryanodine receptors (IP$_3$R and RyR, respectively) (380). The ER is also critical for trafficking, folding and post-translational modification of proteins destined for secretion or plasma membrane insertion (381). Peptides with a signal recognition particle (SRP) are translocated into the ER as ribosomal translation occurs. Upon termination of translation, chaperone proteins including BiP (GRP78), GRP94, calnexin, and calreticulin, facilitate peptide folding (380). The β cell ER lumen has a protein concentration exceeding 100 mg/ml and processes approximately 1 million proinsulin polypeptides per minute (382). In addition to chaperone-mediated folding of insulin, the ER also promotes oxidation of its three disulfide bridges by protein disulfide isomerase (PDI) (381). Only properly folded proteins may transition along the secretory pathway. The ER routinely disposes of soluble misfolded proteins, up to 30% of newly synthesized polypeptides, by ER-associated degradation (ERAD), in which they are retro-translocated into the cytosol, ubiquitinated, and degraded by the proteasome (380, 381); insoluble aggregates of protein are disposed of by autophagy (382, 383). The β cell ER must handle continual fluctuations in protein load whilst maintaining quality control.

Demand for insulin increases in accordance with the extracellular glucose concentration. Acute exposure to high concentrations of glucose can promote a twenty-fold increase in insulin biosynthesis (384). Proinsulin represents 30-50% of the total protein in the β cell, thus such increases produce dramatic changes in ER protein content (382). At elevated glucose concentrations, the ER must rapidly increase its capacity to fold and oxidize proinsulin. A lack of equilibrium between ER protein load and folding capacity is referred to as “ER stress”, which triggers signaling pathways critical to cellular adaptation to stress (377). Transient activation of ER stress responses protects the cell from an ER protein overload by attenuating translation of most peptides including insulin, promoting ER chaperone translation, and promoting ERAD. Such transient activation may be referred to as the unfolded protein response (UPR). The ER stress response has 3 distinct pathways, each initiated by an ER transmembrane protein (PERK, IRE1, and ATF6) (380). Activation of the UPR promotes general translation attenuation, increased production of ER chaperone proteins, and ERAD (380). The UPR is a protective temporary means restoring of ER homeostasis (385) that is critical in response to constant fluxes in insulin production in the β cell.
1.4.2.2.1.2 Pathological ER stress and apoptosis in β cells

Sustained activation of ER stress responses is not conducive to cell viability. β cells are particularly sensitive to translational attenuation (381). Prolonged activation of ER stress responses in the β cell may occur due to a combination of elevated demand for insulin production and an elevated load of misfolded protein in the ER. Protein over-expression in β cells of transgenic mice (30, 386, 387) triggers ER stress-induced apoptosis due to high biosynthetic load. Sustained ER protein overload of misfolded protein leads to β cell dysfunction and progressive loss of β cell mass, as illustrated in MUNICH and AKITA mice (377, 380, 382, 388, 389). Metabolically stressed human β cells demonstrate markers of ER stress (91, 390) and activation of associated apoptotic signaling (30, 380, 391). Superimposing additional internal or external stressors on the β cell may exceed the capacity of the UPR to restore ER homeostasis and preserve β cell function. Internal stressors in addition to protein overload and misfolding include changes in $[\text{Ca}^{2+}]_{\text{ER}}$, required to sustain chaperone function (392), and oxidative stress, such as that produced in the ER upon extensive oxidation of disulfide bridges or in the absence of sufficient reducing equivalents or antioxidants (393). ER stress-mediated apoptosis is exacerbated in rodent β cells expressing amyloidogenic isoforms of islet amyloid polypeptide (IAPP) (394, 395) and leads to reduction of β cell mass in the HIP rat (97). External stressors that promote ER stress include elevated extracellular glucose (91, 396-398), elevated free fatty acids, cytokines (399-402), nitric oxide (403), and reactive oxygen species (236, 398, 404, 405). Many of these stresses are exacerbated in the islet transplantation environment. Prolonged activation of ER stress signaling leads to activation of pro-apoptotic pathways. Prolonged ER stress responses promote CCAAT/enhancer-binding homologous protein (CHOP) (229), caspase-12 (380), and JNK activation (406, 407) in β cells.

1.4.2.2.1.3 Oxidative stress

As a highly metabolically active cell, the β cell produces high levels of reactive oxygen (ROS) and nitrogen (RNS) species. ROS are primarily generated by complexes I and II of the mitochondrial respiratory chain and by NADPH oxidases (408). ROS are generated upon catalysis of the three disulfide bonds per proinsulin molecule and one disulfide bond per proIAPP molecule in the ER (409). The β cell expresses low levels of antioxidants compared to other cell types and is highly susceptible to oxidative stress (244). Oxidative stress results when a
cell’s antioxidant capacity is insufficient to buffer ROS. Increasing metabolic demand increases ROS generation by increased mitochondrial respiration and by increased prohormone disulfide bond catalysis. As an example of β cells under constant secretory demand, human type 2 diabetic β cells show markers of oxidative stress (90, 410, 411). Islet isolation (412) and reperfusion injury (413) upon islet revascularization introduce additional oxidative stress in islet transplantation. Oxidative stress in islet transplantation and type 2 diabetes may be alleviated by antioxidant (181, 414) or ROS scavenging (415) therapies. Prolonged oxidative stress promotes β cell apoptosis primarily mediated by CHOP and JNK signaling (416).

1.4.2.2.1.4 Oxidative and ER stress pro-apoptotic signaling

ER and oxidative stress are intricately linked. Elevated production of proinsulin generates not only ER but also oxidative stress. Elevated mitochondrial metabolism generates oxidative stress by ROS production but also modulates ER stress through calcium signaling interaction with the ER. As such, the CHOP and JNK pro-apoptotic signaling pathways activated by ER stress are also activated by oxidative stress. CHOP promotes Ca^{2+}-dependent apoptosis by transcriptional activation of proapoptotic genes (DR5, TRB3, BIM, Doc1, CA-VI, Doc4, Doc6), induction of ROS, repression of the anti-apoptotic factor Bcl-2, translocation of Bax to mitochondria, and depletion of glutathione (381, 382). Islets from mice lacking functional CHOP (CHOP^{−/−}) have decreased apoptosis and increased expression of UPR genes and CHOP deletion delays the onset and severity of the diabetic phenotype (417, 418). Additional mechanisms of apoptotic induction, both mitochondrial-dependent and ER-specific, have been attributed to particular branches of the ER stress pathway. ATF4 may also promote apoptosis by suppression of Bcl-2 (384). IRE1α recruits TRAF2 and ASK1 to promote MAPK/JNK signaling (384, 419). IRE1α/TRAF2 may also promote the clustering and activation of caspase-12 (419). Procaspe-12 is localized to ER membranes and undergoes cleavage during ER stress in murine cells (420). Active caspase-12 is thought to promote the caspase cascade by cleaving caspase-3 in mice, although its role in promoting apoptosis is controversial and the human orthologue is a pseudogene (391, 421). Pharmacologically-induced ER stress has given additional insight into mechanisms by which ER stress may promote apoptosis (420). Such agents cause mitochondrial cytochrome c release and loss of mitochondrial transmembrane potential and implicate the BH3 proteins Bax and Bak in ER stress-induced apoptosis. Finally, perturbed ER Ca^{2+} homeostasis may contribute to apoptosis following induction of ER stress. ER Ca^{2+} stores can both sensitize mitochondria to pro-apoptotic
stimuli and activate cytoplasmic cascades (420). Islets lacking the ER-stress responsive ER Ca^{2+} channel Wsf1 are particularly susceptible to ER-stress-induced apoptosis (422).

### 1.4.2.1.5 Impaired prohormone processing in islet transplants

Proinsulin and proIAPP are synthesized as inactive precursors that undergo proteolytic processing to generate their mature forms. Both proinsulin and proIAPP are synthesized by ribosomes on the rough ER (RER) and translocated into the ER as synthesis occurs. These proteins are then trafficked through the regulated secretory pathway. Prohormone processing occurs in the secretory granules by the prohormone convertases PC1/3 and PC2 which cleave adjacent to pairs of dibasic residues. Proinsulin is cleaved in immature secretory granules by PC1/3 between residues 32 and 33 (423). It is later cleaved in mature secretory granules by PC2 between residues 65 and 66 (424). Subsequent removal of dibasic pairs at each cleavage site by carboxypeptidase E (CPE) yields mature insulin and C-peptide (425). ProIAPP cleavage also begins in immature secretory granules by C-terminal PC1/3 cleavage (426). PC2 then cleaves the resulting N-terminally extended form at the N-terminal cleavage site and acts in mature secretory granules (427). Mature IAPP is yielded following CPE removal of dibasic residues and N-terminal amidation by peptidylglycine α-amidating monooxygenase (PAM).

Prohormone processing is inefficient in the type 2 diabetic β cell (86). This impairment may result from an intrinsic β cell defect or decreased processing time when immature granules are secreted under high metabolic demand. Defects in prohormone processing are illustrated by hyperproinsulinemia and elevated ratios of secreted proinsulin:insulin and proinsulin:C-peptide in both type 2 diabetic human patients (86) and in human islet transplant recipients (316, 376).

### 1.4.2.1.6 Islet amyloid

Islet amyloid refers to a localized amyloidosis specific to the pancreatic islets. Classically, islet amyloid deposits have been identified in insulinomas (428, 429) and in islets of type 2 diabetic patients (97, 430, 431). Islet amyloid is identified in histological sections by fluorescence upon thioflavin S staining or by apple green birefringence upon Congo red staining. Islet amyloid is composed primarily of aggregated IAPP. Additional minor components include N-terminally
unprocessed IAPP (432), heparan sulfate proteoglycans (433), apolipoprotein E (433), and serum amyloid P (433).

IAPP is a 37 amino acid peptide secreted from β cells in response to glucose or other secretagogues along with insulin, in a molar ratio (IAPP:insulin) of ~1:100 (433). Under normal physiological conditions, IAPP is secreted as a soluble hormone and circulates in serum at concentrations in the low picomolar range (433). IAPP has central and peripheral effects that protect against obesity and diabetes. IAPP has anorexigenic effects by stimulation of noradrenergic neurons (434) in the area postrema of the hindbrain (435) and by potentiating the anorexigenic effects of leptin in the hypothalamus (436, 437). The peripheral effect of IAPP on energy balance is to slow gastric emptying (433, 437) and thereby reduce post-prandial glucose excursions (438). A non-amyloidogenic analogue of IAPP, pramlintide, is used clinically as an adjunct therapy to improve glycemic control and promote weight loss (439). IAPP also inhibits bone resorption (440-442). IAPP may also inhibit glucagon secretion (443) and act as a vasodilator (436), although the physiological relevance of these studies is questionable due to the high concentration of IAPP used. The physiological effects of IAPP are thought to be mediated by the calcitonin receptor in combination with receptor modifying proteins (RAMP) RAMP1, RAMP2 or RAMP3 (444). In normal individuals, IAPP is a secreted peptide and does not aggregate into islet amyloid deposits (445).

Islet amyloid forms in conditions under which the β cell is under metabolic stress. Such conditions include elevated insulin resistance (446, 447), exposure to a high fat diet (448), obesity (449), insulinoma (428, 429), and type 2 diabetes (97, 430, 431). Islet amyloid has been reported in 20-99% (97, 430, 431) of islets of type 2 diabetic patients but in only 3-11% of islets in non-diabetic controls (431). The importance of islet amyloid to type 2 diabetes is underscored by observations that the degree of islet amyloid deposition may correlate with severity of the disease in humans, with reduced β cell mass in humans and non-human primates, and with the development of hyperglycemia in transgenic rodents (98, 448, 450-452). Extensive amyloid deposition in primate islets around the time of diabetes onset provides additional support for the role of amyloid in progression of disease (453-455). A missense mutation (S20G) in human IAPP that enhances amyloidogenicity and cytotoxicity (456, 457) is associated with severe, early
onset type 2 diabetes (458, 459). The appearance of amyloid pathology in the islet correlates strongly with β cell dysfunction and loss.

A role for islet amyloid in β cell dysfunction is further supported by the lack of disease pathology in species expressing non-amyloidogenic forms of IAPP. Feline, primate, and human IAPP are amyloidogenic by virtue of a β-sheet prone region located between amino acids 20-29 (460, 461), although other regions of the peptide potentiate amyloidogenesis (460-463). In rodents, three proline substitutions in the 20-29 region act as β sheet breakers (460, 461, 464, 465); consequently, synthetic rodent IAPP does not form fibrils in vitro, and is not toxic to β cells. Porcine IAPP is also minimally fibrillogenic, which is consistent with the lack of diabetes incidence in sus scrofa (466). The conditions that predispose IAPP aggregation into islet amyloid are not fully understood. Elevated expression and secretion of IAPP are not sufficient to promote amyloid formation. As an example, mice expressing the amyloidogenic human IAPP transgene failed to develop amyloid or diabetes despite a five-fold elevation in IAPP content and IAPP serum levels above those in wild-type mice (446). Nevertheless, amyloid-prone forms of IAPP contribute to islet pathology under conditions of metabolic stress.

Islet amyloid has toxic effects on the islet. Amyloid formation in cultured human islets (2, 3, 467) and murine islets expressing the human IAPP transgene (468) correlates with selective apoptotic loss of β cells. Synthetic human IAPP induces apoptosis in transformed β cell lines (469-475). Adenovirally expressed human IAPP may induce apoptosis in transformed cell lines, depending on the level of expression (3, 476). IAPP has additionally been shown to be preferentially cytotoxic to cultured β cells relative to α cells (38). As discussed in a previous section, pre-fibrillar species of misfolded IAPP are the principal mediators of toxicity (96). These pre-fibrillar entities do not necessarily correlate with “oligomeric species” of IAPP purportedly detected by the A-11 antibody, with which we have considerable concerns (12). It should be noted that the polyclonal A-11 antibody was raised to colloidal-gold linked oligomers of a different pro-amyloidogenic protein, amyloid β, and not to IAPP.

IAPP toxicity has been proposed to occur by either intracellular or extracellular mechanisms. In an intracellular model of toxicity, prefibrillar species may alter the permeability of organelle membranes to disrupt cellular calcium homeostasis and to cause mitochondrial dysfunction.
An intracellular mechanism of cytotoxicity would most likely occur due to membrane permeabilization by IAPP species upon formation in the secretory granules rather than proIAPP earlier in the secretory pathway. Although lipid membranes may induce fibrillization of proIAPP (478), proIAPP species do not alter membrane permeability (479). In contrast, IAPP pre-fibrillar species induce membrane permeabilization (480, 481). An alternate mechanism of intracellular toxicity is ER stress pro-apoptotic signaling activated upon aggregation of misfolded proIAPP species in the ER (482). Islets from type 2 diabetic humans show an accumulation of polyubiquitinated proteins (483), consistent with elevated levels of proIAPP in the ERAD pathway, as well as increased CHOP immunostaining (30), consistent with ER stress-associated apoptotic signaling. Islets from the HIP rat, which expresses the human IAPP transgene in its β cells, show the same phenomena (97, 483). It is possible that ER stress signaling in this model is unrelated to IAPP toxicity, as amyloid deposition in cultured human islets is not associated with ER stress (468).

Extracellular mechanisms of IAPP toxicity include IAPP-induced membrane permeabilization (480, 481), altered cation channel activity (484), or death receptor-mediated apoptosis (37). Downstream events are mediated by caspase (38, 485) and JNK activation (485) and mitochondrial dysfunction (486). Seeding of IAPP amyloid formation by membrane heparan sulfate interaction with N-terminally extended proIAPP (487) likely potentiates extracellular formation of prefibrillar IAPP species and associated toxicity. Extracellular toxicity is reproduced upon incubation of synthetic IAPP (469-475) with transformed β cell lines. A strong argument may be made for extracellular toxicity, as administration of extracellular peptide inhibitors (2) of IAPP amyloid formation reduce amyloid load and promote β cell survival cultured human islets. IAPP may also promote toxicity indirectly by induction of pro-inflammatory signaling. Human IAPP potentiates β cell production and secretion of IL1-β by activation of the NLRP3 inflammasome (40). IL1-β signaling potentiates β cell dysfunction under conditions of metabolic stress (293, 345).

Recent evidence suggests that islet amyloid deposition contributes to pathology in transplanted islets. Amyloid deposits have been shown to develop rapidly in transplanted human islets (290, 488). A recent case report points to extensive amyloid deposition within islets in a patient with a marginally-functioning graft (489). Islet amyloid deposition correlates with loss of glycemic
control in transplanted murine islets expressing the human IAPP transgene (490). Islet amyloid may contribute to graft dysfunction by promotion of apoptosis, inducing β cell dysfunction independent of amyloid deposition (491), inhibition of local diffusion of nutrients and metabolites, and potentiation of inflammation. Transplanted islets, particularly under conditions of hyperglycemia, secrete disproportionate amounts of IAPP (492). Enhanced production and secretion of IAPP, however, is unlikely in itself to be sufficient to increase amyloid deposition and toxicity (446).

Factors within the transplantation environment likely act synergistically to potentiate amyloid formation and toxicity. IAPP may increase peripheral insulin resistance (493-502), shown to be elevated in islet transplant recipients (376), and its secretion may thus exacerbate β cell dysfunction. Conditions related to islet isolation and transplantation may promote islet amyloid deposition and toxicity. Collagenase digestion of islets disrupts islet vasculature and may expose basement membrane and proteoglycans, which may promote the deposition of islet amyloid. Advanced glycosylation end-product-modification of IAPP potentiates its amyloidogenicity and cytotoxicity, suggesting that prolonged hyperglycemia in type 2 diabetes or following islet transplantation may potentiate the detrimental effects of IAPP on β cell function and mass (503, 504). Inflammation triggered by islet transplantation may elevate levels of the acute phase protein serum amyloid P (505), a known component of islet amyloid, and thus promote islet amyloid deposition. Rapid amyloid deposition and associated toxicity in islet transplantation makes islet amyloid a viable therapeutic target to improve graft function and sustain graft mass in islet transplantation.

1.5 Summary

β cell replacement by islet transplantation offers a potential cure for type 1 diabetes that can slow the progression of the devastating micro- and macrovascular complications of diabetes beyond that of best medical therapy. This procedure, however, is still far from ideal. Improvements in graft function and survival are essential to improve long-term transplant outcomes. Much focus on islet loss has centered around immune-mediated mechanisms of graft destruction, but accumulating evidence from animal studies and clinical islet transplantation suggests that a number of non-immune factors also likely play an important role in graft failure, for example ER stress, islet amyloid, and ischemia. Some of these stressors – notably ER stress and islet amyloid
are also thought to play a role in β cell loss in type 2 diabetes. Interestingly, β cell dysfunction in transplanted islets resembles that of the type 2 diabetic islet, including impairments in first phase glucose-stimulated insulin secretion, defective proinsulin processing, and gradual decline of β function and mass. Dysfunction and death of transplanted β cells resembles closely that of type 2 diabetes, and the striking similarities between the type 2 diabetic islet and the transplanted islet may have implications for therapy for islet transplant recipients and for tracking graft function.

1.6 Thesis hypothesis and objectives
There are many similarities between β cell dysfunction in type 2 diabetes and in islet transplantation, particularly in terms of islet amyloid pathology and metabolic dysfunction. In light of this, the overall hypothesis of this thesis is that islet amyloid and CHOP, a pro-apoptotic pathway activated by ER and oxidative stress in β cell dysfunction, are important non-immune modulators of graft dysfunction and therapeutic targets in islet transplantation. To address this hypothesis, the following objectives were pursued.

Objective 1: To determine the role of human islet amyloid polypeptide in islet graft failure.

Aim 1: To determine whether islet amyloid is a significant contributor to graft failure.

Specific Aim 1.1.1: To determine whether transplantation of a marginal islet mass promotes amyloid formation;
Specific Aim 1.1.2: To determine whether amyloid load is elevated in human islet transplants that have failed as compared to those which have maintained normoglycemia;
Specific Aim 1.1.3: To determine whether the presence of islet amyloid in donor islets can predict graft outcome.

Aim 2: To determine whether compounds present during and following islet transplantation promote amyloid aggregation and contribute to graft failure.

Specific Aim 1.2.1: To determine whether heparin can accelerate graft failure by promoting amyloid deposition.
Aim 3: To determine whether knockdown of IAPP by RNAi and/or inhibition of amyloid formation by small molecule inhibitors protects against graft failure.

   Specific Aim 1.3.1: To determine whether knockdown of IAPP by RNA silencing protects against amyloid formation and improves graft function and survival;  
   Specific Aim 1.3.2: To determine whether peptide fragments derived from human IAPP and shown to inhibit IAPP aggregation in vitro reduce amyloid formation and associated toxicity.

Objective 2: To determine whether xenotransplantation of islets expressing non-fibrillogenic forms of IAPP improves transplantation outcomes.

   Specific Aim 2.1: To determine whether pig IAPP is fibrillogenic and cytotoxic;  
   Specific Aim 2.2: To determine whether pig islets form islet amyloid following transplantation.

Objective 3: To determine whether CHOP negatively impacts graft function and survival, and whether suppression of CHOP expression improves islet transplant outcomes.

   Specific Aim 3.1: To determine whether CHOP immunoreactivity is elevated in marginal mass grafts;  
   Specific Aim 3.2: To determine whether CHOP<sup>−/−</sup> islets have improved function and survival in marginal mass islet transplantation;  
   Specific Aim 3.3: To determine whether suppression of CHOP expression in islets by RNAi improves islet transplant outcomes.
Chapter 2. Methods and materials

2.1 Materials
Clinical grade heparin (10,000 IU/ml) was obtained from the Ike Barber Human Islet Transplant Laboratory or the British Columbia Children’s Hospital Pharmacy. INS-1 (832/13) cells, a transformed rat β-cell line, were a gift of Dr. C. Newgard (Duke University Medical Center, Durham, NC). SiRNA-containing liposomes were prepared by our collaborators, Drs. C. Tam and P. Cullis (University of British Columbia). Histological sections from human pancreas and islets for allograft and autograft donors were received from Dr. G. Korbutt (University of Alberta) and Dr. H. Rilo (University of Cincinnati), respectively. Histological sections from neonatal porcine islet transplants were obtained from Dr. G. Korbutt (University of Alberta). Histological sections of native adult porcine pancreas and of engrafted porcine islets were obtained from Dr. M. Trucco (University of Pittsburgh).

2.2 Preparation of peptide stock
Rat and human IAPP were obtained from Bachem (Torrance, CA). Porcine IAPP and additional human IAPP were also synthesized in the laboratory of Dr. D. Raleigh (State University of New York, Stony Brook, NY). Prior to use, IAPP was solubilized in 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) and incubated for one hour at room temperature. Solubilized IAPP (1 mg/ml) was divided into 0.1 ml aliquots, which were frozen for 1 hour at -20°C and subsequently kept at -80°C overnight. Aliquots were lyophilized for 2.5 hours. Following lyophilization, aliquots were kept in a desiccator at -20°C.

2.3 Fluorometry
The kinetics of IAPP aggregation into amyloid fibrils were measured by thioflavin T fluorescence using the assay described by Park and Verchere (487) and adapted from Naiki et al (506) and Kudva et al (507). Lyophilized peptide was added to wells containing 10 μM thioflavin T (Sigma-Aldrich, Oakville, ON) in cell media (described in the following section) or 10 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 0.1% Triton X-100. The plate was sealed with Parafilm® and fluorescence measured at 37 °C using a Fluoroskan (Labsystems, Vista, CA) fluorometer with filters set at 444 (excitation) and 485 (emission) nm and bandwidth slits of 12 and 14 nm, respectively. Upon binding to amyloid fibrils, thioflavin T fluoresces with excitation
and emission maxima of 450 and 482 nm. Measurements were taken every minute in the first 2 hours and every 1-10 minutes thereafter. Duplicate measurements were taken for each condition. Each experiment was repeated on a minimum of 3 occasions.

2.4 Circular dichroism
Circular dichroism studies presented in this thesis were performed in the laboratory of Dr. D. Raleigh according to published techniques (466).

2.5 Transmission electron microscopy
Transmission Electron Microscopy studies were performed in the laboratories of Dr. D. Raleigh (466) and Dr. P. Fraser (508) according to their published techniques. Aliquots (4 µl) were removed from solutions containing aggregating peptide, placed on a carbon-coated 200-mesh copper grid or pialoform and carbon coated grid and incubated at room temperature. Excess solution was removed by blotting with filter paper. Grids were negatively stained with saturated uranyl acetate.

2.6 Cell culture
INS-1 cells were grown in Roswell Park Memorial (RPMI) 1640 (Gibco-BRL, Burlington, ON) supplemented with 10% fetal bovine serum (FBS), 11 mM glucose, 10 mM Hepes, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 µM β-mercaptoethanol, 100 U/ml penicillin (Gibco-BRL), and 100 U/ml streptomycin (Gibco-BRL). Cells were maintained at 37°C in a humidified environment supplemented with 5% CO₂. Cells were grown for two passages prior to use and used in assays between passages 59 and 65.

For toxicity experiments, cells were seeded at a density of 60,000 cells per well in 96-well plates and cultured for 24 hours prior to addition of peptide. Lyophilized rodent, porcine and human IAPP were solubilized in RPMI culture media and added directly to cells. After 24 hours, alamarBlue® (Biosource International, CA) was diluted ten-fold in culture media and cells were incubated for 3 hours at 37°C. Fluorescence (excitation 530; emission 590 nm) was measured by a Fluoroskan Ascent plate reader (Thermo Labsystems, Helsinki, Finland).
For transferase-mediated dUTP nick-end labeling (TUNEL assays), INS-1 cells were seeded at a density of 100,000 cells per well in 8-well chamber slides and cultured for 48 hours prior to addition of peptide. Porcine and human IAPP were solubilized in RPMI culture media and added directly to cells. After 14 hours, cells were fixed in 4% paraformaldehyde (20 min), permeabilized with 0.5% Triton X-100 in PBS, and incubated with TUNEL reaction mixture (Roche Diagnostics, Laval, QC, Canada) for 1 hour at 37 °C. Cells were co-stained with Hoechst-33342 for 10 minutes. Images were taken using an Olympus BX-61 fluorescent microscope. Image quantification was performed using Image Pro 6.2 (Media Cybernetics, Bethesda, MD). IAPP was tested at concentrations between 20 and 200 μM. Experiments were performed in triplicate.

For western blot analysis, INS-1 cells were plated at 350,000 cells/well in 12-well plates and grown until 50% confluence. Cells were incubated with fresh media containing dimethyl sulfoxide (DMSO) or thapsigargin (Sigma-Aldrich, Oakville, ON) stock in DMSO for 12 or 24 hours. Maximum DMSO concentration was 0.1% vol. Cells were lifted from 12-well plates using 0.25% trypsin. Cells were centrifuged for 2 min at 1200 rpm and washed twice with phosphate-buffered saline. Lysis solution of 25 µL PMSF (Sigma-Aldrich), 25 µL aprotinin (Sigma-Aldrich), 3.3 µL pepstatin (Sigma-Aldrich), 100 µL leupeptin (Sigma-Aldrich), and 846.7 µL NP40 (Roche, Mississauga, ON) was added in a volume double that of the cell pellet. Cells were kept on ice and vortexed for 10 sec every 5 min for 25 min. Cells were spun at 12,000 rpm at 4°C for 5 min and supernatant was collected. Cell lysate protein concentration was determined using BCA protein assay (Thermo Scientific, Nepean, ON). Ten millitres of lysate diluted in H₂O was incubated at 37°C with 200 µL assay buffer for 30 min. Absorbance (560 nm) was measured using a Multiskan plate reader (Thermo Labsystems, Nepean ON).

2.7 Animals

Animals used in these studies included FVB/N-Tg(Ins2-IAPP)RHFSoel/J (hereafter called hIAPPTg0 mice), FVB/NJ (hereafter called hIAPP0/0 mice), B6.129S-Ddit3tm1Dron/J (hereafter called CHOP-/- mice), NOD.CB17-Prkdcscid/J (hereafter called NOD.scid mice), and wild-type C57BL/6 mice (hereafter called Bl/6 mice), each of which were originally obtained from the Jackson Laboratories (Bar Harbour, Maine) and bred in-house in the Child and Family Research Institute (CFRI) Animal Care Facility. All animal experiments were approved by the Animal
Care Committee of the University of British Columbia in compliance with Canadian Council on Animal Care (CCAC) guidelines and were performed at CFRI.

Genotyping of hIAPP\textsuperscript{Tg0} mice was performed using genomic DNA obtained by an ear punch sample taken from weaned mice (4-6 weeks of age). Ear punches were digested with Proteinase K (20 mg/ml; Gibco, Invitrogen Canada, Burlington, ON) in SET buffer (20 mM Tris, 5 mM EDTA, 1% SDS) at 37°\textdegree C while shaking at 1300 rpm. DNA was precipitated using 4.9 M NaCl/245 mM KCl and ice cold 95% ethanol, followed by resuspension in sterile ddH\textsubscript{2}O.

Polymerase chain reaction (PCR) primer sequences and thermocycler programs for hIAPP\textsuperscript{Tg0} mice were obtained from the Jackson Laboratory. Gene targets were amplified by PCR at 94°\textdegree C for 3 minutes, 35 cycles at 94°\textdegree C for 30 seconds, primer annealing temperature for 1 minute and 72°\textdegree C for 1 minute, followed by 72°\textdegree C for 2 minutes. PCR products were separated by gel electrophoresis on a 1.5% agarose gel (Ultrapure, Gibco) with ethidium bromide (0.5 \mu g/ml, Sigma-Aldrich, St Louis MO).

2.8 Islet isolation

2.8.1 Human islet isolation

Human islets were isolated at the Ike Barber Human Islet Transplant Laboratory at Vancouver General Hospital (509) or the University of Illinois Chicago (UIC) Medical Centre. Upon receipt, islets were filtered to remove large tissue fragments. Filtered islets were further purified by hand-picking under a light microscope. An additional light source was used to enhance the pink tint of islets to aid with purification. Islets were maintained in CMRL (Mediatech, Manassas, VA) supplemented with penicillin (50 U/ml), streptomycin (50 U/ml), gentamycin (50 \mu g/ml), Glutamax® (2 mM), and additional glucose as required by experimental design. Islets were cultured at 37°\textdegree C for up to three weeks in a humid atmosphere of 95% air 5% CO\textsubscript{2}. Islets that were not to be used immediately were maintained in culture at 22°\textdegree C until required for transplantation.

2.8.2 Murine islet isolation

Mice were anesthetized by intraperitoneal injection of Avertin (0.02 ml/g body weight). Pancreata were harvested and retrogradely perfused via the pancreatic duct using collagenase (1000 units/ml, type IX, Sigma) dissolved in Hank’s Balanced Salt Solution (HBSS; Cat. 14185,
Gibco). Pancreata were digested with collagenase for up to 14 minutes at 37°C followed by 1 minute of vigorous hand-shaking at room temperature. Islets were purified by filtration using 70 μm nylon cell strainers (BD Biosciences, Mississauga ON). Following filtration, islets were maintained overnight in Ham’s F-10 medium (Gibco) at 37°C in a 5% CO₂ incubator.

2.9 Islet culture
2.9.1 Human islet culture
Human islets were cultured at 37°C for up to 3 weeks in CMRL supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), gentamycin (50 μg/ml), Glutamax® (2 mM), and additional glucose as required by experimental design. Islet media was replaced every 2-3 days. Following culture, islets were washed and frozen at -80°C for later protein studies or fixed in formalin, preserved in agar, and processed in paraffin for immunohistochemistry.

2.9.2 Murine islet culture
Murine islets were cultured at 37°C for up to 1 week in RPMI media supplemented with penicillin (100 U/ml), streptomycin (100 U/ml), Glutamax® (2 mM), and additional glucose as required by experimental design. Following culture, islets were washed and frozen at -80°C for later protein studies or fixed as for human islets.

2.10 In vitro glucose-stimulated insulin secretion
Isolated murine islets (minimum of 20 per well) were plated in sets of 6 wells per condition in a 96 well plate. Three wells per condition were allocated to low (1.67 mM) glucose incubation and three to high (16.7 mM) glucose stimulation. All wells were pre-incubated in Krebs-Ringer bicarbonate buffer (KRB) supplemented with 10 mM HEPES (pH 7.4), 0.25% BSA and 1.67 mM glucose for two hours at 37°C. Subsequently, wells were incubated in fresh 1.67 or 16.7 mM glucose KRB for 1 hour at 37°C. Media and islets were collected from each well to measure secreted insulin and islet insulin content, respectively.

2.11 Islet transplantation
Recipients of islet transplants were rendered diabetic with a single intraperitoneal injection of streptozotocin (STZ; Sigma-Aldrich) four days prior to transplantation surgery. STZ dose varied according to murine strain from 200 to 250 mg/kg. Blood glucose was checked between 9 am
and noon on the 2 days prior to surgery. Mice were used as recipients if they had 2 consecutive glucose readings greater than 20 mM. On the day of transplantation, mice were anesthetized using isoflurane. Human or murine islet grafts were transplanted into the subcapsular space of the left kidney. Following transplantation, glycemia was monitored daily for the first week, following which it was monitored 1-2 times per week. Random blood glucose measurements were taken from the tail vein between 10 am and 12 pm using a OneTouch® Ultra Glucometer (LifeScan).

2.12 Intraperitoneal glucose tolerance tests
Mice were fasted for four hours prior to intraperitoneal glucose tolerance test (IPGTT). A 50% solution of dextrose was delivered by intraperitoneal (ip) injection (1.5 mg/g of body weight). Blood glucose was taken from the tail vein at 0, 15, 30, 60 and 120 min post-injection. Blood was also collected from the saphenous vein at 0 hours in EDTA-coated microvette tubes (Sarstedt Inc, Montreal QC). Plasma was extracted by centrifugation. Insulin was measured using a mouse insulin ELISA (Mercodia, Burlington, ON) according to the manufacturer’s instructions. Incubation consisted of 2 hours at 600 rpm and fluorescence measured at 450 nm using a Multiskan plate-reader.

2.13 Immunohistochemistry and other histological staining
Specimens for immunohistochemistry were fixed in formalin and processed for histology. Paraffin-embedded sections (5 μm) were mounted on glass slides and de-paraffinized by consecutive incubation in xylene, 95% ethanol, 70% ethanol, and distilled water. Sections were blocked in phosphate-buffered saline (PBS) containing 2% normal goat serum (Vector Laboratories). Primary antibodies were prepared in PBS containing 1% BSA. Primary antibodies, dilutions, and incubation conditions are listed in Table 1. Various secondary Alexa-488 and Alexa-594 fluorescently-labeled antibodies (Invitrogen) were used at a dilution of 1:100. Secondary antibodies were incubated for 1 hour at room temperature. Appropriate negative controls were performed and antibody specificity confirmed by the manufacturers.

TUNEL staining was performed using In Situ Cell Death Detection kit, TMR red (Roche 12156792910). Sections for TUNEL staining were treated for antigen retrieval by microwaving for 5 minutes at 40% power in 0.1 M citrate buffer. Sections were incubated for 60 min at 37° C.
with a 1:10 solution of TUNEL enzyme:substrate. Thioflavin S staining was used to stain for amyloid. TissueTrol® amyloid sections (Sigma) were included as a positive control for thioflavin S staining. For thioflavin S staining, following secondary antibody incubation, slides were immersed in 0.5% thioflavin S solution for 2 minutes, rinsed in 70% ethanol and water, and wet-mounted using Vectashield mounting media (Vector Laboratories). Images were captured using an Olympus Bx61 fluorescent microscope and analyzed using Image Pro 6.2 software (Media Cybernetics Inc).

**Table 1. List of primary antibodies.**

<table>
<thead>
<tr>
<th>Primary or Secondary Antibody</th>
<th>Target</th>
<th>Manufacturer</th>
<th>Antibody Characteristics</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
<th>Incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary</td>
<td>Insulin</td>
<td>Dako (A0564)</td>
<td>Polyclonal guinea pig-derived antibody</td>
<td>1:100</td>
<td>None</td>
<td>1 hour at 22°C</td>
</tr>
<tr>
<td>Primary</td>
<td>Glucagon</td>
<td>Dako (A0565)</td>
<td>Polyclonal rabbit-derived antibody</td>
<td>1:75</td>
<td>None</td>
<td>1 hour at 22°C</td>
</tr>
<tr>
<td>Primary</td>
<td>Ki-67</td>
<td>Vector (VP-K451)</td>
<td>Polyclonal rabbit-derived antibody</td>
<td>1:100</td>
<td>10 mM citrate</td>
<td>Overnight at 4°C</td>
</tr>
<tr>
<td>Primary</td>
<td>Cleaved caspase-3</td>
<td>Cell Signalling (9661)</td>
<td>Polyclonal rabbit-derived antibody</td>
<td>1:40</td>
<td>10 mM citrate</td>
<td>Overnight at 4°C</td>
</tr>
<tr>
<td>Primary</td>
<td>CHOP</td>
<td>Santa Cruz (Sc-7351)</td>
<td>Monoclonal mouse-derived antibody</td>
<td>1:100</td>
<td>10 mM citrate</td>
<td>1 hour at 22°C</td>
</tr>
</tbody>
</table>

### 2.14 Porcine IAPP sequencing

Primers (Invitrogen, Burlington, ON) were designed according to EST sequence (Q29119) from porcine hypothalamus homologous to the predicted sequence for mature (1-37) porcine IAPP. The primer sequences used were: Forward, CTGAATTCCAAAGGATTGTACTGGGAA, ACCAAAAACACTGGGTATTACTGATAGGAAGA; Reverse, GTGCGTTGACCTCTAAAGGGTGAAAGTA, CTGGCAGCAAACATGGGACACA. RNA was isolated from neonatal porcine islets. Reverse transcription was performed using SuperScript First-Strand (Invitrogen). PCR was performed using Accuprime TaqDNA Polymerase High Fidelity (Invitrogen).
2.15 Insoluble IAPP/protein assay
Cultured human islets were lysed with 100 µl NP40 lysis buffer containing the previously mentioned protease inhibitors. Cells were kept on ice and vortexed for 10 sec every 5 min for 25 min. The lysate was ultracentrifuged at 60,000 rpm at 4°C for 1 hour using a Beckman ultracentrifuge with a Beckman TLA-100-3 rotor (Beckman Instruments, Gagny, France). The soluble fraction was separated from the insoluble fraction and stored at -80°C. The pellet was solubilized in 0.1 ml 70% formic acid, sonicated until the solution was clear, and vortexed for 1 minute. The fraction was neutralized using 1.9 ml of 1M Tris and diluted 1:3 in filtered distilled water. Total protein (soluble plus insoluble fractions) was determined using the BCA assay (described above). IAPP content in the insoluble fraction was determined using the Total Amylin ELISA (Millipore, Billerica, MA).

2.16 IAPP western blotting
Lysates were electrophoresed on a polyacrylamide gel using Tris-tricine buffer for separation of small proteins and transferred to 0.45-µm polyvinylidene fluoride (PVDF) membranes (15 V, 20 min) using a Bio-Rad semidry electrophoretic transfer cell (Trans-Blot SD). The membranes were blocked with 5% skim milk for 1 hour at room temperature and washed and incubated for 1 hour with a 1:1000 dilution of primary anti-IAPP (7323 or 4145) at 1:1,000 room temperature, followed by 1 hour incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Amersham) diluted 1:5,000 at room temperature. Immunodetection was performed using an enhanced chemiluminescence detection kit (Amersham).

2.17 CHOP western blotting
Gels were made using 1.0 mm spaced plates with 10 wells/gel. Forty micrograms of protein were loaded into each well and run at 70 V for 15 min and 110 V for 60-120 min depending on the position of the leading band. Proteins were wet transferred onto PVDF membrane using 110 V for 60 min. Membranes were blocked using 2.5% skim milk in tris-buffered saline containing 0.1% Tween-20. CHOP antibody (Santa Cruz 7351), cleaved caspase-3 (Cell signaling D-175), and β-actin (Sigma 9E10) antibodies were diluted 1:200, 1:1000 and 1:1000, respectively. Secondary antibodies containing horseradish peroxidase were diluted 1:1000 for all. Immunodetection was performed using an Amersham ECL Plus™ Western Blotting Detection Reagents enhanced chemiluminescence detection kit (GE Healthcare).
2.18 Data and statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical significance was determined using Student’s t-test or analysis of variance (ANOVA) and Neuman-Keuls post-hoc test, where appropriate. P < 0.05 was considered statistically significant. Data analysis was performed using Prism v 4.0 (GraphPad Software Inc, La Jolla CA).
Chapter 3. Role of islet amyloid in islet transplant dysfunction

3.1. Introduction

The long-term success of human islet transplantation is limited. Seventy-five percent of patients who initially achieve insulin independence require insulin within 2 years post-transplant (129). Although allo- (159, 163, 175, 193, 348, 510-515) and auto-immune (175, 512, 513, 516-518) mediated destruction of β cells is a major contributor to β cell loss in islet transplants, evidence from clinical data suggests that non-immune factors also contribute to long-term graft failure. Two key observations lead us to this hypothesis. First, long-term graft failure occurs in human islet autograft recipients. An autograft is one in which one’s own islets are re-implanted following pancreatectomy and islet isolation. Autograft recipients do not require immunosuppression, as the graft is not subject to alloimmune rejection. This patient population, primarily composed of patients with chronic pancreatitis (519) and patients undergoing pancreas resection for tumour removal (520), does not typically include individuals with type 1 diabetes (519) nor do patients have serum autoantibodies against β cell epitopes (521, 522). Human and primate autografts display progressive graft dysfunction (205, 207, 208, 210-212, 348), albeit at a slower rate than human allografts, in the absence of allo- or auto-immune mediated graft destruction. The second critical observation is that the rate of decline in serum C-peptide levels was a fraction of the rate of loss of insulin independence in the International Trial of the Edmonton Protocol (129). It seems unlikely that the slow rate of decline in C-peptide would reflect rapid immune-mediated clearance of β cells. In human islet transplant recipients, rapid elevation in serum numbers of cytotoxic T lymphocyte precursors by 12 weeks following transplantation was associated with extremely low levels of C-peptide 14 weeks later (511). In islet allograft recipients with auto-antibodies present prior to transplant, C-peptide levels were significantly reduced in the first 6 weeks following transplantation (176). Rather, the gradual loss of the ability of the transplanted β cell to maintain glycemic control suggests a progressive impairment in insulin production, reminiscent of that seen in type 2 diabetes.

The available evidence is not persuasive for a critical role of immune-mediated destruction in progressive graft dysfunction. Autoimmunity is an unlikely cause. Development of islet auto-antibodies following transplantation is associated with detrimental graft outcomes (176). In the International Trial of the Edmonton Protocol, 82% of islet transplant recipients were auto-antibody negative at 1 year post-transplantation, at which point 40% of recipients had lost insulin
independence (129). It is conceivable that autoimmune attack in chronic islet rejection is a T-cell mediated process and thus does not correlate with serum levels of auto-antibodies (511, 523). This possibility, however, is inconsistent with observations from human and islet recipient autopsy data. In autopsies of 3 failing human islet grafts (205), there was an absence of inflammatory markers and a lack of immune cell infiltration in transplanted islets. A similar finding was reported for slowly progressive dysfunction of cynomolgus macaque islet allografts that were adequately immunosuppressed (207); these grafts did not demonstrate islet invasive lymphocytic infiltration characteristic of islet allograft rejection. Moreover, optimally immunosuppressed allografts failed at a similar rate to autografts performed in the same study (207). Taken together, these findings make a strong case for the involvement of non-immune factors in long-term graft dysfunction.

It is now well-recognized that defects in β cell function and mass are critical contributors to the pathogenesis of type 2 diabetes. The β cell in type 2 diabetes is characterized by multiple defects in insulin secretion and gradual cell loss by apoptosis. Defective first-phase glucose-stimulated insulin secretion is detectable in patients with impaired fasting glucose or impaired glucose tolerance (80) and is nearly absent at onset of type 2 diabetes (524, 525). β cell function progressively declines, illustrated by a gradual loss in glucose-stimulated insulin secretion and reduced insulin secretion in response to non-glucose secretagogues such as arginine (81, 87). Insulin secretory patterns are also abnormal (526, 527). In type 2 diabetic patients, both diurnal and ultradian oscillations in serum insulin concentration are decreased early in the disease process due to impaired pulsatile insulin release (84, 85, 528). Finally, proinsulin processing is also known to be impaired in type 2 diabetes. Basal and stimulated proinsulin levels and the proinsulin:insulin ratio are significantly elevated in type 2 diabetic patients (86).

In addition to progressive loss of β cell function, β cell mass is also lost over the course of the disease. In autopsy studies, type 2 diabetic patients have greatly reduced β cell mass compared to controls matched for the degree of obesity (73, 529). Prior to the onset of diabetes, fractional β cell volume is reduced by approximately half in individuals with impaired fasting glucose (529). Loss of β cell mass in type 2 diabetes is associated with increased β cell apoptosis, rather than decreased replication or neogenesis (529). Causes of β cell death in type 2 diabetes include
glucolipotoxicity (300, 526, 530-533), ER stress (390, 534, 535), oxidative stress (90, 536-538), local islet inflammation (293, 345), and islet amyloid species (433, 477).

Many features of the transplanted β cell are reminiscent of those seen in type 2 diabetes. Like type 2 diabetic patients, islet transplant recipients demonstrate impaired first phase glucose-stimulated insulin secretion (357, 539) and reduced maximal secretory capacity (539, 540). Furthermore, these impairments are progressive in nature (540). Similar findings have been reported in syngeneic murine islet transplants (541, 542). Impaired proinsulin processing in islet transplant recipients (316, 376, 543), demonstrated by an elevated proinsulin:C-peptide ratio, provides further evidence of β cell dysfunction in transplantation. This ratio is a surrogate measure of the proinsulin:insulin ratio used for type 2 diabetes. In islet transplant recipients, C-peptide is used as the denominator so that the ratio is not influenced by exogenous insulin, which many patients require. Impaired proinsulin processing in islet transplants may be associated with decreased graft PC2 immunostaining (543), suggestive of an intrinsic β cell processing defect.

Reduced β cell mass relative to normal individuals is another common feature between type 2 diabetes and islet transplants. In humans, an adequate graft mass in islet allotransplantation is considered to be above 10,000 IE/kg (316), although islet recovery from a single pancreas donor is commonly insufficient to provide recipients with this amount of islets. Patients who receive less than 10,000 IE/kg have greater impairment in β cell function than those that receive greater than 10,000 IE/kg (316). Transplantation of a marginal mass of islets in animals models predisposes recipients to defective glucose-stimulated insulin secretion and poor glycemic control (326, 331, 544). MRI imaging of autologous islet transplantation into baboon liver (545) and human islets into the liver of immunodeficient mice (546) shows a progressive decrease in transplanted islet mass over several weeks following transplantation. Consistent with this decrease in mass, apoptotic cells but no replicating cells were detected in marginal mass human islet grafts in immune-deficient, diabetic mice (543). Thus, islet transplant recipients tend to receive an inadequate islet mass that continues to decline following transplantation.

Islet amyloid is a pathology common between transplanted and type 2 diabetic islets, which causes significant β cell dysfunction, apoptosis, and loss of islet mass. Indeed, in the marginal mass graft studies described in the previous paragraph, the authors also detected intra- and
extracellular amyloid associated with “pre-apoptotic” β cells within the graft (543). Westermark and colleagues first described amyloid deposition in human islets within 2 weeks after transplantation into diabetic, immune-deficient mice (290, 488). This same group followed up this observation with a case report in which they identified extensive amyloid deposition within transplanted human islets of a patient with a marginally-functioning graft (489). Transplantation of murine islets with β cell expression of a human IAPP transgene (Tg-hIAPP) provides additional support for the detrimental effect of islet amyloid on graft function. Transplanted Tg-hIAPP islets initially restore glycemic control following transplantation but develop modest hyperglycemia by two weeks post-transplantation, while wild-type grafts maintain normoglycemia (490). Islets expressing non-amyloidogenic forms of IAPP do not develop amyloid deposits following transplantation and consistently maintain long-term glycemic control (208, 466). Islet amyloid deposition and its associated toxicity is common to both type 2 diabetes and islet transplantation, although deposition and toxicity are accelerated in transplantation. In this Chapter, we investigated factors underlying amyloid formation and toxicity in transplanted human and Tg-hIAPP murine islets. Furthermore, we evaluated several strategies to prevent amyloid formation, with the ultimate goal of a translatable therapeutic strategy. Finally, we evaluated whether islet amyloid load or prevalence within histological sections of donor pancreas or isolated islets might have predictive value in human autograft and allograft function.

3.2 Methods
3.2.1 Islet transplantation
Please refer to Chapter 2 for a description of human and murine islet transplantation into streptozotocin (STZ)-diabetic, immune deficient mice.

3.2.2 Islet allograft cohort
In collaboration with Dr. G. Korbutt (University of Alberta), we obtained histological sections of pancreas fixed prior to islet isolation for subsequent islet allotransplantation. We received histological sections from a total of 19 islet allograft donors that were used for islet transplantation into 11 recipients. It should be noted that all of the 11 allograft islet recipients received at least 2 and as many as 4 islet infusions. We did not obtain any information pertaining to donor characteristics, such as age, BMI, or gender. Recipient graft function was assessed by the area under the insulin response curve in response to an intravenous glucose challenge
(AIRglu). We received the most recently available AIRglu data for 91% (10 out of 11) allograft recipients. Additionally, we were informed as to whether patients had maintained graft function since the last islet infusion or if their grafts were dysfunctional such that they once again required insulin therapy.

3.2.3 Islet autograft cohort
In collaboration with Dr. H. Rilo, formerly of the University of Cincinnati, we obtained histological sections of pancreas and isolated islets from a cohort of patients on which islet autografts were performed following total pancreatectomy. We received materials from 21 separate islet autograft procedures, all of which were retrieved at the time of islet isolation. For 52% of the cohort, we received both pancreas and isolated islets. For the remainder of the cohort, we received isolated islets for 33% of the donors and pancreas specimens only for the remaining 14%. In terms of clinical information, we did not obtain any information pertaining to donor characteristics, such as age and gender, underlying health conditions, or reason for undergoing islet auto-transplantation. In terms of measurements of graft function, we were only provided HbA1c at 1 year following transplantation. In spite of these limitations, we characterized analysis of islet amyloid load and prevalence in this cohort and attempted to correlate the limited clinical data with our findings.

3.2.4 Quantification of islet amyloid load and prevalence in donor islets
Donor islets and pancreatic sections were stained for amyloid by thioflavin S as described in Chapter 2. We quantified amyloid load and prevalence as defined by Hull et al (547). Islet amyloid severity is defined as the proportion of total islet area occupied by thioflavin S-positive staining. Islet amyloid prevalence is defined as the proportion of islets that had detectable amyloid.

3.3 Results
3.3.1 Expression of human IAPP in transplanted islets promotes β cell dysfunction
To determine the effect of human IAPP expression on function of an optimal mass islet transplant, we transplanted 250 islets from mice with β cell-specific expression of the human IAPP transgene (hIAPP\textsuperscript{Tg/0}) or their wild-type littermate controls into STZ-diabetic immune-deficient (NOD.scid) recipients. Blood glucose was monitored weekly (Figure 1A). There was
no significant difference in blood glucose between hIAPP\textsuperscript{Tg0} and wild-type mice as determined by repeated measures two-way ANOVA (p = ns). We compared glucose tolerance and proinsulin processing between the genotypes to determine whether human IAPP expression had an effect upon β cell function. To assess glucose homeostasis, we performed i.p. glucose tolerance tests on graft recipients at 1 and 3 months post-transplantation. At 1 month post-transplantation, hIAPP\textsuperscript{Tg0} graft recipients had blood glucose values significantly greater than recipients of wild-type grafts at 60 and 120 minutes following i.p. glucose injection (p < 0.05; Figure 1B). The area under the curve was significantly elevated in hIAPP\textsuperscript{Tg0} graft recipients (1854 ± 168 versus 2398 ± 188 mM/min, p < 0.05). At 3 months post-transplantation, transgenic grafts had significantly elevated glucose at 60 minutes following i.p. glucose injection (p < 0.05, Figure 1C). The area under the curve remained significantly elevated between the genotypes (1108 ± 52 versus 1714 ± 287, p < 0.05) but was not increased for either group as compared to 1 month post-transplant.

Proinsulin processing efficiency, assessed by the proinsulin to insulin ratio (PI/PI+I), may be used as a marker of β cell function (86, 316). To assess PI/PI+I, fasting serum was collected from wild-type (n=7) and hIAPP\textsuperscript{Tg0} (n=9) graft recipients at 6 weeks following transplantation. Recipients of hIAPP\textsuperscript{Tg0} islets tended to have less insulin (91.8 ± 7.9 versus 141.1 ± 26.3 pM, p = 0.07; Figure 1D) but similar amounts of proinsulin (4.0 ± 0.3 versus 4.2 ± 0.8 pM, p = ns; Figure 1E). The PI/PI+I was significantly higher in hIAPP\textsuperscript{Tg0} graft recipients (4.3 ± 0.4 versus 2.8 ± 0.3%, p < 0.05; Figure 1F). Taken together, these data demonstrate that glucose tolerance and proinsulin processing are impaired in recipients of hIAPP\textsuperscript{Tg0} grafts.

We performed histological analysis of amyloid load in optimal mass islet grafts by thioflavin S staining. Representative images are shown in Figure 2. As expected, no amyloid was detected in grafts of wild-type islets. Grafts from hIAPP\textsuperscript{Tg0} donors had variable amyloid histology, with 69% (9 of 13) of recipients having detectable amyloid. Thirty eight percent of grafts had greater than 0.1% of graft area occupied by amyloid, while a single graft had greater than 1% of graft area occupied by amyloid. The mean proportion of the graft occupied by amyloid was 0.22 ± 0.12% (Figure 2E).
Figure 1. Optimal mass grafts of islets expressing human IAPP restore glycemic control but have impairments in glucose tolerance and proinsulin processing.

STZ-diabetic NOD.scid mice were transplanted with 250 wild-type (n=13) or hIAPP<sup>Tg</sup>/<i>/0</i> (n=14) islets. Non-fasted blood glucose (A) was measured daily for the first week, three times in the second week, and weekly thereafter for three months following transplantation. Recipients of hIAPP<sup>Tg</sup>/<i>/0</i> (n= 12) grafts have significantly impaired glucose tolerance compared to recipients of wild-type grafts (n=10) at 1 month following transplantation (B). A similar trend was observed (hIAPP<sup>Tg</sup>/<i>/0</i>, n= 4; wild-type, n = 8) at 3 months following transplantation (C). Plasma was collected at 6 weeks post-transplantation for analysis of insulin (D), proinsulin (E), and the proinsulin/insulin ratio (F). Data are expressed as mean ± SEM. Differences between groups were assessed by repeated measures two-way ANOVA for (A) and Student’s t-test for all other results. p < 0.05 (*) was considered significant. ip, intraperitoneal; STZ, streptozotocin; TX, transplantation.
Figure 2. Optimal mass grafts of islets expressing human IAPP develop islet amyloid.

Histological analysis was performed on optimal mass wild-type (n = 11) and hIAPP<sup>Tg</sup>/0 (n = 12) grafts removed by survival nephrectomy at 3 months following transplantation. Representative images following insulin (red) and thioflavin S (ThioS; blue) staining are shown for wild-type (A, C) and hIAPP<sup>Tg</sup>/0 grafts in (B, D). Amyloid was only detected in hIAPP<sup>Tg</sup>/0 grafts (E). Differences between groups were assessed by Student’s t-test. p < 0.05 (*) was considered significant. Size bar = 100 μm.
3.3.2 Deposition of amyloid within transplanted marginal mass grafts of hIAPP<sup>Tg<sup>0</sup></sup> islets is associated with graft failure

To determine the effect of human IAPP expression on function of an islet graft mass under greater metabolic demand, we transplanted 150 (rather than 250) islets from transgenic hIAPP<sup>Tg<sup>0</sup></sup> mice or their wild-type littermate controls into STZ-diabetic NOD.scid recipients. Maintenance of glycemic control varied amongst graft recipients (Figure 3A). Three of thirteen hIAPP<sup>Tg<sup>0</sup></sup> grafts (23%) failed between days 6 and 12 following achievement of glycemic control. All remaining hIAPP<sup>Tg<sup>0</sup></sup> grafts and all wild-type grafts maintained glycemic control, although three of the hIAPP<sup>Tg<sup>0</sup></sup> grafts tended to have glycemic values at the upper limits of normal. Blood glucose in marginal wild-type and hIAPP<sup>Tg<sup>0</sup></sup> grafts was 7.8 ± 0.5 mM and 9.4 ± 0.7 mM, respectively (p < 0.05), at two weeks following transplantation. Kaplan-Meier analysis (Figure 3B) revealed a significant difference (p < 0.05) between marginal wild-type and hIAPP<sup>Tg<sup>0</sup></sup> grafts to maintain glycemic control following transplantation. Intraperitoneal glucose tolerance tests were performed at 4 weeks following transplantation in a subset of normalized marginal mass recipients (Figure 3C). Random fasting blood glucose was significantly elevated in hIAPP<sup>Tg<sup>0</sup></sup> grafts recipients above that of wild-type recipients in this subset (12.5 ± 0.8 mM versus 7.3 ± 0.3 mM, p < 0.05); there was no significant difference in blood glucose between recipients of the same genotype of islets for which IPGTT was or was not performed (wild-type recipients, 7.3 ± 0.3 mM versus 8.0 ± 0.6 mM, p = ns; hIAPP<sup>Tg<sup>0</sup></sup> recipients, 12.5 ± 0.8 mM versus 11.5 ± 2.3 mM, p = ns). In the intraperitoneal glucose tolerance test, blood glucose was significantly elevated in hIAPP<sup>Tg<sup>0</sup></sup> graft recipients at 1 hour following i.p. glucose injection. Area under the glucose curve was also significantly greater for recipients of hIAPP<sup>Tg<sup>0</sup></sup> grafts.

We next analyzed graft amyloid load (Figure 4E). Of marginal mass grafts, 62% of transgenic grafts developed amyloid. In these grafts, amyloid occupied a mean 1.2 ± 0.9% of graft area. Amyloid tended to be greater in marginal mass than in optimal mass grafts, although this was not significant. Amyloid deposition was highly variable amongst graft recipients, with 54% having amyloid area of greater than 0.1% of graft area and 23% having area greater than 1% of graft area. Amyloid in hIAPP<sup>Tg<sup>0</sup></sup> grafts positively correlated (r<sup>2</sup> = 0.66, p < 0.001) with random plasma glucose at day 10 following transplantation (Figure 4F). In this Figure, we confirmed that this relationship between amyloid-positive graft area and blood glucose remained significant upon exclusion of a point lying well upward and to the right of all other points (r<sup>2</sup> = 0.83, p < 0.0001).
Figure 3. Recipients of marginal mass grafts of islets expressing human IAPP have impaired glucose tolerance.

STZ-diabetic NOD.scid mice were transplanted with 150 wild-type (n=19) or hIAPP\textsuperscript{Tg0} (n=13) islets. Non-fasted blood glucose was measured daily for the first week, twice in the second week, and weekly thereafter for one month following transplantation. Blood glucose is shown in (A). Kaplan-Meier analysis is shown in (B). All grafts normalized in the first week following transplantation. Three of 13 hIAPP\textsuperscript{Tg0} grafts failed within the first 2 weeks following achievement of glycemic control. Recipients of hIAPP\textsuperscript{Tg0} grafts (n = 3) graft recipients have significantly impaired glucose tolerance compared to recipients of wild-type islets (n=4) at 1 month following transplantation (C). Data are expressed as mean ± SEM. Differences between groups were assessed by Student’s t-test. *, p<0.05; **, p<0.01.
Figure 4. Recipients of marginal mass grafts of islets expressing human IAPP develop amyloid associated with graft failure.

Histological analysis was performed on optimal mass wild-type (n = 19) and hIAPP\textsuperscript{Tg0} (n = 13) grafts removed for histology at 1 month following transplantation. Representative images following insulin (red) and thioflavin S (ThioS; blue) staining are shown for wild-type (A, C) and hIAPP\textsuperscript{Tg0} grafts in (B, D). Quantification of amyloid load is shown in (E). There was a significant (p < 0.05) correlation between amyloid load and blood glucose (F). Data are expressed as mean ± SEM. Differences between groups were assessed by Student’s t-test. p < 0.05 (*) was considered significant. Size bar = 100 \(\mu\text{m}\).
3.3.3. Amyloid formation is associated with human islet graft failure

To determine whether amyloid correlates with graft dysfunction, as it does for hIAPP\textsuperscript{Tg0} islets, NOD.scid mice transplanted with human islets were assessed for the presence of amyloid by thioflavin S staining (Figure 5). Upon graft harvest, mice whose grafts had maintained normoglycemia throughout the duration of the experiment had a significant elevation in the proportion of graft area occupied by amyloid (0.70 ± 0.3\% versus 6.1 ± 3.5 \%, p<0.05). Grafts which had maintained normoglycemia also tended to have a greater proportion of the graft occupied by β cells than those mice that failed to maintain normoglycemia (24.8 ± 3.8\% versus 16.5 ± 4.3\%, p = ns). These results demonstrate that amyloid deposition is greater in failed human islet grafts in this model and is associated with a loss of graft insulin immune-positive area.
Figure 5. Amyloid formation is associated with human islet graft failure.

Human islets were transplanted into streptozotocin-diabetic NOD.scid recipients. Grafts were retrieved following graft failure or at approximately 2 months following transplantation. Thioflavin S (ThioS) positive area (A) was significantly increased and insulin-immunopositive β cell area (B) tended to be reduced in recipients of grafts with blood glucose values >15 mM at the time of graft harvest. Data are expressed as mean ± SEM. Differences between groups were assessed by Student’s t-test. P < 0.05 (*) was considered significant.
3.3.4. Increased amyloid area within human islet grafts is associated with a lower islet transplant mass

To determine whether increased amyloid formation is associated with a lower transplanted mass of human islets, as it is for hIAPP\(^{Tg0}\) islets, we compared insulin-positive \(\beta\) cell area and thioflavin S (amyloid) positive area within grafts of normoglycemic recipients of 300 or 500 human islets (Figure 6). Recipients of 300 islets had a significantly elevated proportion of the graft occupied by amyloid (0.44 ± 0.12\% versus 0.11 ± 0.03\%, p <0.05). The proportion of graft area comprised of insulin-positive \(\beta\) cells was not significantly different between groups (20.4 ± 2.8\% versus 26.6 ± 2.8\%, p = ns), although there was a trend towards a lower proportion of \(\beta\) cell area in recipients of fewer islets. Recipients of fewer islets also tended to have a greater impairment in proinsulin processing (1). This finding suggests that increased secretory stress on a smaller graft mass promotes \(\beta\) cell dysfunction and amyloid deposition even when glycemic normalization is achieved.
Figure 6. Increased amyloid area is associated with lower transplanted graft mass.

Grafts from mice transplanted with 300 (n=16) or 500 (n=16) human islets were stained for amyloid (ThioS; thioflavin S, A) and insulin (B) and analyzed for positivity as a percentage of total graft area. Representative images for 300 (C) and 500 (D) islet grafts are shown. Arrows indicate amyloid as assessed by thioflavin S staining. Insulin immunostaining is shown in red and amyloid is shown in cyan. Data area expressed as mean ± SEM. Differences between groups were assessed by the Mann-Whitney test. p < 0.05 (*) was considered significant. Scale bar = 100 μm.
3.3.5. Islet amyloid in human islet allograft donors

3.3.5.1. Analysis of amyloid severity and prevalence in the islet allograft cohort

The islet allograft cohort studied is described in the Methods section of this chapter. We analyzed amyloid severity and prevalence in islets from donors whose islets were used in clinical allograft recipients. Amyloid severity for individual donors is shown in Figure 7A. The mean amyloid load was 0.64 ± 0.19% of islet area. Ninety-five percent of donors had thioflavin S-positive staining within islets. Twenty-six percent of donors had greater than 1% of mean islet area occupied by amyloid. The least, median, and greatest amyloid loads within islets containing amyloid were 0.01%, 0.38%, and 3.20%, respectively. Islet allograft donors in this cohort had a three-fold increase in amyloid load relative to the islet autograft cohort. Amyloid prevalence is shown in Figure 7B. The mean amyloid prevalence was 29.5 ± 5.9%, approximately two-fold greater than for the islet autograft cohort. The least, median, and greatest amyloid prevalences were 0%, 26.2%, and 78.0%. Amyloid prevalence correlated positively and non-linearly with amyloid severity in islets from allograft donors (Figure 7C; $R^2 = 0.768$), as has been previously shown in islets of mice expressing the human IAPP transgene (548). After log transformation, the equation between these two parameters was $\log_e(\text{severity}) = 0.674 \times \log_e(\text{prevalence}) + 3.747$ (R$^2 = 0.678$; p < 0.0001).

3.3.5.2 Correlation of amyloid load and prevalence with available clinical data

Limited clinical data were available for this cohort. Recent AIRglu data was available for 7 recipients for whom we had characterized amyloid load and prevalence in islets prior to isolation for the most recent islet infusion. There was a lack of correlation between AIRglu and either amyloid severity (Figure 8A; $r^2 = 0.0002$, p = 0.97) or prevalence (Figure 8B; $r^2 = 0.02$, p = 0.79).

We also compared amyloid severity and prevalence between recipients with optimal graft function and those requiring insulin supplementation. These data were only available for the most recent islet infusion. Data was available for 7 patients for whom we also had donor pancreas histological sections for the most recent islet infusion. Four of these patients had optimal graft function, while 3 required insulin supplementation. Patients requiring insulin supplementation tended to have increased donor islet amyloid load at time of transplantation, although this difference was not significant (Figure 9A; 0.24 ± 0.20% versus 0.91 ± 0.26%, 0.24 ± 0.20% versus 0.91 ± 0.26%, 0.24 ± 0.20% versus 0.91 ± 0.26%, 0.24 ± 0.20% versus 0.91 ± 0.26%, 0.24 ± 0.20% versus 0.91 ± 0.26%.
Amyloid prevalence was significantly increased in islet recipients requiring adjunctive insulin therapy (Figure 9B, 10.8 ± 8.8% versus 56.4 ± 5.7%, p<0.05). Taken together, these data suggest that the greater the amyloid load and prevalence in donor islets prior to transplantation, the greater the likelihood of graft dysfunction.
Figure 7. Amyloid severity and prevalence in islet allograft donors.

Amyloid load (A) and prevalence (B) were determined in the islet allograft cohort. Amyloid severity was determined by quantification of mean thioflavin S (ThioS) positive area within islets. Islets were immunostained for insulin to illustrate islet boundaries. Amyloid prevalence was determined as the proportion of islets staining positively for thioflavin S. Image analysis was performed using islets in histological sections of donor pancreas fixed prior to islet isolation. Amyloid prevalence correlated positively and non-linearly with amyloid severity in islets from allograft donors (C; $R^2 = 0.768$). After log transformation, the equation between these two parameters was $[\log_e(\text{severity}) = 0.674 \times \log_e(\text{prevalence}) + 3.747]$ ($R^2 = 0.678; p < 0.0001$).
Figure 8. Lack of correlation between acute insulin response to glucose and islet amyloid in islet allografts.

Linear regression analysis of the relationship between AIRglu and amyloid severity (A) and prevalence (B). No correlation was observed. AIRglu, incremental area under the glucose curve above the baseline in an intravenous glucose tolerance test.
Figure 9. Amyloid prevalence in islets prior to transplantation is predictive of loss of insulin independence.

Amyloid severity (A) and prevalence (B) from the last islet infusion received by a given patient were stratified according to whether a patient required adjunctive insulin supplementation at one year following islet transplantation. **, p < 0.01.
3.3.6. Islet amyloid in human islet autograft donors

3.3.6.1. Amyloid load characterization for the islet autograft cohort

The islet autograft cohort is also described in the Methods section of this chapter. As for allografts, we analyzed amyloid severity and prevalence in the islet autograft cohort. We determined amyloid severity for both islets in the native pancreas and isolated islets (Figure 10). In native pancreas specimens, the mean amyloid load was 0.23 ± 0.17% (Figure 10A). Of these donors, 50% had detectable amyloid. Amongst donor islets containing amyloid, the mean amyloid severity was 0.46 ± 0.32%, with lowest, median and greatest amyloid severities of 0.001%, 0.03% and 2.3%, respectively. The mean amyloid severity in isolated islets was 0.17 ± 0.08% (Figure 10B), which was not significantly different from the severity in native islets. Of these specimens, 50% had any detectable amyloid. Amongst isolated islets containing amyloid, the mean amyloid severity was 0.35% ± 0.15%, with least, median and greatest amyloid severities of 0.003%, 0.11% and 1.4%, respectively. A comparison of amyloid severity in isolated islets and native islets (Figure 10C) was uninformative due to an amyloid severity of 0 in both isolated and native islets in 5 of 9 donors for whom both isolated and native islets were available.

Amyloid prevalence for the autograft cohort is shown in Figure 11. In native pancreas specimens, the mean amyloid prevalence was 17.0 ± 7.6% (Figure 11A). Of these donors, 50% had islets containing amyloid; the mean prevalence amongst donors with detectable amyloid was 34.1 ± 13.1%. The least, median, and greatest amyloid prevalence within native pancreatic islets were 1.0%, 4.0%, and 17.0%. The mean amyloid prevalence amongst isolated islets (Figure 11B) was 17.7 ± 6.3% and amongst donors with detectable amyloid, prevalence was 35.4 ± 9.6%. The least, median, and greatest amyloid prevalence within the isolated islets were 3.2%, 31%, and 91.2%. Linear regression revealed a positive correlation between amyloid prevalence between islets in native pancreas and isolated islets for donors in which we had both (Figure 11C, n = 11, r² = 0.75 , p < 0.0005). Amyloid severity and prevalence were compared within the autograft cohort (Figure 12). We combined results from analysis of native pancreatic and isolated islets and found that there was a positive and non-linear correlation between amyloid severity and prevalence (R² = 0.523). After log transformation, the equation between these two parameters was [logₑ(severity) = 0.391 x logₑ(prevalence) + 4.041] (R² = 0.662; p < 0.0001).
3.3.6.2. Lack of correlation of amyloid load and prevalence in donor islets with HbA1c

Metabolic data (HbA1c) was available at 1 year following transplantation for only 6 patients. The mean HbA1c was 6.7 ± 0.4% for these patients. Correlation of these data with amyloid load and prevalence is shown in Figures 13A and 13B, respectively. No significant correlation was found for either amyloid load (r² = 0.31, p = 0.25) or amyloid prevalence (r² = 0.51, p = 0.11). Of donors with corresponding metabolic data, 83% had little to no amyloid. Based upon these data, we cannot draw meaningful conclusions regarding the influence of pre-existing donor amyloid load and prevalence upon metabolic outcomes in islet autotransplantation.
Figure 10. Amyloid severity in islet autograft donors.

Amyloid load was determined by quantification of mean thioflavin S (ThioS) positive area within islets. Islets were immunostained for insulin to illustrate islet boundaries. Amyloid severity was determined using (A) native pancreas specimens and (B) islets isolated prior to transplantation. Lack of significant correlation between amyloid severity in matched donors
between islets in native pancreas and isolated islets is shown in (C). It should be noted that 5 data points are clustered at the intersection of the X and Y axes in (C).

Figure 11. Amyloid prevalence in islet autograft donors.
Amyloid prevalence was determined by the proportion of islets from a given donor that stained positively for thioflavin S (ThioS). Amyloid prevalence was determined using (A) native pancreas specimens and (B) islets isolated prior to transplantation. Amyloid prevalence correlated positively between native pancreatic islets and isolated islets (C; \( p = 0.0005 \)).
Figure 12. Correlation between amyloid load and prevalence in islet autograft donors.

Amyloid prevalence correlated positively and non-linearly with amyloid severity in islets from autograft donors ($R^2 = 0.523$). After log transformation, the equation between these two parameters was $\log_e(\text{severity}) = 0.391 \times \log_e(\text{prevalence}) + 4.041$ ($R^2 = 0.662; p < 0.0001$).
Figure 13. Lack of correlation between HbA$_{1c}$ in islet graft recipients and amyloid in transplanted islets.

Amyloid load and prevalence failed to correlate with HbA$_{1c}$ for islet autograft recipients. HbA$_{1c}$, glycated hemoglobin.
3.3.7. Hexapeptide IAPP derivatives inhibit amyloid formation and protect against amyloid-induced apoptosis

A library of hexa-peptide derivatives corresponding to regions of the mature IAPP (1-37) sequence were synthesized and tested for their ability to inhibit aggregation of synthetic human IAPP in vitro by electron microscopy in the laboratory of Dr. P. Fraser (University of Toronto). We tested the most promising inhibitor, L-ANFLVH, for its effects on amyloid formation and apoptosis in cultured human islets (Figure 14). L-ANFLVH significantly reduced amyloid deposition in cultured human islets (control, 0.65 ± 0.10% versus L-ANFLVH treated, 0.22 ± 0.06%, p < 0.01). Apoptosis in islets was assessed by two independent histological methods, TUNEL and cleaved caspase-3 staining. L-ANFLVH significantly reduced apoptosis as assessed by TUNEL (control, 24.6 ± 1.2% versus L-ANFLVH treated, 14.4 ± 2.3%; p<0.05) and by cleaved caspase-3 (control, 2.26 ± 0.25% versus L-ANFLVH treated, 1.28 ± 0.24%; p<0.05). Negative control peptides that did not inhibit synthetic human IAPP amyloid formation in vitro did not protect against the deposition of amyloid or against apoptosis in cultured human islets.

![Graphs A, B, and C]

Figure 14. A hexapeptide fragment of IAPP inhibits amyloid formation and apoptosis in cultured human islets.

The proportion of islet area occupied by thioflavin S (ThioS) positive amyloid is shown in (A). Apoptosis was assessed as percentage of TUNEL-positive islet cell nuclei (B) and the proportion of islet area occupied by cleaved caspase-3 positive staining (C). Culture experiments were performed using islets from 4 human donors. Data are expressed as mean ± SEM. Differences between groups were assessed by Student’s t-test. p < 0.05 (*) was considered significant. ClC3, cleaved caspase-3.
3.3.8. RNA silencing of human IAPP protects against amyloid deposition and toxicity in cultured human islets

Human IAPP siRNAs were previously characterized in transformed β cell lines and the sequence which achieved the greatest knockdown of IAPP was cloned into an adenovirus (Ad-hIAPP-siRNA) (3). Human islets were cultured for 2 weeks in the presence of this adenovirus (Figure 15A). In treated islets, amyloid deposition was significantly reduced as compared to islets not treated with adenovirus (control, 0.43 ± 0.05% versus Ad-hIAPP-siRNA-treated, 0.16 ± 0.04%, p < 0.01). Apoptosis was also significantly reduced in human IAPP siRNA-treated islets (3).

Clinical translation of adenoviral vectors is limited, particularly due to immunogenicity, lack of high specificity for β cells in vivo, and potential toxicity. Lipid nano-particle delivery systems for siRNA have been shown to be highly effective, minimally toxic, and translatable (549). We have shown these particles to be efficient in delivering fluorescently-labelled siRNA homogenously throughout islets in a concentration-dependent manner (see Chapter 6). The use of this technology to deliver siRNA against human IAPP would be a novel strategy for therapeutic modulation of IAPP and amyloid deposition within islets and may have great potential for clinical translation. In collaboration with Drs. P. Cullis and C. Tam (University of British Columbia), we characterized five siRNA against human IAPP in cell lines (data not shown) and selected the two most effective. One of the two effective siRNAs (sihIAPP3) was the same sequence as used in the adenoviral construct in Figure 15A (549). The other was a novel sequence (sih4; AACCATCTGAAAGCTACACCCATTG). Human islets from 4 donors were treated with lipid nano-particles containing sihIAPP3, sihIAPP4 or scrambled siRNA. These islets were treated in 16.7 mM glucose rather than 11.1 mM glucose to enhance amyloid deposition, as shown by (32) and our own data (Figure 15A). Although there was no significant reduction in amyloid load or apoptosis (Figure 15B), there was a strong trend towards decreased amyloid deposition in islets treated with both si-hIAPP. Si-hIAPP4 also tended to protect against apoptosis (Figure 15C,D).
Figure 15. siRNA-mediated inhibition of hIAPP protects cultured human islets from amyloid deposition and toxicity.

Human islets (n=3) treated with an adenoviral vector expressing siRNA against human IAPP were cultured for 2 weeks in 11.1 mM glucose-containing medium (A). Human islets (n=4) in (B-D) were treated with lipid nano-particles (10 mg/ml) in 16.7 mM glucose. Quantification of amyloid load is shown in (A) and (B). Quantification of TUNEL-positive nuclei expressed as a proportion of islet cell nuclei (C) and cleaved caspase-3 positive area (D) is shown for nanoparticle treated islets. Data are expressed as mean ± SEM. Differences between groups were assessed by Student’s t-test or one-way ANOVA. **, p<0.01. si-scramble, scrambled siRNA; si-hIAPP3, and si-hIAPP4, human IAPP siRNA 3 and 4; ThioS, thioflavin S; ClC3, cleaved caspase-3.
3.4. Discussion

Human IAPP aggregation has a detrimental effect upon transplanted islets. Using hIAPP<sup>Tg<0</sup> islets, we showed that expression of this peptide promotes mild elevations in blood glucose and glucose intolerance even when an optimal mass of islets is transplanted. Notably, impaired blood glucose homeostasis was present in recipients of hIAPP<sup>Tg<0</sup> islets even when amyloid was not detectable, and moreover the presence of islet amyloid did not worsen blood glucose in recipients of 250 hIAPP<sup>Tg<0</sup> islets. This finding is consistent with the development of diabetes in another strain of hIAPP<sup>Tg<0</sup> mouse that does not develop detectable islet amyloid (491). The potentially deleterious effects of islet amyloid formation are more notable in a marginal mass islet graft. Marginal mass grafts of hIAPP<sup>Tg<0</sup> islets had a significantly increased probability of hyperglycemia and graft failure, both of which correlated with the degree of amyloid deposition in the graft. Our data elaborated upon a recent study in which it was demonstrated that the presence of islet amyloid deposition correlated with the development of hyperglycemia (490). hIAPP<sup>Tg<0</sup> islets over-express amyloidogenic IAPP, which may lead to supra-physiological concentrations of human IAPP in the circulation. Human islet studies thereby have greater physiological and clinical relevance. Using transplanted human islets, we determined that in human islet grafts which failed to maintain glycemic control, there was increased amyloid deposition and a trend towards reduced β cell mass. Further, we determined that transplantation of fewer islets promotes amyloid deposition within human islet grafts. Amyloid load is highly variable amongst human islet donors. Consistent with the detrimental effect of islet amyloid in transplantation, we determined that a greater amyloid load prior to transplantation correlated with graft dysfunction in recipients. Moreover, we noted that islets used in a cohort of clinical islet allografts had an elevated incidence of amyloid prior to transplantation, unlike the islet autograft cohort. Finally, we demonstrated that two promising therapeutic strategies protect against amyloid deposition and amyloid-associated toxicity in cultured human islets. Although these strategies need to be further validated in vivo, they show great promise for clinical translation in the development of anti-amyloid therapeutics.

The success of clinical islet transplantation is limited by progressive loss of graft function. Despite its considerable success in slowing the progression of diabetes-related complications, its broader implementation is dependent upon improvement in long-term graft viability and function. Though patient satisfaction is high and quality of life improved upon successful islet
transplantation, both deteriorate rapidly upon a return to insulin therapy (550, 551). A slow decline in production of C-peptide and graft failure in non-immunosuppressed autograft recipients are highly suggestive that non-immune mechanisms contribute to long-term graft failure. Amyloid deposition has been previously described in transplanted human islets (543, 552) but never correlated with pathology. In this chapter, we show that human islet amyloid polypeptide contributes substantially and rapidly to β cell dysfunction in transplantation in human islets. This finding is consistent with a case report of extensive amyloid deposition in a failing human islet graft (489). Furthermore, it validates a previous study in which amyloid deposition within transplanted human IAPP-overexpressing transgenic mouse islets was associated with recurrence of hyperglycemia within weeks of transplantation (490). Rapid human IAPP-associated pathology in transplanted human islets demonstrates that islet amyloid is a significant contributor to graft dysfunction at physiological levels of IAPP expressed in human islets rather than simply attributable to transgenic over-expression at supraphysiological levels.

Amyloid deposition in the type 2 diabetic islet begins prior to the onset of overt disease and is associated with progressive apoptotic loss of islet β cell mass. In autopsy studies of human pancreas, islet amyloid load and prevalence are substantially increased in both lean and obese type 2 diabetic individuals, consistent with a 70% reduction in islet β cell volume (529). Prediabetic individuals with impaired fasting glucose had a 50% reduction in islet β cell volume compared to non-diabetic individuals but had similar islet amyloid load and prevalence to non-diabetic controls (529). Autopsy studies present a limited snapshot of disease pathology and do not follow the progressive pathology in individuals. The contribution of islet amyloid deposition to progression of type 2 diabetes is better illustrated in the baboon, a primate with pro-amyloidogenic IAPP (454) that spontaneously develops human-like type 2 diabetes and associated islet amyloid pathology (453, 553). In a non-diabetic, normoglycemic baboon, up to 17% of islet area may be occupied by islet amyloid (454). Islet amyloid severity increases as baboons age (454). Between 36 and 51% of islet volume is occupied by amyloid upon onset of hyperglycemia. Amyloid load within the islet continues to increase as hyperglycemia worsens, suggesting that amyloid formation continues to contribute to islet pathology. When amyloid load exceeds 51% of baboon islet volume, β cell volume within the islet drops dramatically (454); this recapitulates the decrease in β cell volume associated with progression from impaired fasting glucose to diabetes and with increased amyloid deposition in the aforementioned human autopsy
study (529). These findings are also reproduced in the HIP rat, a transgenic rat which overexpresses IAPP in the β cell under control of the rat insulin promoter (97). The hemizygous HIP rat is a hIAPP-expressing transgenic model that closely approximates human type 2 diabetes (554) and develops a phenotype in the absence of a high fat diet or pharmacological stimulation. In this model, a tripling of islet amyloid load between 2 and 5 months of age precedes the onset of hyperglycemia and rapid decline in islet β cell volume that begin at 5 months of age (98). A significant increase in β cell apoptosis is noted at 3 months of age, correlating with a rapid increase in islet amyloid deposition. Taken together, these data illustrate that islet amyloid deposition precipitates β cell dysfunction and loss in type 2 diabetes and continues to contribute to disease pathology as diabetes progresses.

The transplanted β cell recapitulates several features of the type 2 diabetic β cell. In addition to amyloid pathology within the graft (543, 552), these features include a blunted first phase of secretagogue-stimulated insulin secretion (357, 539), a progressive decline in insulin and C-peptide secretion (129), impaired prohormone processing leading to hyper-proinsulinemia (316, 376, 543), and decreased PC2 immunostaining (543). Additional similarities are the progressive loss of transplanted islet mass following successful engraftment (545, 546) and the improvement of transplanted islet function with therapeutic agents used in the treatment of type 2 diabetes (211, 246, 298, 311, 312, 555-559). The progressive decline in the function of transplanted β cells is accelerated relative to type 2 diabetes. In type 2 diabetes, progression from a state of impaired fasting glucose to overt diabetes tends to occur over 3 to 4 years (560). Amyloid aggregation in aforementioned animal models of type diabetes occurs over months. The time course of its deposition is likely longer in humans, considering the low amyloid load in humans with impaired fasting glucose (529) and that progression to overt disease occurs over several years. In islet transplantation, the decline in islet function is rapid, such that graft failure occurs within 1-2 years of transplantation (129). Correspondingly, islet amyloid fibrils have been observed in transplanted human islets as early as two weeks following transplantation (552). Furthermore, in our optimal mass islet transplantation model, human-IAPP expressing islets demonstrated a slight but significant increase in blood glucose values within the second week following transplantation. This correlated with impaired glucose tolerance upon an intraperitoneal glucose challenge. Islet amyloid species contribute markedly to β cell pathology in both transplanted and type 2 diabetic islets. The accelerated onset of amyloid pathology and
deposition in islet transplantation suggest that islet amyloid and associated pre-fibrillar IAPP aggregates are important non-immune factors that contribute to long-term islet graft failure.

The causes of accelerated amyloid pathology in islet transplantation remain unclear. Islet injury associated with islet isolation or the peri-transplantation environment may enhance islet amyloid deposition. Pancreatic digestion by collagenase may damage the integrity of islet basement membrane and capillary beds. Moreover, islet endothelium dies rapidly during overnight islet culture prior to transplantation. Together, these factors lead to exposure of endogenous heparan sulfate proteoglycans (487, 561) that may seed islet amyloid formation. Islet isolation (236) and peri-transplant inflammation stimulate β cell apoptosis. Localized release of contents of apoptotic cells may elevate intra-islet extracellular IAPP concentrations and also promote its aggregation. Many factors in or associated with the transplantation environment may contribute to and potentiate β cell dysfunction. Transplanted β cells are subject to metabolic stress, particularly when engrafted islet mass is low (202) and in the presence of recipient hyperglycemia. Many factors in the transplant environment may exacerbate β cell dysfunction (202, 262, 263, 265, 355, 360, 562-565), leading to impaired prohormone processing and elevated release of N-terminally extended proIAPP, which may bind to heparan sulfate proteoglycans to seed amyloid formation by IAPP (487). Alternately, rapid amyloid formation within grafts may stem from decreased IAPP clearance upon loss of islet vasculature in islet isolation. Furthermore, it has not been established whether vasculature formed from endothelial cells at a heterologous site of transplantation maintain the characteristic fenestrations of islet endothelium and thus normal rates of IAPP clearance upon re-establishment of islet vasculature. Finally, as insulin may inhibit amyloid formation, an increased ratio of secreted proinsulin:insulin in the face of impaired insulin processing may destabilize IAPP and promote its misfolding. Although there are many potential mechanisms underlying increased amyloid deposition in transplantation, the actual mechanism remains to be elucidated.

Amyloid prevalence in donor islets prior to transplantation is predictive of long-term graft function. Although not statistically significant, there was also a strong tendency for amyloid severity to predict outcomes that was likely limited by the small sample size. Taken together, these preliminary data suggest that a larger multi-centre study evaluating whether stratification of recipients according to amyloid in donor islets is predictive of long-term graft function is
warranted. Amyloid severity correlates with disease severity (566) or glycemic control (567) in type 2 diabetes in human autopsy studies (568). Thus, findings in type 2 diabetes support our predictive findings in transplanted human islets. Amyloid imaging in vivo has been performed in humans and animal models of Alzheimer’s disease by positron emission tomography (PET) using a radiolabelled isotype of the Pittsburgh compound (PiB) (569). As this technology becomes more widely available, monitoring the rate of amyloid deposition in transplanted islets will likely be an even stronger predictor of long-term graft outcome.

Thioflavin S-positive area in donor islets provides an estimate of amyloid plaque load but not pre-fibrillar, thioflavin S-negative IAPP species. The predictive value of amyloid plaques on disease severity is not fully supported in Alzheimer’s disease. Some autopsy studies in humans have shown association between amyloid plaque load and degree of cognitive impairment (570, 571), while others have failed (572) or detected amyloid in normal individuals without cognitive impairment (573). A lack of association was found over time as detected by PET-PiB imaging over time despite Alzheimer’s disease progression in a case report of single individual (569). Severity of Alzheimer’s disease is earlier (574) and better predicted by the presence of prefibrillar “oligomeric” species (572, 575-577) as detected by an oligomer-specific antibody rather than for fibrillar amyloid. In our hands, impaired glucose tolerance was present even in recipients of hIAPP\textsuperscript{Tg\(0\)} islets whose grafts failed to develop amyloid deposits, supporting the idea that undetectable, pre-fibrillar IAPP aggregates contribute to disease and graft failure.

Several studies support the notion that a pre-fibrillar species is the predominant toxic species formed by human IAPP (96, 578). Zhao et al claimed to have identified IAPP oligomeric species in type 2 diabetic human islets (579) using the previously described A-11 antibody, although concerns have been raised regarding the specificity of this A-11 antibody (12) and whether this it has been adequately characterized for IAPP (476, 580). Furthermore, this antibody is well known not to work in paraffin-embedded tissues (579) and in our own studies we have been unable to replicate the findings of Zhao et al. (data not shown). The A-11 antibody has only been used in a single study in Alzheimer’s disease (577), while other antibodies are more commonly used (572, 575, 576). The development of an antibody that truly binds the toxic IAPP oligomeric species would be of great value to the field. In the meantime, we have shown that fibril load and prevalence do have predictive power in islet graft success in transplantation.
Although the amyloid fibril end-product may not be the predominant toxic species affecting β cells, its presence demonstrates the prior aggregation of pre-fibrillar species. Moreover, as IAPP aggregates up to a hundred-fold faster than amyloid β, the time course from the appearance of prefibrillar species to mature fibrils is much more rapid in IAPP pathology than in other amyloid diseases. Nevertheless, alternate methods of characterization of islet amyloid might increase the predictive power of graft success and allow a measure of IAPP aggregates species that correlate better with toxicity. Several currently available methodologies could potentially provide such evidence. Serum amyloid P (SAP) is a known component of amyloid plaques. It may detected by immuno-histochemical staining and while it localizes well with thioflavin-staining (581, 582), it may bind to areas that do not stain positively by thioflavin dyes (583). Moreover, it may be monitored in vivo. In patients with systemic amyloidosis, serum SAP levels decrease significantly (584) and SAP localization within tissues may be determined by scintigraphy (584-587). Detection of fibril load will likely become possible using the previously described method of PET imaging in conjunction with PiB (588). Future developments in the ability to detect amyloid oligomers and amyloid in vivo imaging techniques will refine our ability to monitor IAPP amyloid pathology in both islet transplantation and type 2 diabetes.

Islet amyloid prevalence and load tend to be greater in conditions in which β cell dysfunction is observed (431, 567, 568, 589, 590). For example, islet amyloid is found in 92% of insulinomas (589). Between 20 and 99% of type 2 diabetic patients (431, 567, 568, 590) have islet amyloid in human autopsy studies; islet amyloid severity is approximately 12 to 35% (567, 590). Moreover, islet amyloid deposition is increased with severity of diabetes (568). In this study, we noted dramatic differences in amyloid load and prevalence between autograft and allograft donors. The islet autograft cohort analyzed in this study was highly representative of a non-diabetic population based upon these citations, with a mean islet amyloid severity of less than 0.2% of islet area and islet amyloid prevalence within a given pancreas of approximately 17%. The islet allograft population had a mean amyloid severity of 0.6% and a mean prevalence of approximately 30%. While the severity of amyloid present in islets used in clinical allografts was less than that of type 2 diabetes, it appears to be markedly elevated relative to islets used in our autograft population. Furthermore, following transplantation, amyloid deposition within human islets may become extensive (489). Although we were not provided with islet donor
characteristics for either population, these data suggests that the donor populations are very different between the two cohorts.

Islet auto-transplantation is most commonly performed following pancreatectomy to alleviate pain in chronic pancreatitis. Chronic pancreatitis is a malabsorption syndrome, leading to thin body habitus and wasting. The mean age of onset of chronic pancreatitis is 40, suggesting that this population is middle-aged. While diabetes mellitus may occur as a consequence of long-standing pancreatitis, chronic pancreatitis patients tend to lack features of the metabolic syndrome associated with type 2 diabetes, in which islets typically develop amyloid pathology. In contrast, islet allograft donors tend to be older and obese (144, 146, 147). Thus, it is perhaps not surprising that islets used in auto-transplants have less amyloid than those used in allo-transplants.

In islet allo-transplantation, cadaveric donor organs are scarce (213), with the highest quality organs being allocated to pancreas transplantation (144). Only 10% of cadaveric multiorgan donors are eligible for pancreas transplantation, while 48% of donors were suitable for islet transplantation (145). Donors for islet transplantation may be as old as 70 years (219) and BMI often exceeds 30 (146, 147). Islet isolation efficacy and yield also favors the use of older, larger donors. Islet isolation is more difficult with the pancreas of a low BMI donor (214, 215, 217) and digestion time is reduced with a pancreas from a larger BMI donor (216). Furthermore, pancreata from high BMI pancreata yield more islets (218). Reports on the function of islets from obese donors are conflicting (216, 217), although it is known that pancreata from obese donors tend to be fatty and thus poorly perfused and more susceptible to ischemia/reperfusion injury (216). Islets from obese donors may be more susceptible to metabolic exhaustion and long-term dysfunction upon transplantation. Donors are excluded if there is a known history of diabetes (214) but many obese donors are likely “pre-diabetic” with undiagnosed metabolic syndrome. Hyperglycemia in a donor has been shown to have poor outcomes following pancreas transplantation (145, 223). Islets from type 2 diabetic humans have abnormal glucose-stimulated insulin secretion and do not reverse hyperglycemia in transplanted mice (205). Donor age also influences glycemic outcomes in transplantation. Once donor age exceeds 45 years, there is increased risk of poor glycemic control and early graft dysfunction in whole pancreas transplant (221). Islet transplantation may be biased against by the use of lower quality organs than pancreas transplantation, which may thus lower likelihood of success of the procedure. Islets
used in allo-transplantation may be preferentially obtained from donors older and more obese donors that may be predisposed to amyloid formation, dysfunction and graft failure.

As islet amyloid contributes significantly to graft pathology, therapeutic manipulation of IAPP expression or islet amyloid deposition should improve long-term graft survival and function.

Currently, there are no amyloid inhibitors in clinical trials or clinically approved for treatment of any local or systemic amyloidoses. The most promising anti-amyloid therapy to date has been anti-amyloid vaccination in mouse models of Alzheimer’s disease (591) and in early human clinical trials (592), demonstrated to promote amyloid plaque clearance and improve cognitive function. Amyloid immunotherapy has not yet passed clinical trials due to significant pathology in recipients (592). Many relevant pre-clinical strategies have been proposed to protect against amyloid. Direct inhibitors of IAPP aggregation have been characterized to varying extents. These include Congo red (38), [(N-Me)G24, (N-Me)I26]-human IAPP-GI (593), acid fuschin (594), epigallocatechin-3- O-gallate (595), phenolsulfonphthalein (596), curcumin (597), and IAPP fragments and their derivatives (598, 599). Interestingly, a non-amyloidogenic derivative of full length human IAPP (pramlintide) is clinically available but has not been tested for its inhibitory properties against IAPP aggregation. The non-amyloidogenic rat IAPP has also been shown to inhibit IAPP fibrillogenesis in vitro (464). Given that IAPP may cause toxicity by intracellular mechanisms, use of molecular chaperones has been proposed to improve proper folding efficiency of proIAPP (394) in the ER. This might be accomplished by gene therapy or by the use of chemical chaperones (397, 406, 600). As IAPP has been proposed to promote toxicity by means of inflammation (40), inhibition of IAPP-stimulated inflammatory processes may also improve graft outcomes. Additional means of protecting β cells from amyloid toxicity include suppression of β cell pro-apoptotic signaling (37, 38). While many strategies have been proposed, further testing is required for all of the above mechanisms in pre-clinical models. In this chapter, we demonstrated human islet protection from amyloid deposition and associated toxicity by the use of a hexapeptide inhibitor of IAPP aggregation and by silencing IAPP mRNA expression.

One promising strategy for islet graft protection against islet amyloid toxicity that we propose is the use of amyloid inhibitors. While many molecules have been proposed to inhibit IAPP fibrillogenesis and cytotoxicity in biochemical and cell culture models (38, 464, 593-599), we are the first to show that this strategy is effective in human tissue. We screened promising hexa-
peptide sequences derived from the primary amino acid sequence of IAPP that were effective in suppressing IAPP amyloid formation in biochemical assays. The hexa-peptide ANFLVH is the most promising candidate for further *in vivo* testing. Within the sequence of full length, mature IAPP, the ANFLVH sequence falls between amino acid residues 13-17. It does not lie adjacent to or within the primary pro-amyloidogenic GAILSS region (residues 24-28). To prevent the formation of pre-fibrillar species by human IAPP, ANFLVH may stabilize full-length IAPP in its native, soluble random coil conformation, as has been shown for other effective IAPP hexapeptide fragments (508). Interestingly, a similar hexapeptide derivative (LANFLV) promotes β sheet formation with IAPP (1-37) and seeds amyloid formation (463). Therefore, a histidine residue (residue 18 in mature IAPP) in the effective inhibitory hexapeptide may play a critical role in preventing IAPP aggregation. This histidine residue promotes β-sheet structure and fibril formation in solution when it is deprotonated at physiological pH (462); its pro- fibrillogenic effects are lost when the residue is charged at low pH. The histidine residue within the hexapeptide may competitively inhibit interaction between histidine at residue 18 within the mature peptide for interaction with other IAPP (1-37) residues critical to formation of β sheet structure.

Therapeutic formulation of a hexapeptide for drug delivery is much simpler than for a larger peptide derivative, such as the proposed IAPP amyloid inhibitor IAPP-GI (593). However, peptides are not ideal for *in vivo* use due to rapid proteolytic degradation. Preliminary studies of the half-life of ANFLVH in serum suggested a half-life of less than two minutes. Many peptidomimetic strategies have been proposed to increase peptide stability *in vivo*. These include N-terminal modification and the use of D-enantiomers. We showed that the D-enantiomer of the L-ANLFVH sequence has remarkably similar properties to L-ANFLVH in its ability to inhibit IAPP fibril formation and toxicity. Furthermore, the half-life of this compound in serum was increased to several hours in preliminary studies. By development of a highly stable and highly effective inhibitor of islet amyloid formation, we have developed a strong candidate drug for *in vivo* protection of β cells in type 2 diabetes and islet transplantation.

A second strategy that we proposed to protect islets against islet amyloid toxicity in transplantation is the silencing of IAPP expression. RNA silencing of gene expression has entered the realm of clinical application. The first clinical trial using siRNA began in 2004, in a
patient population affected by blinding choroidal neovascularization (601). SiRNA was administered by direct intraocular injection. The most recent report in the treatment of this particular disease demonstrates that siRNA therapy is well tolerated and results in disease stabilization or improvement (602). The first effective systemic delivery of siRNA to humans resulting in specific RNA and protein level knock-down of M2 subunit of ribonucleotide reductase (RRM2) in solid tumours was achieved by lipid nano-particle delivery (601) and published less than a year ago. Positive results in clinical trials, achieved without systemic toxicity, open the gateway for RNA targeting for an endless possibility of gene targets.

Our initial studies on human IAPP knockdown in human islets were performed using an adenoviral vector. We effectively knocked down IAPP and correspondingly limited islet amyloid deposition and protected against its toxicity in cultured human islets. The possibility of clinical translation into humans using adenoviral vectors is limited, however, by their immunogenicity and potential toxicity (603-605). Adenoviral vectors are currently in human clinical trials for gene therapy (606), for which mild influenza-like symptoms were associated with delivery. Lipid nano-particle delivery may be achieved with an absence of systemic toxicity. In this chapter, we show that use of this technology (549) to deliver highly stable siRNA to cultured human islets also reduces islet amyloid deposition and toxicity when the target is human IAPP. Given IAPP toxicity in transplanted human islets, this represents a novel and highly translatable approach to protect β cells against the toxic effects of human IAPP.

A particular novelty of islet cell transplantation, as compared with other organ transplants, is that overnight culture of islets prior to transplantation is typically performed, allowing sufficient time for ex-vivo treatment. The current limitation of this system is the lack of β cell specificity of lipid nano-particle delivery. Systemic delivery would thereby also reduce IAPP expression in other cell types. Lack of IAPP expression in a transgenic mouse has minimal metabolic effects, with mild improvement in glucose tolerance (607), and therefore systemic knockdown of IAPP production is unlikely to have significant physiological effects. Additional reported effects include increased susceptibility to chemically-induced diabetes (608) and reduced nociception (609). β cell-specific lipid nano-particle delivery systems would eliminate risks of IAPP knockdown in other organ systems and decrease the dose required to achieve effective silencing in the β cell. IAPP replacement therapy is possible using the clinically available non-
amyloidogenic analogue of human IAPP, pramlintide. Increased β cell specificity of lipid nano-particle delivery is currently in development by means of ligand associated nano-particles. We conclude that our results in human islets using both adenoviral and lipid nano-particle delivery systems show great promise for in vivo modulation of amyloidogenic protein expression.

In summary, in this Chapter, we show that islet amyloid deposition is detrimental to graft function and exacerbated under conditions of metabolic stress. Pre-existing amyloid in an allograft donor cohort may have value for prediction of long-term graft outcome. We demonstrate several therapeutic strategies with clinical applicability to protect human islets against the toxic effects of human islet amyloid polypeptide.
Chapter 4. Effect of heparin on amyloidogenesis and toxicity of human IAPP

4.1 Background

Heparin is used in clinical islet transplantation to prevent loss of islets by the instant blood-mediated inflammatory reaction (IBMIR) upon infusion, thus preserving β cell mass (149, 334). Islets are exposed to heparin (149, 610) prior to infusion and again in serum up to 7 days following transplantation (153, 311). Islet surface heparinization protects islets against IBMIR (149) and is in clinical trials for islet transplantation.

Heparin directly promotes the fibrillogenesis of many amyloidogenic species, including proIAPP (561), IAPP (611, 612), acylphosphatase (613), serum amyloid A (614), β-2 microglobulin (615), gelsolin (616), Aβ 1-28 (617), the prion fragment PrP 185-208 (617), and α-synuclein (618). IAPP amyloid formation in the presence of heparin results in increased fibril density and fibril compaction (619). Further evidence of a role for heparin in amyloid formation is the inhibition of heparin binding to IAPP by the amyloid inhibitor Congo red (611) and removal of heparin-IAPP complexes from solution by centrifugation (611). Heparin is thought to act as a scaffold for amyloid formation (613). Heparin increases the nucleation rate of amyloid fibrils at low concentrations and the elongation rate of fibrils at higher concentrations (620). Heparin may also stabilize fibrils such that it prevents de-polymerization, as has been shown for β-2 microglobulin (621). Thus, heparin exposure to islets likely promotes aggregation of IAPP.

Heparin may promote IAPP aggregation in islets by multiple mechanisms. First, it may promote fibrillization of mature IAPP by direct interaction, as suggested by interaction with its positively charged arginine residue at residue 11 (622). Heparin may also seed amyloid formation by its interaction with a processing intermediate of proIAPP. Islet amyloid is comprised primarily of islet amyloid polypeptide (IAPP) (623) but N-terminally extended (C-terminally processed) pro-IAPP is also detected in islet amyloid plaques (432). N-terminally unprocessed proIAPP has a putative heparin-binding domain that mediates proIAPP binding to heparin and heparan sulfate (487), comprising 3 basic amino acids at its N-terminal cleavage site. Binding of heparin to this domain promotes amyloid formation by proIAPP species (561), which may subsequently seed IAPP aggregation into amyloid fibrils (624). Glycosaminoglycan binding domains exist in other mature amyloidogenic proteins (561, 625, 626) and have been shown to promote amyloid
deposition (625). Sulfated glycosaminoglycans such as heparan sulfate are recognized components of amyloid deposits (487). Given the known cytotoxic effects of aggregating human IAPP, the potent effects of heparin on its aggregation likely impact its toxicity to β cells. In this study, we investigated the effects of heparin on amyloid aggregation and toxicity in cell culture, human islet culture, and in vivo transplantation studies.

4.2 Methods
4.2.1 Time course of IAPP toxicity
A time course of IAPP toxicity was performed according to the method of Bucciantini et al (627) and Rigacci et al (628). IAPP was rapidly solubilized in 90% of a final volume of cell culture media to obtain a concentration of 48.9 µM. It was subsequently aliquoted into tubes containing media alone or media and heparin, such that tubes contained 40 µM hIAPP, 40 µM hIAPP plus 41 IU/ml heparin, or 40 µM hIAPP plus 410 IU/ml heparin. These aliquots were kept at 37°C in a water bath throughout the experiment. One hundred microlitres of each aliquot was transferred in duplicate to a 96 well plate so as to monitor IAPP fibrillization by thioflavin T kinetics at 37°C throughout the experiment. Concentrated thioflavin T stock was added to each well to make a final concentration of 10 µM. INS-1 cells were plated in 96 well plates such that for every time point, fresh INS-1 cells were at 70-80% confluence. Immediately after preparing hIAPP-containing solutions, INS-1 media was removed from 8 wells and media in 2 wells per condition was replaced with fresh media or an aliquot of the three solutions described above. At 0.5, 1, 2, 3, 6, and 12 hours after IAPP solubilization, an aliquot of each solution was added to fresh wells containing INS-1 cells. Each of these solutions were left on cells for 9 hours to allow toxic species within the aliquot to impact the viability of INS-1 cells. After 9 hours, images were taken of all wells. At this time, media in wells was replaced with fresh media containing 10% alamarBlue®. Cells were incubated with alamarBlue® for 4 hours, after which time fluorescence was read as described in Chapter 2.
4.3 Results

4.3.1 Heparin accelerates the formation and the final yield of IAPP amyloid fibrils

We assessed the effect of heparin on amyloid fibril formation using a thioflavin T kinetics assay as previously described (487) (Figure 16). IAPP (40 μM) was incubated alone or in the presence of increasing concentrations of heparin (41 IU/ml, 410 IU/ml). The lowest concentration, 41 IU/ml, was chosen to mimic concentrations of heparin used clinically prior to islet infusion. Islets are exposed to between 3,000 and 5,000 IU/ml of heparin (149, 610) in a volume of 120 ml (610). A ten-fold greater concentration was also used to determine whether the effects of heparin on IAPP occurred in a concentration-dependent manner. Each condition was performed in duplicate and repeated on five separate occasions. Data was corrected for the fluorescence of buffer containing thioflavin T alone, or in the case of heparin addition, for the fluorescence of buffer containing thioflavin T and the appropriate concentration of heparin alone. Even the highest concentration of heparin used did not alter fluorescence of solution in the absence of IAPP as compared to buffer with thioflavin T alone (0.042 ± 0.001 versus 0.042 ± 0.001, p = ns).

When IAPP (40 μM) was incubated alone (Figure 16), the lag phase lasted 0.55 ± 0.10 hours. The slope of the upstroke of fibril growth was 0.52 ± 0.17 AFU/hour. Maximal fluorescence was achieved by 0.93 ± 0.12 hours, at which point fluorescence above the corrected baseline was 0.16 ± 0.04 AFU. In the presence of either 41 or 410 IU/ml heparin, the initial thioflavin-corrected fluorescence was slightly but significantly increased, reflective of previous observations that thioflavin T fluorescence is slightly increased upon binding prefibrillar aggregates with β-sheet structure (629-631). The lag phase was significantly reduced in the presence of both concentrations of heparin. Heparin (410 IU/ml) reduced the lag phase by six-fold to 0.09 ± 0.03 hours. At the time of onset of the rapid fibril growth phase, corrected fluorescence was increased two-fold in the presence of 410 IU/ml heparin. The slope of fibril-induced fluorescence was also significantly greater in the 41 IU/ml heparin-treated group compared to hIAPP alone, and was 4- and 2.5-fold increased in the presence of 41 and 410 IU/ml heparin, respectively. Peak fluorescence was significantly increased between 2- and 3-fold in the presence of both concentrations of heparin. IAPP in the presence of heparin tended to have higher fluorescence at two hours following initiation of aggregation, although this was not statistically significant.
Figure 16. Heparin decreases the lag time of fibril formation and increases load of fibril formation as assessed by thioflavin T fluorescence kinetic assay.

Thioflavin T fluoresces upon binding to amyloid fibrils. Synthetic human IAPP (40 μM) was incubated alone (black) or in the presence of heparin (purple: 41 IU/ml; blue: 410 IU/ml. Heparin alone did not alter thioflavin T fluorescence (not shown). Assay conditions are described in Chapter 2 (Methods).

Electron microscopy was performed to provide representative images of amyloid fibrils in the absence or presence of heparin at different time points (Figure 17). No fibrillar structures were noted immediately following IAPP solubilization and addition of heparin. By 4 hours, IAPP fibrils formed in the presence of both concentrations of heparin Figures 17E and H) but not in buffer containing 40 μM IAPP alone (Figure 17B). By 24 hours post-solubilization, denser fibril meshworks were observed at both concentrations of heparin (Figures 17F and G) while no fibrils were noted in buffer containing 40 μM IAPP alone (Figure 17C). By Thioflavin T data, we confirmed that 40 μM IAPP formed fibrils in the absence of heparin, although this was not observed in TEM images shown in Figure 17. TEM images do, however, confirm that heparin (41 and 410 IU/ml) enhances IAPP fibril formation.
Figure 17. Heparin increases fibril load as assessed by transmission electron microscopy.
Synthetic human IAPP (40 μM) was solubilized with or without heparin (41 or 410 IU/ml).
Aliquots were removed immediately after solubilization and at 4 or 24 hours following
solubilization, and then incubated on grids for electron microscopy. Grids were dried, negatively
stained with uranyl acetate, and imaged. Scale bar represents 200 μm. Fibrils are demonstrated
by black arrows.
4.3.2 Heparin accelerates the extent and onset of IAPP toxicity

To determine the effect of heparin on IAPP toxicity, we incubated INS-1 rat insulinoma cells with synthetic human IAPP at increasing concentrations of heparin (Figure 18). Viability was assessed by alamarBlue® reduction assay (Figure 18A) and apoptosis was assessed by TUNEL (Figure 18B).

Human IAPP significantly reduced INS-1 viability as assessed by alamarBlue® incubation with 20 or 40 µM hIAPP for 24 hours (control viability, 100.0%; 20 µM hIAPP, 72.4 ± 5.3% of control viability; 40 µM hIAPP, 58.8 ± 9.1%; p < 0.01 and p < 0.001, respectively). Heparin had no effect on INS-1 cell viability even at the highest concentration of heparin used (control, 100%; 410 IU/ml heparin, 99.5 ± 2.7%; p = ns). A concentration-dependent effect of heparin in reducing cell viability in the presence of hIAPP was observed in cells treated with 20 and 40 µM hIAPP. At 40 µM hIAPP, heparin significantly increased the toxicity of IAPP at a concentrations of 410 IU/ml (40 µM hIAPP, 58.8 ± 9.1%; 40 µM hIAPP + 410 IU/ml heparin, 23.7 ± 6.2%; p < 0.05) as compared to cells treated with heparin alone.

Human IAPP did not significantly increase apoptosis in INS-1 cells as assessed by TUNEL staining after incubation with 10 or 20 µM hIAPP for 16 hours, although a trend towards increase apoptosis was observed in the latter (control, 2.3 ± 0.9% TUNEL positive cells; 10 µM hIAPP, 3.0 ± 0.2%; 20 µM hIAPP, 7.0 ± 4.5%). Any effects of heparin alone on INS-1 cells were not significant even at the highest concentration of heparin used (control, 2.3 ± 0.9% TUNEL-positive nuclei; 410 IU/ml heparin, 0.21 ± 0.16%; p = ns). Heparin did not increase IAPP toxicity at 10 µM hIAPP (Figure 18). At 20 µM hIAPP, however, heparin significantly increased the toxicity of IAPP at concentrations of 41 IU/ml (34.3 ± 11.8%, p < 0.05) and 410 IU/ml (36.5 ± 8.2%, p < 0.01) as compared to cells treated with heparin alone.
Figure 18. Heparin enhances toxicity of synthetic human IAPP.

INS-1 cells treated with IAPP with or without heparin were assessed for viability by Alamar blue reduction assay (A) and apoptosis by TUNEL (B). For Alamar blue, cells were treated with human IAPP without (white) or with heparin (green, 4.1 IU/ml; purple, 41 IU/ml; blue, 410 IU/ml heparin) for 24 hours. For TUNEL, cells were treated with identical conditions but incubated for 16 hours. Cells were incubated with synthetic human IAPP at 10, 20, or 40 μM. Statistical analysis was performed by two-way ANOVA. * p < 0.05; ** p < 0.01.
To better delineate the effects of heparin on IAPP-induced toxicity in INS-1 cells, we performed a time course of toxicity according to the method of Bucciantini et al (627) and Rigacci et al (628). The experimental setup is illustrated in Figure 19 and described in the Methods section of this chapter. Essentially, IAPP was solubilized in cell media and at various time points following solubilization, aliquots were removed and assessed for amyloid load by thioflavin T fluorescence or added to INS-1 cells to determine the effect on cellular viability 9 hours following addition of a given aliquot. It should be noted that in this experiment, in contrast to section 4.3.1, kinetics of IAPP aggregation were assessed in cell media as opposed to Tris buffer. Cell viability was assessed at each time point by alamarBlue® fluorescence.
Figure 19. Experimental design to assess the onset of heparin-potentiated IAPP toxicity relative to fibril formation.

The experimental design allowed comparison of the aggregation state of IAPP, as indicated by thioflavin fluorescence, with toxicity at a given time point. Aliquots were added to wells containing INS-1 cells at ~75% confluence. Heparin concentrations were 41 and 410 IU/ml.
The effects of heparin on IAPP fibrillization in cell media (Figure 20A) are similar to the effects in Tris buffer until the plateau phase (Figure 16). The two concentrations of heparin (41 IU/ml, 410 IU/ml) had nearly identical effects on IAPP fibrillization. Heparin (410 IU/ml) significantly decreased the lag phase relative to 40 μM hIAPP alone (control, 2.23 ± 0.07 hours; heparin 0.48 ± 0.08 hours; p < 0.0001). There was no significant difference in slope, although heparin tended to make the slope steeper (control, 0.25 ± 0.11; heparin 41 IU/ml 0.66 ± 0.22; heparin 410 IU/ml 0.72 ± 0.16, p = 0.18). Curves reached a smooth plateau that, unlike fibrillization in Tris buffer, did not reach maximal fluorescence immediately and from which fluorescence did not decline over time. A final maximal fluorescence was observed by 6 hours following solubilization, at which time fluorescence yield in the presence of heparin tended to be greater than with IAPP alone, although this was not significant (control: 0.69 ± 0.08; 41 IU/ml heparin: 1.61 ± 0.42; 410 IU/ml heparin: 1.60 ± 0.24, p = ns).

The effects of heparin on cell toxicity were immediate (Figure 20B), prior to fibril formation as assessed by thioflavin T fluorescence (Figure 20A). In wells treated immediately following IAPP solubilization, heparin significantly increased toxicity at both concentrations (control, 83.6 ± 7.5% control viability; 41 IU/ml heparin, 45.4 ± 1.4%; 410 IU/ml heparin, 43.4 ± 0.8%; p<0.01). The same trend persisted even as heparin promoted the rapid upstroke of fibril formation over the first hour. By 2 hours following IAPP solubilization, there was no significant difference between any of the groups consistent with the heparin-IAPP thioflavin curves reaching a steady-state plateau (p = 0.30). By 6 hours post-solubilization, heparin-treated IAPP was significantly less toxic than IAPP alone (control, 85.1 ± 5.4%; 41 IU/ml heparin, 109.4 ± 1.2%, p< 0.01 compared to control; 410 IU/ml heparin, 101.4 ± 1.4%, p < 0.05 compared to control).
Figure 20. Heparin accelerates the onset and disappearance of IAPP toxicity.

The effect of heparin on human IAPP fibril formation and associated toxicity was investigated as described in the experimental design shown in Figure 19. Fibril formation was monitored by thioflavin T fluorescence (A). Aliquots were removed immediately following solubilization of IAPP in cell media and addition of heparin. Aliquots were incubated with 10 mM thioflavin T at 37°C to monitor fibril formation. Toxicity to INS-1 cells was monitored by alamarBlue® reduction assay (B). Cell media was replaced with fresh media containing 10% alamarBlue® at 9 hours following incubation with IAPP and alamarBlue® fluorescence measured 4 hours later. IAPP (40 μM) was incubated without (black) or with heparin (purple: 41 IU/ml; blue: 410 IU/ml).
4.3.3 **Heparin promotes islet amyloid deposition in cultured human islets**

To determine the effects of heparin on IAPP deposition in human islets, we cultured human islets in 11.1 mM glucose with or without heparin (41 IU/ml and 410 IU/ml; Figure 21). After two weeks, islets were fixed and prepared for histology. The proportion of islet area staining for amyloid was significantly increased in heparin-pretreated islets (control, 0.17 ± 0.03%; heparin 410 IU/ml, 0.52 ± 0.11%; p <0.05). The proportion of islets containing amyloid, or amyloid prevalence, was significantly increased by both concentrations of heparin (control, 45.6 ± 4.6%; heparin 41 IU/ml, 65.0 ± 4.4%, p<0.05; heparin 410 IU/ml, 70.1 ± 4.5%; p<0.01).
Figure 21. Heparin enhances amyloid deposition in cultured human islets.

Representative islets are shown in (A). Islets were immunostained for insulin (red) and stained for amyloid with thioflavin S (cyan). Islet amyloid load, or the proportion of an individual islet staining positive for amyloid, without (white) or with heparin (purple: 41 IU/ml; blue: 410 IU/ml) is shown in (B). Amyloid prevalence, or the proportion of total islets staining positively for amyloid, is shown in (C). Data represent the mean of 15 islet culture experiments. *p<0.05; ** p< 0.01. Space bar = 100 μm.
To confirm heparin-induced amyloid deposition in cultured islets by a non-histological method, we sought to adapt an assay developed for the detection of amyloid β deposits in insoluble deposits in the brains of Alzheimer’s disease (632). The underlying principle of the assay is that amyloid fibrils are insoluble but may be solubilized in formic acid. We treated human islets in parallel with islet culture experiments performed for histology (Figure 22) under identical conditions. After 2 weeks of culture, islets were washed and subjected to a NP40 lysis procedure. Islets were ultracentrifuged to separate soluble and insoluble (containing amyloid and IAPP aggregates) fractions. Following ultracentrifugation, the insoluble pellet was solubilized in formic acid and sonicated; it was subsequently neutralized and diluted. We determined levels of IAPP in the insoluble fraction by ELISA. Levels of IAPP were normalized to total protein in both the soluble and insoluble fractions. Amyloid load determined from this assay significantly correlated with that evaluated by thioflavin S (p < 0.01; Figure 22B), validating this assay. As shown in Figure 22C, heparin did not significantly increase IAPP deposition into the insoluble fraction (p = ns); however, a trend towards heparin-promoting amyloid deposition into the insoluble fraction was observed.
Figure 22. Heparin enhances amyloid deposition in cultured human islets as assessed by an ELISA-based assay.

The assay is outlined in (A). We validated this assay by comparison to matched histological (thioflavin S) analysis. Islets from 10 human donors were cultured in quadruplicate. Following 2 weeks of culture, islets were fixed for histology (duplicate per condition) or processed according to the assay outlined in (A) (duplicate per condition) and linear regression performed. IAPP content (fmol IAPP/µg total protein) is shown for human islets (n=12 donors) cultured in 11.1 mM glucose alone or with heparin (41 IU/ml, purple; 410 IU/ml, blue) for 2 weeks (C). No statistical significance was determined amongst groups, although there tended to be a difference between untreated and heparin (410 IU/ml)-treated islets.
4.3.4. Effect of heparin on apoptosis in cultured human islets

To determine the effect of heparin on apoptosis in cultured islets, we quantified apoptosis by histological analysis using two markers, TUNEL and cleaved caspase-3 (Figure 23). Surprisingly, despite increasing islet amyloid formation, heparin protected against islet cell apoptosis in a concentration-dependent manner, as assessed by both markers. Heparin (410 IU/ml) significantly decreased apoptosis compared to control islets as assessed by both TUNEL (control, 14.5 ± 1.5% versus heparin 410 IU/ml, 8.1 ± 2.0% TUNEL positive nuclei, p<0.05) and cleaved caspase-3 (control, 10.2 ± 1.6% versus heparin 410 IU/ml, 6.6 ± 1.2% cleaved caspase-positive islet area, p<0.05). To further investigate the anti-apoptotic effects of heparin, we assessed its protective effects in INS-1 cells exposed to various pro-apoptotic stimuli (Figure 24). Heparin (410 IU/ml) significantly protected against thapsigargin- (p < 0.05) and brefeldin A-induced apoptosis (p = 0.001), but had no effect on staurosporine- or etoposide-induced apoptosis. Heparin (41 IU/ml) protected against brefeldin-induced apoptosis (p < 0.05).
Figure 23. Heparin abrogates IAPP toxicity in cultured human islets as assessed by TUNEL and cleaved caspase-3 immunohistochemistry.

Representative islets stained for DAPI (blue) and TUNEL (red) are shown for (A) untreated and heparin-treated (B: 41 IU/ml; C: 410 IU/ml) conditions. * p<0.05 as determined by one-way ANOVA followed by Neuman-Keuls post-hoc test. Clc3, cleaved caspase-3. Scale bar = 100 μm.
Figure 24. Heparin protects against apoptosis in INS-1 β cells.

Cells were treated for 18 hours without heparin or with heparin (41 IU/ml, blue; 410 IU/ml heparin, purple) in the presence of pro-apoptotic stimuli: thapsigargin (0.1 μM, A), etoposide (0.5 mM, B), brefeldin A (10 μM, C) and staurosporine (1 μM, D). Each condition was performed in triplicate. Viability was assessed by Alamar Blue fluorescence assay. *, p<0.05, ** p < 0.01.

4.3.5 Functional evaluation of the effects of heparin pre-treatment on human islets in a transplantation model

To evaluate the function of human islets after culture with or without heparin, we transplanted islets into STZ-diabetic, immunodeficient (NOD.scid) mice (633-636). In vivo dosing with heparin for an extended period of time was not feasible due to the heightened risk of bleeding and likelihood of morbidity in mice. Therefore, we pre-cultured human islets in 11.1 mM glucose for 1 week prior to transplantation. The experimental model is illustrated in Figure 25.

Islets were treated with or without heparin (410 IU/ml). Due to limited human islet availability and the limited number of transplantations that could be performed on a given day, we chose to
use a single concentration of heparin. We chose the higher concentration of heparin employed in our previous studies for maximal effect of heparin on amyloid formation. Islets treated in parallel were fixed after 1 week of transplantation to evaluate the effects of heparin on amyloid formation and apoptosis prior to transplantation. We transplanted a suboptimal number of islets per diabetic recipient (200 islets) so as to exacerbate metabolic stress on islets. Following transplantation, blood glucose was monitored daily in recipient mice for the first 2 weeks and every 2 days thereafter. Body weight was checked weekly. Graft normalization of blood glucose was defined as three consecutive blood glucose values <15 mM. Mice were sacrificed after 4 consecutive glucoses > 20 mM in the first 4 days, or after 2 consecutive blood glucoses > 20 mM thereafter. Experiment duration was thirty days so as to minimize the chance of recovery of endogenous β cell mass following STZ treatment in diabetic recipients. Survival nephrectomy, in which the graft-containing kidney is removed, was performed on mice remaining normoglycemic after 30 days to confirm that this had not occurred. All mice became diabetic after survival nephrectomy, demonstrating that endogenous pancreatic β cells had not regenerated following STZ treatment.
Figure 25. Experimental design for islet transplantation experiments following pre-transplant culture with heparin.

Human islets (n=4 human islet donors) were cultured for 7 days without heparin or with heparin (410 IU/ml) in 11.1 mM glucose. At the end of the islet culture period, islets were either fixed and processed for histology or transplanted into STZ-diabetic NOD.scid mice. Transplanted mice were sacrificed following 2 consecutive blood glucose values > 20 mM. If a recipient remained normoglycemic at 28 days following transplantation, the graft was removed by survival nephrectomy. Following survival nephrectomy, mice were sacrificed following 2 consecutive blood glucose values > 20 mM.

We performed pre-transplant analysis of islets fixed at the time of transplantation (Figure 26A). At this time, heparin tended to increase thioflavin S-positive area and amyloid prevalence but this was not statistically significant. Apoptosis at time of transplantation was assessed by TUNEL and cleaved caspase-3 staining. There was no significant change in apoptosis induced by heparin treatment when assessed by TUNEL or cleaved caspase-3 (Figures 26B and C).
Figure 26. Histology of human islets after 1 week in culture in the presence of heparin.

Islets from 4 human donors were cultured for 1 week without or with heparin (410 IU/ml) in 11.1 mM glucose and then transplanted or fixed for histological assessment of amyloid and apoptosis. Amyloid was quantified with thioflavin S (ThioS) staining (A); apoptosis was assessed by TUNEL (B) and cleaved caspase-3 staining (C). No significant differences were found, although amyloid area tended to be higher and apoptosis tended to be lower in heparin-treated islets. ClC3, cleaved-caspase-3.
Following transplantation, the proportion of recipients of heparin pre-treated islets in which blood glucose was normalized was significantly lower than recipients of untreated islets (untreated, 18.8 ± 12.0% versus heparin pre-treated, 64.5 ± 14.6%, p<0.05; Figure 27).

![Graph showing the percentage of grafts that failed to normalize with and without heparin treatment.](image)

**Figure 27. Pre-transplant culture of human islets in heparin promotes graft failure.**

Two hundred human islets per well were cultured for 1 week prior to transplantation without heparin or with heparin (410 IU/ml). After 1 week in culture, islets were transplanted into STZ-diabetic NOD.scid mice. The proportion of grafts which failed to normalize was significantly different between untreated (white) and heparin-pretreated (grey) human islets. Normal blood glucose was defined as three consecutive values <15 mM. Human islets were obtained from 4 human donors. This experiment was repeated with islets from 4 donors. *, p<0.05.
We retrieved grafts after 4 consecutive days of blood glucose > 20 mM immediately following transplantation, or thereafter following 2 consecutive days of glycemia > 20 mM, or at 30 days post-transplantation at the time of nephrectomy. Amyloid load within the graft tended to be increased in heparin pre-treated islets (control, 0.30 ± 0.13%; heparin pretreated, 0.64 ± 0.23%; p= ns; Figure 28A), although this was not statistically significant. We quantified cell apoptosis within the graft area by TUNEL and cleaved caspase-3 staining. There was no significant difference in the proportion of TUNEL-positive cells (Figure 4-28B) or in cleaved caspase-3 area within the grafts (Figure 28C) between the untreated and heparin-treated groups.

Figure 28. Graft histology following transplantation of human islets pre-cultured with heparin.

Grafts were removed 4 weeks following transplantation by survival nephrectomy or following two subsequent days on which random plasma glucose measured > 20 mM. Grafts were analyzed for amyloid load (A) and apoptosis by TUNEL (B) and cleaved caspase-3 (C). ThioS, thioflavin S; ClC3, cleaved caspase-3.

To replicate in an islet culture model what occurs within grafts following the removal of heparin we cultured human islets for 3 weeks in media alone, media containing heparin (410 IU/ml), or media containing heparin (410 IU/ml) for 1 week followed by 2 weeks in media alone (Figure 29). Apoptosis, as assessed by TUNEL, was significantly reduced in islets treated for 3 weeks with heparin as compared to control islets (control, 15.3 ± 2.6% versus 3 week heparin, 4.3 ± 0.8%; p=0.01). There was no difference between control islets and islets cultured for only 1 week in heparin, suggesting that short-term treatment of islets with heparin has an anti-apoptotic effect only while heparin is present and that this effect is not sustained following removal of heparin.
**Figure 29.** Removal of heparin following 1 week of culture does not affect amyloid formation but does increase islet cell apoptosis.

To attempt to mimic preculture of islets in heparin and subsequent transplantation, human islets were cultured for 3 weeks without heparin, with heparin (410 IU/ml) for the first week only, or with heparin (410 IU/ml) for 3 weeks. Islets were fixed for histology. Analysis of (A) amyloid load (thioflavin S staining) and (B) TUNEL is shown. These experiments were repeated in 3 sets of human islets.

### 4.4 Discussion

In this chapter, we examined the effects of heparin on islet amyloid fibrillogenicity and toxicity in several models. We showed that heparin promotes amyloid formation by synthetic human IAPP. Correspondingly, heparin also exacerbated IAPP toxicity to cultured cells. Furthermore, heparin accelerated both the onset and disappearance of toxicity. Heparin promoted amyloid deposition in cultured human islets and exposure to heparin promoted graft failure following transplantation.

Inhibition of IBMIR is critical to successful islet engraftment. IBMIR is thought to be an important cause of islet loss during the early post-transplantation period (149), during which time up to 75% of transplanted islets are lost (254). Strategies proposed to prevent IBMIR include transplantation of freshly isolated islets (245), islet surface modification (637), depletion of platelets prior to transplantation, and treatment with Resolvin E1 (638), thrombomodulin (156), melgatran (149), active-site inactivated FVIIa (149), nicotinamide (639), activated protein C (640), tyroban (640), or anti-TF antibodies (148). Heparin is the currently employed...
pharmaceutical agent to minimize IBMIR. It has been shown to be protective in animal models (641). Indeed, heparin may have direct anti-apoptotic (642, 643) and anti-inflammatory (644) effects on cells in addition to its anti-coagulant properties. Heparin (153, 311) or its low molecular weight analogue, enoxaparin (252, 311), has been administered up to 7 days following islet transplantation to minimize the risk of portal vein thrombosis, which may lead to areas of focal ischemia (285) and necrosis in the liver (254). In human islet transplant recipients, the infusion of heparin and insulin during the peri-transplant period promotes insulin independence (311), although the authors did not consider the effects of heparin and insulin separately and the protective effect may thus have been mediated by insulin treatment. Furthermore, while the combined heparin-insulin infusion promoted islet function and prevented islet loss following transplantation, 80% of these grafts still failed by 2 years following islet transplantation. These grafts failed more slowly than untreated grafts, but this seems likely due to the substantially lower graft mass and rate of engraftment in untreated islets. A lower starting graft mass ultimately predisposes grafts to earlier dysfunction in the face of metabolic stress.

Heparin seeds amyloid formation of multiple pro-amyloidogenic proteins (613-618), including both proIAPP (561) and IAPP (611, 612, 619). We demonstrated that heparin decreases the lag phase of thioflavin T amyloid kinetic formation, which is likely due to acceleration of formation of prefibrillar structures. In buffer, the highest concentration of heparin promoted a slight but significant increase in thioflavin fluorescence during the lag phase, consistent with previous observations that thioflavin T fluorescence is slightly increased upon binding prefibrillar aggregates with β-sheet structure (629-631). In support of this hypothesis, heparin promotes dimerization of amyloid precursor protein (645), the precursor to amyloid-β, and has been proposed to promote formation of multimeric oligomers by light chain immunoglobulins (646). Heparin increased the slope of the rapid growth phase (12) of fibril formation. Heparin acts as a scaffold for amyloid formation (621, 646-648), by way of stabilizing β-sheet structure (647, 649). The highly negatively charged sulphate groups of heparin promote β sheet formation within IAPP by interaction with the positively charged arginine at residue 11, as heparin promotes fibrillization of IAPP (11-37) but has no effect upon fibril formation of IAPP (12-37) (622).
In TEM images of IAPP incubated in the presence of heparin, heparin increased the final amyloid fibril load. Amyloid load tended to be increased in the presence of heparin as assessed by thioflavin T fluorescence when IAPP was dissolved in cell media, although significance was not reached. These findings are consistent with previously reported findings that heparin increased IAPP fibril density and fibril compaction (619). IAPP aggregation kinetics in Tris buffer do not support this conclusion due to a declining thioflavin fluorescence of IAPP in the presence of heparin following a maximal. When IAPP was dissolved in Tris buffer, heparin significantly increased the intensity of thioflavin fluorescence above that of hIAPP alone; however, after 2 hours, fluorescence did not differ between the groups. Interestingly, the same trend was observed for thioflavin kinetics of proIAPP in the presence of another glycosaminoglycan, dermatan sulphate (650). This phenomenon was not observed when thioflavin kinetics were performed in cell media. Furthermore, TEM data show an increase in fibril load that becomes increasingly dense over 24 hours. TEM has been demonstrated to be more reliable than thioflavin probes for assessing the fibrillar states of amyloidogenic proteins (580, 651). One possible explanation is that as fibril compaction occurs in the presence of heparin (619), thioflavin T is excluded from binding to β sheets of internally compacted fibrils. Given that this phenomenon was only observed in Tris buffer, fibril structure or density may differ somewhat in cell media. The increased final fibril load in the presence of heparin observed in TEM images and the trend towards increased thioflavin fluorescence in cell media are consistent with an effect of heparin to stimulate amyloid formation in islet culture. We demonstrated that heparin increases amyloid severity and prevalence in cultured human islets. As heparin enhances IAPP amyloid formation in human islets, it is important to consider the effects of heparin and enhanced amyloid deposition on transplanted islets.

Heparin has been reported to have no effect (617) or a protective effect (617, 652-656) on the toxicity of other amyloidogenic proteins. Enoxaparin, a low molecular weight heparin derivative, reduces plaques and β amyloid accumulation in a mouse model of Alzheimer’s disease (657). As described above, heparin also promotes fibrillogenesis of these proteins. Since toxic pre-fibrillar species may mediate amyloid toxicity (96, 627, 658) rather than mature amyloid fibrils, rapid formation of amyloid in the presence of heparin may deplete cytotoxic prefibrillar species which otherwise would have mediated toxicity. Methylene blue (659) and ellagic acid (660) promote fibrillization of amyloid β and protect against amyloid-associated toxicity (660, 661). Methylene
blue also inhibits oligomer accumulation (659), consistent with depleting pre-fibrillar species by driving amyloid formation. We found that heparin strongly promoted IAPP toxicity in a cultured β cell line, in contrast to the effects of heparin on other amyloidogenic proteins.

Heparin dramatically promoted toxicity immediately following IAPP solubilization, in contrast to IAPP alone. The effects of heparin on toxicity occurred prior to fibrillization of IAPP, as monitored by thioflavin t kinetics, suggesting that heparin promotes IAPP toxicity by promoting the formation and stability of pre-fibrillar aggregates. Heparin-IAPP-mediated toxicity disappeared soon after thioflavin kinetics reached the steady state plateau at which point mature fibril meshworks are observed by electron microscopy. Consistent with the effects of ellagic acid on amyloid β (660), mature fibrils appeared not to promote amyloid-mediated toxicity. The rapid kinetics of IAPP cytotoxicity observed in the presence of heparin may be explained by the time course of IAPP aggregation, which is up to a hundred-fold faster than other amyloidogenic proteins. Rapid stabilization of prefibrillar species may allow these species to reach a threshold concentration for cytotoxicity, which is not reached upon slower aggregation. Alternately, heparin exacerbation of IAPP toxicity may be due to a particular structural interaction unique to these molecules.

The contrasting effect on IAPP cytotoxicity in the presence of heparin relative to other amyloidogenic species may be related to protein-specific interactions with heparin. Glycosaminoglycan (GAG)-interactions with amyloidogenic species depend on the spacing of negatively charged residues or the charge density of a specific GAG. For example, heparin and heparan sulphate interact with IAPP while the highly sulphated GAG chondroitin sulphate does not (487). Chondroitin does not promote amyloid formation by IAPP, does not exacerbate its toxicity, and is not found in amyloid plaques. Cells are protected against amyloid-β toxicity in the presence of heparin but amyloid-β toxicity is promoted in the presence of dextran sulphate, a GAG with a greater number of sulphate moieties per disaccharide (654). Interactions also depend upon primary sequence of the pro-amyloidogenic protein or peptide. We speculate the effects of heparin on IAPP toxicity are specific to its interactions with IAPP. IAPP species have also been proposed to exert their toxicity via death receptors (37). Cell surface levels of death receptors may vary between cell types or a subtype of receptor may be highly expressed in β cells relative to other cells. The structure of IAPP pre-fibrillar species may also be specific to interaction with
certain receptors such that the β sheet structure of other pro-amyloidogenic proteins would not stimulate receptor activity.

Surprisingly, we noted that heparin had a strong anti-apoptotic effect in human islet culture. This effect was noted after 2 weeks in culture but not following one week of culture. It is possible that heparin promotes an early wave of amyloid toxicity soon after its incubation with islets, as we measured apoptosis after 2 weeks in culture. In early islet culture, islets are more viable and likely secreting much more insulin and IAPP. Prolonged human islet culture induces pro-apoptotic signaling in islets and by two weeks, β cell replication is decreased and apoptosis increased (229). Furthermore, maintenance of human-derived β cell lines in culture leads to rapid de-differentiation and decreased insulin secretion (229). If IAPP secretion is greatly decreased by several weeks in culture, other direct (non-amyloid)-related effects of heparin may decrease islet cell apoptosis, masking any deleterious effects of IAPP.

A more likely explanation for the anti-apoptotic effects of heparin in human islets despite increased amyloid deposition is that the simultaneous activation of anti-apoptotic signaling counteracts the toxic effects of islet amyloid. This phenomenon has been demonstrated using exendin-4 in hIAPPTg0 islets; exendin-4 promoted extracellular amyloid deposition by increasing hIAPP release but protected against its toxicity by activating of anti-apoptotic AKT signaling (662). Stimulation of IAPP secretion by an alternate mechanism without anti-apoptotic properties leads to increased amyloid deposition and increased apoptosis within the islet (662). There are many reports of heparin’s anti-apoptotic (642, 643) and anti-inflammatory (311) properties in addition to its primary role as an anticoagulant. Indeed, we demonstrated a protective effect of heparin against several pro-apoptotic stimuli. One plausible mechanism underlying heparin anti-apoptotic signaling in cultured human islets is by activation of the epidermal growth factor (EGF) receptor (EGFR). EGRF signaling is critical to normal β cell function (663-666). Heparin-binding EGF (HB-EGF), over-expressed in damaged or diseased epithelial tissues (423) and produced by the islet (664, 667, 668), can activate the EGFR in the presence of heparin (669) to promote anti-apoptotic signaling.

We used an in vivo transplantation model to evaluate the function of islets following culture with heparin. We did not treat graft recipients with heparin due to the risk of morbidity at doses of
heparin required to promote sufficient amyloid deposition to detect by thioflavin S fluorescence. This model was not designed to replicate perfectly conditions in human islet transplantation, but rather to determine the effects of heparin-induced amyloid formation on subsequent islet function. Short-term incubation of islets with heparin does, however, mimic peri-transplant exposure to heparin and heparin dosing up to 7 days following transplantation in human islet transplantation. We were limited by human islet availability, which restricted the number of transplant experiments that could be performed. Based upon these limitations, we opted to use the highest dose of heparin as it induced the greatest amount of amyloid deposition. Thioflavin S is relatively insensitive to amyloid formation relative to electron microscopy, but the latter cannot be reliably quantified over large areas. Amyloid formation was significantly greater in the presence of heparin by the end of two weeks culture, but was not significant by 1 week. This finding may be due to the relative insensitivity of thioflavin S and illustrates the limitations of performing amyloid quantification in tissue. Thioflavin S is a less sensitive means of detecting amyloid fibrils in tissue as compared to TEM, but whole islet amyloid quantification is not feasible by TEM. After 2 weeks in culture, heparin-treated islets have a significant increase in thioflavin S-positive amyloid; one week of culture likely does not promote sufficient amyloid deposition to achieve a significant difference using an insensitive probe. In the amyloid-beta field, many antibodies exist for the detection of various monomeric, prefibrillar, and fibrillar states of amyloid-beta; this is not the case for the IAPP, which is limited to thioflavin or Congo red dyes for histological amyloid quantification (12).

Following transplantation, islets pre-treated with heparin failed significantly faster than control islets. Prior to transplantation, heparin-treated islets had a four-fold increase in amyloid load as compared to untreated islets, although this trend was not statistically significant. Amyloid load in grafts also tended to be increased, although not significantly. As amyloid is toxic to β cells, the majority of amyloid-laden islets may have degenerated due to extensive β cell apoptosis following transplantation. As amyloid fibrils may stimulate innate immune reactions (40) and IAPP amyloid fibrils may be internalized by macrophages (670, 671), some amyloid may have been cleared by infiltrating macrophages following transplantation. Amyloid plaques may also have dis-aggregated following the removal of heparin (672). There was no significant difference in apoptosis within the graft between the untreated and heparin-treated groups. Because the rapid clearance of apoptotic cells are rapidly cleared by macrophages in vivo, the number of apoptotic
cells that can be counted in transplanted islets at any one time is very low, making it difficult to observed differences in islet cell apoptosis between groups.

Our islet transplantation studies suggest that heparin likely has an amyloid-related detrimental function on human islets \textit{in vivo}. This finding is in agreement with our cell toxicity data that showed that heparin enhanced IAPP-mediated aggregation and toxicity, but not with our finding that the proportion of apoptotic cells was not different in heparin-treated islets following 2 weeks culture. Cell toxicity data using synthetic IAPP and beta cell lines should be interpreted cautiously, as supraphysiological concentrations of IAPP are likely used, beta cell lines are imperfect models of primary beta cells, and other \textit{in vivo} components of amyloid plaques are not present. Toxicity may occur at lower IAPP concentrations and may involved different mechanisms in a physiological setting. The lack of apoptosis after 2 weeks in heparin-treated cultured islets may be unrelated to amyloid; it is conceivable that we missed an early wave of heparin-induced toxicity earlier in the islet culture period.

If heparin truly decreased toxicity while promoting amyloid deposition in cultured human islets, as seen with ellagic acid and amyloid β (660) or with exendin-4 and IAPP (662), several mechanisms may account for the progressive loss of graft function observed in transplanted islets following achievement of normoglycemia. First, heparin may deplete toxic oligomeric species, as it stimulates amyloid fibril formation. Removal of heparin would thus allow the formation of more toxic prefibrillar species in transplanted islets. Second, heparin may compete with native GAGs, namely heparan sulphate, for proIAPP or IAPP binding. Heparan sulphate is thought to interact with proIAPP to seed amyloid formation and toxicity of prefibrillar species in the vicinity of beta cells. The presence of heparin might competitively inhibit GAG binding to IAPP species or sequester IAPP into amyloid fibrils to decrease IAPP-mediated toxicity. Removal of heparin would remove this competitive inhibition. By either of these mechanisms, it could be argued that islet apoptosis would merely return to similar levels in the control islets. However, preincubation with heparin increases islet amyloid load. Heparin inhibits amyloid depolymerization (672); the removal of heparin likely destabilizes plaques (672) to promote greater localized concentrations of toxic prefibrillar IAPP species in heparin-pretreated islets than in untreated islets. Finally, it is also conceivable that graft failure following heparin pre-treatment
may be related to loss of islet secretory function rather than beta cell apoptosis or alternatively due to the removal of the strong anti-apoptotic effect of heparin.

We conclude that due to its pro-amyloidogenic effects on IAPP, caution should be taken in the use of heparin in islet transplantation. While the results of this work certainly do not provide sufficient evidence to preclude its use, they provide evidence that pre-transplant exposure of human islets to heparin may seed amyloid formation, which may in turn have a detrimental effect on beta cell function and survival following transplantation. Alternate heparin-related compounds, such as dextran sulphate (334), are also effective in preventing IBMIR. Dextran, in particular, should be investigated for its pro-amyloidogenic effects on IAPP prior to use, as it promotes fibrillogenesis and toxicity of other pro-amyloidogenic proteins (654). Islet surface heparinization (149), currently in clinical trials for human islet transplantation, should also be subject to further studies prior to clinical implementation; the authors of this study showed viability and post-transplantation functional and survival data for heparin-treated rodent, but not human, islets. The authors also demonstrated that islet surfaces that became heparinised included the extensive fenestrated capillary network throughout the islet and mentioned that heparinization of arterial vessels led to subendothelial deposition of heparin (149), which might seed amyloid formation by IAPP. Acute suppression of IAPP amyloid formation (2, 594) or silencing of IAPP expression (3) may be effective strategies to minimize the effects of heparin employed according to current clinical protocols on IAPP fibrillogenesis and toxicity should models mimicking exact clinical conditions substantiate our findings. Should xenotransplantation become a clinical reality, transplantation of islets from species expressing non-amyloidogenic IAPP (466) might obviate this problem, as discussed in Chapter 5.
Chapter 5. Porcine IAPP is minimally fibrillogenic and porcine islet transplants have excellent long-term outcomes

5.1 Introduction
Organ transplantation, on the whole, is highly successful. It improves quality of life, and, for many whole organ transplant recipients, prolongs survival when medical therapy has reached its limits. Islet transplantation has been shown in many studies to improve metabolic control (509, 673-675) and quality of life (550, 551, 673, 674, 676-679) in patients with long-standing or brittle type 1 diabetes. Islet transplantation decreases the frequency of hypoglycemic events (673, 678), which cause significant reduction in quality of life. It also slows the progression of the severe complications of diabetes, including retinopathy and renal disease (115, 680). Despite limited long-term maintenance of insulin independence (129), islet transplantation dramatically reduces insulin dose (128, 681) and slows the progression of microvascular complications (680). An economic analysis of islet transplantation showed it to be cost effective relative to standard medical therapy due to its effects on slowing complications (682).

A limiting factor in islet transplantation remains organ supply and quality. Organ supply is highly limited for most transplantation procedures (213). Supply is particularly limited for islet transplantation as organs are preferentially allocated to pancreas transplantation (145, 147, 683). Furthermore, to maintain long-term insulin independence, a single islet transplant recipient requires islets from multiple donors (684-686). Higher quality organs are allocated for whole pancreas transplants (145-147, 683, 687). A higher quality, more widely available source of islets would greatly increase islet transplantation availability and reduce the supply burden.

Xenotransplantation of pancreatic islets, using pigs or other animals as islet donors, has received increasing interest in recent years, given the limited number of human islets available for clinical transplantation. Pigs are ideal donors, as they may be easily replenished and have similar sized islets to humans. They also lack the degree of ethical constraints as animals kept as pets or of similar speciation to humans. Many properties of porcine islet support the use of pigs as donors in islet xenotransplantation. Neonatal porcine islets show particular promise, as they are easily isolated, are highly resistant to hypoxia and hyperglycemia (688, 689), and have shown remarkable ability to expand their β cell mass following transplantation (690). Notably, porcine islets have been shown to maintain long-term function following intraportal transplantation into
non-human primates with production of detectable porcine C-peptide and restoration of normoglycemia (691-695). In a limited number of case reports, xenotransplantation of porcine islets into diabetic humans has improved glycemic control (696).

Given that expression of the pro-amyloidogenic human IAPP is detrimental to graft function and survival (466, 490), it is critical to consider the amyloidogenic potential of porcine IAPP prior to clinical trials for porcine islet transplantation. There is wide variation amongst species in the fibrillogenicity of IAPP (453, 697-704). The region of human IAPP containing residues 20 through 29 is a key determinant of its ability to form amyloid (705). Human (699) and primate IAPP (701, 703) form amyloid fibrils, are cytotoxic, and form islet amyloid deposits in type 2 diabetes pathogenesis (699). The various forms of rodent IAPP (mouse (704), rat (699), and hamster (698)), unlike human IAPP, do not form amyloid fibrils (698) or pre-fibrillar aggregates (706, 707), and are not cytotoxic (33, 469, 708, 709). The critical difference between human and rodent IAPP is the presence of three proline substitutions at residues 25, 28, and 29 within the mature peptide. Proline is well known to disrupt β-structure and is not compatible with the cross β-structure of amyloid. Cat IAPP incorporates the GAILS sequence although it has a single proline substitution within the 20-29 region; cat IAPP forms amyloid deposits consistent with the development of type 2 diabetes in cats (699). Dog IAPP also contains the GAILS sequence but only forms amyloid fibrils associated with the rare occurrence of canine insulinomas (700). Additional variations amongst species in amino acids 8-20 and 30-37 likely also affect the amyloidogenicity of different species’ forms of IAPP (460, 462, 463, 710). The full sequence of porcine IAPP has not been determined. In this study, we characterized the sequence of mature porcine IAPP (1-37) and examined its fibrillogenicity, cytotoxicity, and whether it formed amyloid in transplanted islets.

5.2. Results
5.2.1. Detection of porcine IAPP in neonatal porcine islets
IAPP immunoreactivity has been reported in fetal and adult porcine β cells (711). To confirm the presence of mature IAPP in porcine islets, we performed western blots with neonatal porcine islet lysates (Figure 30). INS-1 cell lysate was used as a positive control. Porcine IAPP had relatively weak affinity for the primary antibody to IAPP (#7323) used for detection.
Nevertheless, we detected both proIAPP and mature (1-37) IAPP in neonatal porcine islet lysates. This result confirmed that mature porcine IAPP was present in porcine islets.

<table>
<thead>
<tr>
<th>Source</th>
<th>INS-1 cells</th>
<th>Neonatal porcine islets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein loaded (µg)</td>
<td>10.0</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>25.0</td>
<td>50.0</td>
</tr>
</tbody>
</table>

![Western blot image showing bands at 8.2 kDa, 6.0 kDa, and 3.7 kDa](image)

**Figure 30.** Proislet amyloid polypeptide and mature islet amyloid polypeptide are detectable in lysates of neonatal porcine islets.

IAPP immunoreactivity in lysates from control INS-1 cells and neonatal porcine islets was detected by western blot on a tris-tricine gel. ProIAPP (8.2 kDa), intermediate proIAPP (6.0 kDa) and mature IAPP (3.7 kDa) were detectable in control INS-1 cells. ProIAPP and mature IAPP were also detectable in neonatal porcine islet lysate.

**5.2.2. Determination of the sequence of porcine (1-37) IAPP**

To determine whether differences in the primary structure of the porcine polypeptide might explain the lack of amyloid formation in transplanted pig islets, we cloned and sequenced a porcine IAPP cDNA reverse transcribed using primers based on an EST sequence (BF712755) for porcine hypothalamus and RNA extracted from neonatal porcine islets. The sequence of mature porcine IAPP predicts a 37 amino-acid peptide (Figure 31) that is well-conserved in the N- and C-terminal regions of the molecule, but has unique amino acid differences in the mid-portion. The predicted sequence matches partial sequences (3-34) that are deposited in SwissProt (Q29119) and GenBank (AAB05919.1). Comparison of the porcine and human sequences reveal amino acid differences in ten positions, five of which reside within the 20-29 region thought to be critical for amyloid formation. Noteworthy differences include the substitution of a positively charged arginine at position 20 and a proline at position 29. Overall, porcine IAPP contains six residues that are potentially charged at neutral pH while human IAPP contains only three. These sequence differences are predicted to reduce the ability of the peptide to form amyloid as they
increase the net charge and reduce the net hydrophobicity (712), raising the question as to whether porcine IAPP is capable of forming amyloid.

![Image]

A) hIAPP: \(+\text{H}_2\text{N}\)-KCNTATCAT QRLANFLVHS SNNFGAILSS TNVGSNTY-CONH\(_2\)

B) pIAPP: \(+\text{H}_2\text{N}\)-KCNMATCAT QHLANFLDRS RNLGTIFSP TKVGSNTY-CONH\(_2\)

**Figure 31. Primary sequence of human and porcine islet amyloid polypeptide.**

Human IAPP (hIAPP) sequence (A). Porcine IAPP (pIAPP) sequence (B). All peptides have an amidated C-terminus and free N-terminus with a Cys-2 to Cys-7 disulfide-bridge. Residues are numbered according to their position in mature IAPP. Residues differing from the human sequence are indicated in bold.

### 5.2.3. Porcine IAPP is minimally fibrillogenic

We investigated the ability of porcine IAPP to aggregate relative to human and rat IAPP. Thioflavin T binds amyloid fibrils formed by aggregation of IAPP and is a useful probe to assess the time course of IAPP aggregation (12). The experimental curve observed for human IAPP aggregation displayed the characteristic features expected for kinetics of amyloid formation (Figure 32A). Following solubilization, human IAPP exhibited a short lag phase in which no significant change in thioflavin T fluorescence was detected, followed by a rapid rise in fluorescence leading to a plateau phase, as previously described in Chapter 4. No change above baseline fluorescence was observed for rat IAPP or porcine IAPP (both 20 \(\mu\)M) over 14 hours. By this method, we concluded that porcine IAPP did not form amyloid fibrils, even at the supraphysiological concentrations tested.

Amyloid fibrils are rich in \(\beta\)-sheet structure and therefore UV circular dichroism (CD) offers another convenient probe of amyloid formation. CD spectra taken of aliquots removed at the end of thioflavin T kinetic studies confirm that human and porcine IAPP have very different tendencies to form \(\beta\)-sheet structure (Figures 32B and C, respectively). The CD spectrum of human IAPP exhibits a strong minimum at 218 nm, a band associated with \(\beta\)-structure that is typical of the CD spectrum of IAPP-derived amyloid. In contrast, the CD spectrum of porcine IAPP displays a wide band ranging from 208 to 222 nm, indicative of a mixture of helical,
random and β-sheet structures. CD spectra were recorded on independently prepared samples of porcine IAPP on different days and similar results obtained.

Neither CD nor thioflavin T fluorescence provides direct information about the morphology of any aggregates formed. Consequently, transmission electron microscopy (TEM) was used to image the reaction products. TEM micrographs of human IAPP revealed dense clusters of rod-like fibrils with classic amyloid morphology (Figure 32B). These images are typical of those previously reported for human IAPP under similar conditions (460). A few long fibril-like structures were observed after incubation of 32 µM porcine IAPP for 24 h, but these fibrils were much fewer in number and appeared dispersed among a mat of smaller amorphous aggregates. Three independently prepared samples of porcine IAPP were imaged and similar TEMs obtained each time. The images displayed in Figure 32C confirm that porcine IAPP is significantly less amyloidogenic than its human counterpart.
Figure 32. Porcine islet amyloid polypeptide is less fibrillogenic than human islet amyloid polypeptide.

Thioflavin T kinetics at 37°C are shown for human, rat and porcine IAPP (all 20 µM) (A). Data are the mean of three replicates. Transmission electron micrographs are shown for human IAPP (B) and porcine IAPP (C). After 24 hours of incubation, human IAPP samples appear as dense mattes of intertwined amyloid fibrils with the classic linear and unbranched morphology. Porcine IAPP samples incubated for the same length of time show the presence of a few fibrillar strands among a bed of amorphous aggregates. TEM samples were incubated at pH 7.4 in 2% HFIP and 25 °C and peptide concentration was 32 µM. Scale bars represent 100 nm. Far UV CD spectra of human and porcine IAPP is shown in (D). Conformational analysis of human (black circles) and porcine (white circles) IAPP after 12 hour incubation indicates that porcine IAPP has significantly less β-sheet propensity than the human peptide. Human IAPP adopts a classic β-sheet conformation signified by a signal minima at 218 nm and positive ellipticity below 200 nm while porcine IAPP shows a wide band ranging from 208 to 222 nm, indicative of a mixture of helical and β-sheet structures. All reactions were monitored at pH 7.4 in 2% HFIP and 25 °C. Peptide concentration was 32 µM. Spectra are the mean of 3 repeats.
5.2.4. Differences in primary sequence of porcine and human IAPP explain species differences in IAPP cytotoxicity

Given that synthetic porcine IAPP is much less amyloidogenic than human IAPP, we predicted that it would also be less toxic. The effect of porcine IAPP on β cell toxicity was next assessed in transformed (INS-1) β cells by Alamar blue reduction (Figure 33B). As expected, addition of human IAPP (40 μM) substantially reduced INS-1 cell viability after 24 hours (12 ± 5% viability) and further increases in IAPP concentration virtually eliminated any viable cells. In contrast, at the same concentration of porcine IAPP, INS-1 cell viability was significantly greater (93 ± 2%, p < 0.001). To confirm the reduced toxicity of porcine compared to human IAPP, we assessed the induction of apoptosis in INS-1 cells by TUNEL staining after 16 hours of incubation of cells with either peptide (Figure 33A). In the presence of 40 μM human IAPP, the proportion of apoptotic β cells was 44 ± 10% compared to only 6 ± 1% (p < 0.001) following incubation in the presence of porcine IAPP. Even in the presence of a higher concentration of IAPP (100 μM), the proportion of TUNEL-positive β cells was still much higher when cells were exposed to human (90 ± 5%) compared to porcine IAPP (14 ± 3%; p < .001). We confirmed these results with porcine IAPP synthesized by two independent sources. Thus, porcine IAPP is much less fibrillogenic and cytotoxic than its human counterpart.

Figure 33. Porcine islet amyloid polypeptide is less cytotoxic than human islet amyloid polypeptide.

TUNEL staining following 16 hours incubation of INS-1 cells in presence of human or porcine IAPP (A). *Denotes statistically significant difference from porcine IAPP-treated cells (p<0.05). Alamar blue viability assay following 24 hours incubation of INS-1 β cells in presence of human
or porcine IAPP (B). The EC50 for human IAPP was 27 µM compared to 172 µM for porcine IAPP (p<0.05). Solid line: human IAPP; dashed line: porcine IAPP.

5.2.5. Porcine islet grafts do not develop amyloid deposits
Islet amyloid forms rapidly in human islets transplanted into immune-deficient, diabetic murine recipients (14). In a previous chapter, we characterized an association between amyloid deposition and graft failure. We sought to confirm the minimal fibrillogenicity of porcine IAPP and the lack of cytotoxicity with lack of graft failure upon transplantation into diabetic, immune-deficient mice. Adult porcine grafts maintained normoglycemia for up to 195 days post-transplantation, with the exception of one recipient out of six studied (Table 2). This recipient was borderline diabetic at 34 days post-transplantation and never achieved normoglycemia. Neonatal porcine islet grafts were unable to correct diabetes immediately after transplantation but eventually established and maintained euglycemia as transplanted β cells were supplemented by the generation of new β cells within the graft (690) (Table 3). Upon restoration of normoglycemia, neonatal porcine islet grafts consistently maintained glycemic control until time of sacrifice between 4 weeks and 3 months post-transplantation. Transplanted neonatal (n = 10) and adult (n = 6) porcine islets failed to stain positively for the presence of detectable amyloid by thioflavin S even after 12 weeks post-transplantation (Figures 34C and D). Islet amyloid was also not detected in adult porcine pancreas (Figure 34E). Native and transplanted human islets containing thioflavin S-positive amyloid deposits are shown for comparison in Figures 34A and B, respectively.
Figure 34. Lack of amyloid in native or transplanted porcine islets.


Table 2. Blood glucose in murine recipients of adult porcine islet transplants.

<table>
<thead>
<tr>
<th>Experiment ID</th>
<th>Following streptozotocin</th>
<th>2 weeks Post-transplantation</th>
<th>4 weeks Post-transplantation</th>
<th>Final</th>
</tr>
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<tbody>
<tr>
<td>Mouse 1</td>
<td>25.6</td>
<td>13.7</td>
<td>14.2</td>
<td>12.2 (34 days)</td>
</tr>
<tr>
<td>Mouse 2</td>
<td>22.7</td>
<td>9.8</td>
<td>7.2</td>
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<td>5.6</td>
<td>4.8 (87 days)</td>
</tr>
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<td>6.6</td>
<td>4.5</td>
<td>7.7 (195 days)</td>
</tr>
<tr>
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<td>6.2</td>
<td>3.7</td>
<td>4.0 (113 days)</td>
</tr>
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<td>Mouse 6</td>
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<td>4.2</td>
<td>4.2</td>
<td>4.2 (25 days)</td>
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Table 3. Blood glucose in murine recipients of neonatal porcine islet transplants.

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<th>4 weeks Post-transplantation</th>
<th>8 weeks Post-transplantation</th>
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<td>17.1</td>
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<td>11.1</td>
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<td>11.1</td>
<td>6.3</td>
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5.3. Discussion

IAPP-derived amyloid deposits are hallmarks of islet pathology in type 2 diabetes. Whereas in type 2 diabetes islet amyloid formation takes months or years to develop, islet amyloid has been observed to form rapidly in cultured human islets (3), in human islets transplanted into diabetic murine recipients (552), and in islets from transgenic mice with β cell expression of human IAPP (490, 713). In addition, a recent case report described the presence of extensive amyloid formation in transplanted islets, engrafted into the liver obtained at autopsy of a diabetic subject (489). Taken together, these data support the hypothesis that amyloid deposition can play a significant role in reducing the viability of human islet grafts. Transplanted porcine islets, in contrast, formed no detectable amyloid following transplantation. Moreover, the sequence of porcine IAPP predicts a 37 amino acid peptide with substantial differences from human IAPP. Indeed, we found synthetic porcine IAPP to be much less fibrillogenic and less toxic to β cells than its human counterpart. These data are supported by a recent study with porcine IAPP fragments (714). We propose that the lower amyloidogenic potential and toxicity of porcine IAPP is likely to be a significant factor contributing to the better long-term survival and function of transplanted porcine islets in animal models, compared to human islets.

IAPP is found in the pancreatic β cells of most mammals; however, only those species in which an amyloidogenic sequence in the mid-portion of the IAPP molecule is conserved have a propensity to form toxic IAPP aggregates (715). Sequencing of the mature form of pig IAPP revealed that human and pig IAPP differ by 10 residues. Notably, porcine IAPP has a serine-for-proline substitution at residue 29, although feline IAPP, which is also amyloidogenic, also has a proline residue at position at 29. A notable difference between feline and porcine IAPP is that the
critical GAILS sequence is conserved in feline IAPP (699) while two substitutions in porcine IAPP alter this sequence to GTIFS. The A-to-T mutation replaces a hydrophobic amino acid with a polar amino acid. Amyloidogenic peptide sequences have a high proportion of hydrophobic residues (7). A 33% reduction in the hydrophobic resides thereby decreases the amyloidogenic potential of the sequence. The L-to-F substitution does not change the hydrophobicity of this residue but replaces a small side chain with the large aromatic side chain of tryptophan, and likely alters the amyloidogenic capacity of the sequence by steric effects. Other amino acid substitutions within porcine IAPP, including V17D, S20R and N31K, all of which involve the substitution of a neutral residue by a charged residue, are also likely to be important.

To consider evolutionary selection forces on the amyloidogenicity of IAPP, mammals that produce non-amyloidogenic forms of IAPP do not naturally develop type 2 diabetes (716). Although no papers specifically characterize the incidence of type 2 diabetes in rodents, transgenic over-expression, intensive pharmacological modulation, or selective inbreeding is required to generate models of type 2 diabetes; even so, these models may poorly mimic the extremes of the human disease phenotype. In the case of IAPP, development of transgenic models is especially difficult as rodent IAPP may inhibit amyloid formation by human IAPP (450, 464). A similar phenomenon has been observed for amyloid-β in rodent models, as rodents do not express a pro-amyloidogenic form amyloid-β. The optimized mouse model for Alzheimer’s disease is a triple transgenic mouse (717), expressing human isoforms of 2 additional genes in addition to human amyloid β. Humans, cats and primates all have pro-amyloidogenic IAPP sequences and develop type 2 diabetes (453, 699). The S20G mutation, which renders human IAPP more amyloidogenic and more toxic, is associated with earlier onset and increased severity of human type 2 diabetes (458, 459, 718). It is conceivable the underlying evolutionary driving force towards amyloidogenic IAPP in cats, humans and primates was adaptation towards improved stabilization of prohormones and increased processing efficiency in β cell secretory granules. This would be consistent with the presence of “functional” amyloid, formed by other endocrine hormones in the secretory pathway of their respective cells and possibly involved in prohormone trafficking (719). Nevertheless, the anti-amyloidogenic substitutions in porcine IAPP protect against its toxicity and may be associated with the lack of type 2 diabetes amongst pigs.
The lack of amyloidogenicity and toxicity of porcine IAPP support the use of pigs as islet xenograft donors. Given that the number of available islet donors is far exceeded by the number of type 1 diabetic patients who could benefit from islet transplantation (720), new sources of transplantable β cells are sorely needed. An ideal source of xeno-organs would have organs of similar size to humans, lack xenozoonosis, have low incidence of disease, and be easily bred. Moral acceptance by human organ transplant recipients is more easily obtained if this donor is not typically kept as a pet and is not phylogenetically similar to humans, such as the non-human primate. The pig is a mid-size animal, breeds before 1 year of age, and has a typical lifespan of 15 years. Pigs are rarely kept as pets. They are most commonly bred as a meat source and widely consumed, thus large-scale breeding and housing protocols are established and it seems likely that the public would be more accepting of their use as organ donors. Pigs are currently used as a source of heart valves for human cardiac valve replacement surgery and there is minimal public concern in this regard.

Porcine islets are particularly appealing for islet transplantation as they may have superior functional characteristics to human islets. Neonatal porcine islets show particular promise for organ donation, as they are easily isolated, are highly resistant to hypoxia and hyperglycemia (688, 689), and have shown remarkable ability to expand their β cell mass following transplantation (690). Porcine islets are resistant to human autoimmunity against β cells (721). Furthermore, with the advent of transgenic pigs, donors may be bred to optimize β cell resistance to diabetes and metabolic stress (721). Pig islets function well in xenotransplantation models. Neonatal porcine islets have been shown to maintain normoglycemia in immune-deficient murine recipients for more than 100 days, with no spontaneous failure (722), and similarly adult pig islet autografts were recently found to function for up to two years (208). Recent studies confirm long-term glycemic normalization of neonatal porcine islets transplanted into swine (723) and primate models (724). Our findings are in keeping with two previous reports that also did not observe evidence of fibrils or islet amyloid in adult porcine pancreas or transplanted pig islets (208, 711). The current literature supports pigs as a model for islet transplantation.

Despite its promise of widespread organ availability and a source of islets resistant to hypoxia and other sources of injury, caution should be taken in the implementation of xenotransplantation into humans. Alarmingly, 75% of current xenotransplantation treatments in humans are
performed in countries which do not regulate xenotransplantation (725). Early promising studies in the transplantation of porcine islets were performed in children in Mexico (726, 727) without the approval and regulation by a clinical ethics panel (728, 729). Such work in humans, particularly in vulnerable populations such as children, defies rigorous scientific ethics established in the Nuremberg trials (730) to prevent recurrence of highly unethical studies by Mengele (731-733) in Nazi Germany. There are considerable risks involved with xenotransplantation of organs into humans. Xeno-organs stimulate a stronger immune response than human allogeneic transplants (734, 735). Human recipients may require stronger immunosuppression and might develop autoantibodies preventing re-transplantation. Hyperacute rejection of porcine tissues in humans is attributed to α-1,3-galactosyl epitopes on the surface of porcine cells; as humans lack α-1,3-galactosyltransferase, such epitopes trigger a humoral response (736). Hyperacute rejection of organs from α-1,3-galactosyl knock-out pigs is considerably reduced. Interestingly, these animals also have improved glucose tolerance (737), suggesting that islet transplant recipients would be less prone to hyperglycemic episodes and that these islets would be more resilient in the face of hyperglycemia and insulin resistance. Furthermore, modified immunosuppressive regimens have been proposed to reduce episodes of hyperacute rejection (738, 739). Novel devices for immunoprotection (740-742) may also be considered for xenotransplantation. The advantages of such approaches include immune-mediated destruction of tissues if the capsule breaks.

Xenozoonosis is another important concern in the xenotransplantation of organs into humans (743-746). Rigorous screening protocols (747-751) and the production of clean pig facilities (749, 752) would allow prevention of the majority of known pig xenozoonoses. Porcine endogenous retrovirus (PERV) remains a challenge to overcome (721). Transmission of PERV to human cells would allow PERV integration into the human genome, potentially knocking out expression of critical genes or inactivating tumor suppressors. Several strategies (753-756), including selective breeding and the creation of PERV knockout pigs, have been proposed to eliminate the risk of PERV. A further limitation that remains, however, is that we may not have characterized all existing xenozoonoses and would need strategies to deal with unforeseen xenozoonosis in human recipients. One further concern is public opinion, although in several studies xenotransplantation was found to be ethically acceptable to most persons (757, 758). It should be noted that cardiac valves from bovine and porcine sources are routinely transplanted without significant public concern. Strategies have been proposed to the major challenges facing porcine
xenotransplantation but clinical trials should be undertaken with caution and careful consideration of the risks to recipients.
Chapter 6. Role of CHOP in islet graft dysfunction

6.1. Introduction

The success of islet transplantation depends upon the engrafted β cell mass. The Edmonton Protocol established that 10,000 islet equivalents per kilogram of body weight are required to attain insulin independence in a recipient (675). Islet isolation efficiency is limited, with a transplantable yield obtained in less than 50% of isolations (759). Engrafted islet mass is further reduced following transplantation. Up to 60% of islet mass is lost in the first week following transplantation (202), due to IBMIR (149), hypoxia prior to re-establishment of the islet vasculature (760, 761), immunosuppressant toxicity (172, 266, 360, 762-766), islet amyloid (490), and failure to engraft in a heterologous site of transplantation (761). Insulin resistance is elevated in human islet transplant recipients (376), which may be caused by immunosuppressive drugs (368). Excessive metabolic demand on remaining β cells enhances β cell susceptibility to oxidative and endoplasmic reticulum (ER) stress.

The β cell ER is highly developed and processes up to one million insulin molecules per minute. Transient activation of ER stress signaling upon accumulation of misfolded protein is tightly coupled to extracellular glucose concentration to modulate ER protein folding capacity in accordance with secretory demand (767, 768). Prolonged ER stress signaling due to increased metabolic demand is detrimental to β cell function. Prolonged activation of the unfolded protein response (UPR) leads to decreased insulin production and glucose-stimulated insulin secretion and activates apoptotic cascades (769, 770). Elevated production of insulin also increases cellular oxidative stress due to oxidation of its three disulfide bonds in the ER and increased demand for mitochondrial energy production for ER-dependent processes (771). The β cell is particularly susceptible to oxidative stress due to its low levels of antioxidants. CCAAT/enhancer binding homologous protein (CHOP) is a primary mediator of ER- and oxidative stress-induced apoptosis (381, 771-773) and is activated upon prolonged ER stress signaling. CHOP signalling may be additionally activated by cytokines, produced by local inflammation in the islet transplantation environment or by the β cell itself under conditions of metabolic stress (774). Deletion of CHOP enhances β cell function and mass in several models of β cell stress and type 2 diabetes (417). CHOP is elevated in transplanted islets in a syngeneic model in the first week following transplantation (775) and in models of whole organ transplantation (776). CHOP is an attractive
therapeutic target as it has no known critical physiological role in β cell function, unlike other upstream mediators.

6.2 Results

6.2.1. CHOP levels are increased in marginal mass grafts

Chop mRNA is significantly elevated following islet transplantation in both normoglycemic and hyperglycemic grafts and tends to remain elevated throughout the first week post-transplantation (775). We investigated CHOP levels by immuno-histochemical staining. We determined that CHOP levels were significantly elevated in marginal grafts, despite glucose normalization at 4 weeks (Figure 35).

![Figure 35. CHOP immunoreactivity is elevated in marginal mass mouse islet grafts.](image)

CHOP immuno-positive area was quantified within insulin-positive graft area in optimal (n = 3) and marginal (n = 14) syngeneic (Bl/6) mouse islet grafts. All recipients had normalized (<15 mM) blood glucose at 4 weeks following transplantation.

6.2.2. CHOP knockout islets are protected against thapsigargin-induced ER stress

Mice with a disrupted CHOP gene (B6.129S-Ddit3<Superscript>tm1Dron</Superscript>/J) (772) are available from the Jackson Laboratories. We isolated islets from male CHOP<Superscript>−/−</Superscript> mice and C57BL/6J controls, and following overnight culture incubated islets for 24 hours with the ER-stress inducer, thapsigarin (0.01 to 1.0 μM). CHOP immunoreactivity was significantly elevated in wild-type islets at all concentrations of thapsigargin (Figure 36). CHOP immunostaining was negligible in thapsigargin-treated CHOP<Superscript>−/−</Superscript> islets, thus confirming the phenotype. CHOP gene inactivation
significantly protected β cells from thapsigargin-induced apoptosis as detected by TUNEL staining (Figure 36B).

**Figure 36. CHOP\textsuperscript{+/-} islet cells are protected against apoptosis thapsigargin-induced apoptosis**

Islets were isolated from C57B/6 (white bars) or CHOP\textsuperscript{+/-} (gray bars) mice and incubated before 24 hour incubation in thapsigargin at the indicated concentrations. CHOP-positive area was normalized to insulin positive area of islets (%). TUNEL-positive nuclei within the insulin immunopositive area were quantified and normalized to insulin area of islets. * p<0.05 versus control; ** p < 0.01 versus control; # p 0.05 between groups; ## p<0.01 between groups.
6.2.3. CHOP knockout islets have improved insulin response to glucose

We compared insulin content and glucose-stimulated insulin secretion between isolated CHOP\(^{+/−}\) and wild-type islets (Figure 37). Islet insulin content (Figure 37A) did not differ between genotypes. In static incubation with high glucose (30 mM), CHOP\(^{+/−}\) islets secreted significantly more insulin (Figure 37B).

![Graph A and B](image)

**Figure 37. In vitro functional comparison of wild-type and CHOP\(^{+/−}\) islets.**

Islets were isolated from C57B/6 (white) or CHOP\(^{+/−}\) (grey) mice, cultured overnight and then incubated in KRB with either low (3 mM) or high (30 mM) glucose for 2 hours and then subjected to either KRB with 3 or 30 mM glucose for 1 hour. Islet insulin content in islet extracts after acetic acid lysis (n = 9) is shown in (A). Insulin content in KRB following 1 hour incubation in low or high glucose is shown in (B). Insulin was measured by ELISA. Error bars represent SEM. * p < 0.05.

6.2.4. CHOP\(^{+/−}\) islets restore and maintain normoglycemia following syngeneic transplantation

To determine whether the CHOP\(^{+/−}\) phenotype impacted glycemic normalization in optimal mass (150 islet) grafts, we compared the effectiveness of optimal islet mass grafts using islets from CHOP\(^{+/−}\) compared to C57BL/6J control donors. Islets were transplanted into syngeneic, STZ-diabetic recipients. Both types of grafts restored glycemic control following transplantation and maintained control for 4-5 weeks following transplantation (Figure 38). Similar preliminary results were obtained for 300 islet grafts (wild-type, n=1; CHOP\(^{+/−}\), n=2, data not shown), in which blood glucose values were restored to less than 7.0 mM at day 1 following transplantation. All recipients became diabetic following removal of the graft-bearing kidney, demonstrating that recovery of the STZ-treated pancreatic β cells did not occur.
Figure 38. Optimal mass syngeneic transplantation of wild-type and CHOP<sup>−/−</sup> islets.

Bl/6 recipient mice were treated with i.p. streptozotocin (230 mg/kg) to render them diabetic and 4 days later (day 0) transplanted with 150 islets. Glycemic control was restored by wild-type (black; n=14) and CHOP<sup>−/−</sup> (red; n=17) islets and maintained over 4-5 weeks post-transplantation. Non-fasted blood glucose was measured daily for the first week, three times in the second week, and weekly thereafter for three months following transplantation. All recipients became diabetic following removal of the graft-bearing kidney between 27 and 39 days post-transplantation.

6.2.5. Marginal mass CHOP<sup>−/−</sup> islet grafts restore glycemic control more rapidly

To determine the effect of CHOP in the function of a marginal mass islet graft, we compared function of marginal mass islet transplants using islets from CHOP<sup>−/−</sup> mice or C57BL/6J controls, grafted into syngeneic, STZ-diabetic recipients. Grafts of 75 islets overtly failed and grafts of 150 islets normalized rapidly, thus we chose 100 islets as a marginal mass graft. In this model, wild-type mice remained hyperglycemic for the first 1-2 weeks following transplantation and tended to normalize by three weeks post-transplantation. A comparison of mean glycemic values for wild-type and CHOP<sup>−/−</sup> marginal mass grafts is shown in Figure 39A. Kaplan-Meier analysis of these data is shown in Figure 39B. For this analysis, we defined a recipient to no longer be diabetic after two consecutive blood glucose readings less than 15 mM. The median time to normalization for grafts containing 100 CHOP<sup>−/−</sup> islets was 3 days, significantly less than the 12 days required for grafts using wild-type islets.
Islet grafts were removed by survival nephrectomy 4 weeks following transplantation, formalin-fixed, and processed for histology. So as to perform histological analysis at an earlier time point, we repeated marginal mass transplants and harvested the grafts at 10 days post-transplantation. Histological quantification of CHOP, replication (Ki-67) and apoptosis (TUNEL) in grafts at 10 and 28 days post-transplantation is shown in Figures 40A, B, and C, respectively. CHOP was elevated in wild-type grafts at 10 days relative to 28 days post-transplantation. There was no significant difference in replication between any groups, although there was a modest trend towards increased replication in both groups at 28 days’ post-transplantation. This finding is consistent with the finding that acute hyperglycemia stimulates β cell replication in vivo (777). At 10 days’ post-transplantation, apoptosis was significantly reduced in CHOP−/− islets as compared to wild-type islets (p < 0.01). There was no significant difference in apoptosis between groups at 28 days post-transplantation. Wild-type grafts, but not CHOP−/− grafts, showed a significant elevation in apoptosis at 10 days post-transplantation relative to 28 days.
Figure 39. CHOP\(^{+/−}\) islets restore glycemic control more rapidly than wild-type islets in marginal mass islet transplants.

Recipient mice were treated with ip streptozotocin (230 mg/kg) and 4 days later, 100 islets were transplanted (A). Mean glycemic values are shown for wild-type (black; n=14) and CHOP\(^{+/−}\) (red; n =17). Removal of the graft-bearing kidneys was performed 4-5 weeks after transplantation. All recipients became diabetic following nephrectomy. *p < 0.05 by 2-way ANOVA with replication. Kaplan-Meier survival curve comparing groups (B). Diabetes was defined as blood glucose >15.0 mM. An animal was considered no longer diabetic after 2 consecutive blood glucose readings < 15.0 mM. *, p<0.05; i.p., intraperitoneal.
Figure 40. Histological analysis of replicating and apoptotic cells in marginal mass wild-type and CHOP\(^{-/-}\) islet transplants.

A comparison of CHOP immunostaining at 10 days’ post-transplantation in wild-type and CHOP\(^{-/-}\) grafts (A). Insulin immunoreactivity is shown in green, DAPI-staining of cell nuclei in blue, and CHOP immunoreactivity in red. The arrow demonstrates CHOP positive staining. Analysis of CHOP immunostaining was performed on histological sections at 10 days (wild-type, white bars, n= 5; CHOP\(^{-/-}\), grey bars, n=6) and at 28 days post-transplantation (wild-type, n=14, CHOP\(^{-/-}\), n=17; B). Replicating cells were detected by Ki-67 immunostaining in (C). Apoptotic cells were quantified following TUNEL staining (D). Cells were measured within the insulin-positive area of the graft for each stain. * p<0.05; ** p<0.01.
6.2.6. Liposomal delivery of siRNA against CHOP is effective in improving marginal mass islet graft function

We characterized several Stealth siRNA against rodent CHOP in INS-1 cells transfected using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen). A representative western blot showing CHOP expression in INS-1 cells following transfection with two effective CHOP siRNAs is shown in Figure 41. ER stress was induced by thapsigargin treatment. A third siRNA (not shown) had moderate efficacy. In collaboration with Drs. P. Cullis and C. Tam, we optimized siRNA delivery into mouse islets using lipid nano-particles developed by the Cullis laboratory, as shown in Figure 42. Lipid nano-particle delivery was assessed using Cy5-labelled siRNA. Delivery was effective throughout the islet and fluorescent intensity within each islet increased relative to the lipid nano-particle concentration used. This delivery system delivered sufficient siRNA to mouse islets after 24 hour pre-incubation to protect against thapsigargin induction of CHOP (Figure 43). CHOP knockdown by lipid nano-particles was achieved in a concentration-dependent manner.

![Figure 41](image)

**Figure 41.** Lipofectamine-mediated delivery of CHOP siRNA suppresses CHOP expression and thapsigargin-induced activation of caspase 3.

Thapsigargin (Tg) was used to stimulate CHOP induction. Western blot for cleaved caspase-3 demonstrates the absence of apoptotic signaling in the presence of CHOP knockdown.
Figure 42. Uptake of liposomes containing Cy3-labelled siRNA into mouse islets.

Freshly isolated Bl/6 islets were incubated with liposomes for 24 hours. Z-stack images were taken of islets, from which a single slice is shown.

<table>
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<tr>
<th>[Liposome]:</th>
<th>1 μg/ml</th>
<th>10 μg/ml</th>
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<tr>
<td>Construct</td>
<td>si-scramble</td>
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<tr>
<td>Thapsigargin</td>
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CHOP

Actin

Figure 43. Lipid nano-particle delivery of CHOP siRNA suppresses thapsigargin-induced CHOP expression.

Bl/6 murine islets were incubated with liposomes for 24 hours. Islets were then stimulated with 10 μM thapsigargin for 24 hours in the presence of liposomes containing either siRNA to CHOP (si-CHOP) or a scrambled siRNA (si-scramble).

To test this system in a transplantation model, we pre-incubated wild-type islets with lipid nanoparticles (10 μg/ml) containing siRNA against CHOP or a scramble siRNA control. Marginal mass islet transplants were performed into syngeneic, STZ-diabetic recipients. Islets treated with siRNA against CHOP restored normoglycemia (2 consecutive glucose measurements less than 15 mM).
significantly faster than did islets treated with scramble siRNA as assessed by Kaplan-Meier analysis (Figure 44). It should be noted that 3 mice in the scramble group remained persistently diabetic (> 25 mM), had to be sacrificed upon 5% loss of body weight or other signs of ill health in accordance with animal ethics, and were excluded from Kaplan-Meier analysis. One mouse in the siCHOP group became persistently diabetic (> 20 mM for at least 2 consecutive readings) late in the experiment, also had to be sacrificed, and was excluded from Kaplan-Meier analysis. Based upon these data, ex-vivo lipid nano-particle delivery is effective in murine islets. Furthermore, we were able to replicate the phenotype of rapid glycemic normalization in CHOP⁻/⁻ marginal mass islet grafts by transfection of siRNA against CHOP into islets pre-transplantation.

**Figure 44. CHOP siRNA enhances islet transplant function.**

Bl/6 islets were treated in culture with liposomes (10 µg/ml) containing siRNA against CHOP (n=15) or a scrambled siRNA (n=10) for 12 hours prior to transplantation. Mice were sacrificed if their blood glucose remained >25 mM for 4 consecutive days in the first 4 days following transplantation, if blood glucose was >20 mM on two consecutive days afterward, or if they lost greater than 5% of their body weight. Prior to survival nephrectomy, 3 mice in the si-scramble group and one in the si-CHOP group were sacrificed for one of these reasons; these 4 mice were excluded from the Kaplan-Meier analysis. A mouse was considered non-diabetic following two consecutive blood glucose readings <15.0 mM.
6.3. Discussion

In this chapter, we showed that CHOP levels are elevated in marginal mass syngeneic grafts, particularly in the early post-transplantation period prior to glycemic normalization. We confirmed that CHOP primarily mediates ER stress-signaled apoptosis in primary islets and showed that the absence of CHOP improves glucose-stimulated insulin secretion in isolated islets. We showed that islets lacking functional CHOP had more rapid glycemic normalization and reduced apoptosis in a syngeneic, marginal mass islet transplantation model. Furthermore, using a novel delivery system for efficient siRNA delivery, we reproduced these results in wild-type marginal mass grafts pre-treated *ex-vivo* with siRNA against CHOP.

Syngeneic marginal mass grafts are under considerable metabolic stress, demonstrated by elevated markers of oxidative stress and apoptosis, including CHOP (775). The islet is particularly vulnerable within the first two weeks of transplantation, as it recovers from the isolation procedure (236, 778) and islet culture (229), re-establishes its vascular supply (282), and is exposed to hyperglycemic (396, 775), hyperlipidemic (265), and pro-inflammatory (192, 638) environments. An additional stress in human allografts is exposure to diabetogenic immunosuppressive agents (355, 360) at elevated concentrations in the portal vein (267) prior to re-establishment of islet arterial blood supply. In human islets, IAPP may further promote oxidative (32) and ER stress (394, 484, 779). Each of these influences (91, 236, 361, 377, 396, 397, 401, 403) may exacerbate ER and oxidative stress in the metabolically stressed β cell, shifting the balance from homeostatic regulation to pro-apoptotic signaling.

An ideal target to modulate ER stress responses would be activated under pathological circumstances and not critical to physiological cellular function. Furthermore, it should be downstream of both ER and oxidative stress pro-apoptotic signaling. Modulation of upstream UPR pathways interferes with signaling pathways critical to β cell function. For example, ablation of PERK signaling impairs insulin gene expression and protein secretion, in addition to impairing β cell proliferation, altering ER redox state (383, 391, 780), dysregulating synthesis of critical ER chaperones (660), and making cells particularly vulnerable to protein overload (383, 391, 780). Defective PERK signaling in human disease (Wolcott-Rallison disease) and mouse models (PERK<sup>−/−</sup>, eIF2α<sup>S51A</sup>) leads to early-onset diabetes and reduced β cell mass (396, 419).
secondary to β cell apoptosis (396, 781). β cells in these models have dilated ER and a low density of secretory granules (419).

In contrast, excessive PERK activity attenuates global protein synthesis and also promotes β cell apoptosis (782). Sustained IRE1α signaling is detrimental to insulin production and glucose-stimulated insulin secretion (769), while IRE1α and xBP-1 knockout mice have embryonic lethal phenotypes (382). Modulation of the upstream UPR pathways may interfere with transient signaling that is critical to maintenance of β cell function, while prolonged activation will promote pro-apoptotic signaling.

An alternative therapeutic strategy to protect the metabolically-stressed β cell is to enhance its ability to withstand ER and oxidative stress. Anti-oxidant (181) treatment and over-expression of anti-oxidant enzymes (235, 783, 784) promotes graft function in islet transplantation. Such treatment by itself, however, is insufficient to protect against ER stress responses. A more feasible strategy to promote homeostasis in the midst of metabolic stress may be to increase the ER protein folding capacity independently of UPR signaling, for example using “chemical chaperones” such as 4-PBA and TUDCA. Each of these chaperones has been shown to have protective effects on β cell secretory function in the midst of ER stressors (397, 600). Simultaneous redox modulation and chaperone treatment could dampen both ER and oxidative stress. We found, however, that both chemical chaperones were highly toxic to islets (data not shown) at neutral pH and at reported concentrations (397, 600). We proposed that CHOP, as a pro-apoptotic downstream of ER and oxidative stress without a critical role in maintaining normal β cell function, would thereby be an attractive therapeutic target for β cells under metabolic stress.

CHOP, otherwise known as DNA damage inducible transcript 3 (DDIT3), is a transcription factor belonging to the CCAAT/enhancer-binding protein (C/EBP) family (785). Each member of this family contains a bZIP involved in DNA recognition and a leucine zipper that is critical to homo- or heterodimerization. CHOP acts as a dominant negative inhibitor of C/EBP and LAP and inhibits their binding to their specific DNA enhancer elements (785). In addition to ER and oxidative stress, CHOP is activated by growth arrest in proliferating cells (785), amino acid deprivation, cytokine stimulation, and human IAPP (30, 394). CHOP is strongly pro-apoptotic
and may induce apoptosis by multiple mechanisms. CHOP promotes Ca\(^{2+}\)-dependent apoptosis by transcriptional activation of proapoptotic genes (DR5, TRB3, BIM, Doc1, CA-VI, Doc4, Doc6) induction of reactive oxygen species, repression of the anti-apoptotic factor Bcl-2, translocation of Bax to mitochondria, by depleting cellular glutathione, and by decreasing intracellular pH (380, 382, 397, 419, 420).

As we confirmed, cells lacking active CHOP are protected against ER and oxidative stress-mediated apoptosis (381). We also discovered that CHOP may act as a negative regulator of insulin secretion under physiological conditions. We observed that in the absence of CHOP signaling, glucose-stimulated insulin secretion was enhanced. Additional evidence supports a negative regulatory role for CHOP. By interaction with C/EBP-β, CHOP regulates expression of mitochondrial stress genes (786). The CHOP gene has a mitochondrial stress response element (787). CHOP interacts with a co-activator of insulin gene transcription, p300 (788), and may thus have effects on insulin gene transcription, although CHOP\(^{-/-}\) mouse islets did not show differences in insulin content. We suggested that an ideal therapeutic target would be pro-apoptotic, downstream of ER and oxidative stress signaling pathways, and would not be active under physiological conditions. While CHOP is typically only described as a pro-apoptotic signaling mediator, we have identified a potential minor physiological regulatory role in insulin secretion under in vitro conditions that warrants further mechanistic investigation. Nevertheless, inhibition of CHOP signaling did not appear to have a detrimental effect in an in vivo islet transplantation model.

CHOP is implicated in the development of diabetes and β cell dysfunction. In several mouse models of diabetes, CHOP deletion promotes β cell function and delays the onset and severity of the diabetic phenotype (417). A single nucleotide polymorphism (SNP) in the 5’UTR region upstream of CHOP predisposed an Italian cohort to the early development of diabetes (787, 789); this SNP prevents the binding of HOXA7, which may protect against the induction of apoptosis (790, 791), to the CHOP promoter. CHOP expression is elevated in human and murine models of type 2 diabetes (787). CHOP expression is also increased in the β cells of mice transgenic for human IAPP (30, 394), demonstrating an intracellular mechanism by which hIAPP may be cytotoxic. ER accumulation of an elevated load of misfolded proIAPP may promote β cell apoptosis by CHOP induction. It was, however, demonstrated that CHOP expression was not
induced in a different strain of transgenic hIAPP-expressing islets or in cultured human islets that developed amyloid deposits (468); thus CHOP induction in some hIAPP transgenic models may be a phenomenon simply associated with over-expression of a transgene in β cells. Over-expression of proteins in β cells, such as rodent IAPP (30), human IAPP (30), hIGRP (386), and MHCII (387), has been shown to trigger ER stress because of a high biosynthetic load. Nevertheless, intracellular deposits of IAPP fibrils have been visualized by TEM in human β cells (624). IAPP species may perforate cell, and therefore organelle, membranes. ProIAPP species in the ER and IAPP fibrillar species in lysosomes could perforate these organelles to promote cytosol IAPP amyloid deposition. Implication of CHOP in human diabetes and evidence of protection against diabetes upon CHOP inactivation suggest that CHOP is a viable therapeutic target in diabetes as well as transplanted islets.

CHOP activity is stimulated when the β cell can no longer compensate to maintain homeostasis under elevated metabolic demand. CHOP is minimally active under non-stress conditions. Consistent with this, lack of CHOP activity is observed following an optimal mass transplant of human islets into diabetic murine recipients (792). In contrast, prolonged islet culture is detrimental to islet viability and promotes ER stress in human islets (229). CHOP is highly relevant in islet transplantation, a procedure in which considerable islet stress is induced. Isolation and transplantation procedures induce islet injury and apoptosis. β cell mass is significantly reduced by islet isolation yield, loss in islet culture, and loss following transplantation due to failure to engraft, hypoxia, or inflammatory or immune-mediated destruction. The β cell mass that does engraft is immediately exposed to a hyperglycemic environment. Immunosuppressive agents are diabetogenic and toxic to β cells; they may also increase metabolic demand on β cells by increasing peripheral insulin resistance. Adaptation to a non-ideal heterogenous site of transplantation and many detrimental factors in the local environment further promote β cell stress.

Modulation of CHOP could be introduced even prior to islet isolation to promote islet viability during warm and cold ischemic times. Inhibition of CHOP signaling in the immediate post-transplantation period should ideally preserve β cell mass and thereby promote engraftment of a greater initial mass. The benefit of long-term inhibition of CHOP signaling is demonstrated by the remarkable improvement in β cell function and the expansion in mass under conditions of β
cell stress (417). It is possible that long-term inhibition of CHOP signaling would increase β cell mass in transplantation, and this possibility warrants further investigation. The effects of CHOP inhibition on β cell survival could be synergistically enhanced by simultaneous inhibition of other pro-apoptotic signaling pathways activated by stress signaling. ER and oxidative stress signaling activate additional pro-apoptotic signaling pathways, including JNK (381, 384, 391, 419) and cleaved caspase-12 (419, 420, 768). JNK inhibition is relevant to human islet transplantation, as the human ortholog of murine cleaved caspase-12 is a pseudogene (381). JNK inhibition and dominant negative (DN-JNK) have been shown to protect β cells from apoptosis and improve graft function (382, 416). Synergistic inhibition of CHOP and JNK may further increase glycemic normalization, promote graft function, and inhibit apoptosis in transplantation.

A particular novelty of this work is the demonstration of efficient siRNA delivery throughout islets by incubation with lipid nano-particles (549). Alternate delivery systems include viral gene transfer and chitosan-based (793) particle delivery of siRNA. Viral vectors typically used to infect β cells include adenoviruses (3), adeno-associated viruses (794), and lentiviruses (795). Due to their immunogenicity and potential toxicity, viral vectors are limited in their translation to clinical medicine (603-605). The lipid nano-particle system used has been shown to have minimal toxicity at therapeutic doses and to deliver efficient knock-down of targets in vivo (549). Ligand-associated lipid nanoparticles targeting beta cell membrane proteins might have increased beta-cell specific delivery and further enhance the effectiveness of this system to modulate the beta cell in disease states.

In summary, we have demonstrated that CHOP signaling is detrimental to glycemic normalization in marginal mass islet transplantation. We have demonstrated a therapeutic approach to modulate CHOP signaling in the β cell. Targeting of CHOP and other pro-apoptotic signaling pathways activated by ER and oxidative stress may preserve β cell mass and function in the early post-transplantation period of islet transplantation, particularly under conditions of elevated metabolic stress.
Chapter 7. Conclusions

Progressive graft dysfunction is a daunting hurdle in islet transplantation. Much focus on islet loss has centered around immune-mediated mechanisms of graft destruction, many non-immune factors which promote metabolic stress in the transplanted β cell also likely play an important role in graft failure. In this thesis, CHOP and islet amyloid were shown to be detrimental to islet graft survival and function. Modulation of CHOP in the early post-transplant period improved glycemic control and reduced apoptosis, particularly in marginal mass grafts. The effects of islet amyloid are important over the long-term in islet transplantation. From both human and transgenic human IAPP-expressing mouse transplant models, we conclude that islet amyloid deposition is detrimental to graft function, that it inversely correlates with islet graft maintenance of glycemic control, and that its effects are exacerbated under increased secretory demand. Islet amyloid deposition in donor islets prior to transplantation predicts graft outcomes. Islet amyloid deposition is exacerbated by factors related to the transplantation procedure, such as heparin. Strategies investigated to mitigate the detrimental effects of islet amyloid in islet transplantation, such as peptide inhibitors of IAPP aggregation, siRNA-based silencing of IAPP, and xenotransplantation of islets expressing non-fibrillogenic IAPP, are promising in terms of their potential for clinical translation. A summary depicting these strategies is shown in Figure 45.
Figure 45. Strategies to prevent β cell dysfunction and loss in islet transplantation.
There are many future directions for these studies. In terms of CHOP, it is important to investigate which upstream mechanisms in islet transplantation, such as ER stress, oxidative stress, and inflammation, are critical to its induction in marginal mass islet grafts. The role of CHOP has been investigated in the early post-transplant period, but much work remains to elucidate its role over the long-term, such as whether CHOP activity increases in proportion to graft dysfunction. As CHOP has only been investigated in rodent models of islet transplantation, its role in human islet graft dysfunction is of particular relevance, particularly whether its activity is related to the presence of islet amyloid and islet amyloid-related inflammation. Finally, the ability to silence CHOP in vivo is of great relevance to both islet transplantation and type 2 diabetes. The development of beta-cell specific ligand-associated nano-particles would allow silencing of CHOP only in the beta cell and would provide an important model for the use of this technology to silence other relevant genes in the dysfunctional beta cell.

In terms of islet amyloid, there are also numerous directions remaining to be explored. Islet allograft donors were found to be at increased risk for the development of islet amyloid, which is detrimental to graft function. This analysis, however, was performed on a small cohort. Expansion of this cohort and analysis of additional clinical parameters of the donor, recipient, and graft function over time will expand the relevance and importance of this study. Replication of this study in human islets transplanted into mouse models will provide additional support. The therapeutic strategies to modulate IAPP expression or islet amyloid deposition are promising but have only been examined in cultured human islets. The expansion of these studies to in vivo models is critical to provide evidence that these strategies are valid therapeutic approaches for the clinic. Given this evidence, these strategies will not only be applicable to islet transplantation, but also to type 2 diabetes. Finally, many factors in islet transplantation may exacerbate islet amyloid deposition. Further work remains to elucidate the role of immunosuppressive agents in islet amyloid deposition. In terms of heparin, it remains to be determined as to what the effect of islet surface heparinization, currently in clinical trials, is on islet amyloid deposition in human islets and whether alternate means of controlling IBMIR have the same detrimental effect upon seeding islet amyloid. Finally, much work remains to expand our current understanding of why islet amyloid deposits rapidly in human islets and what factors other than IAPP are critical to islet amyloid deposition and toxicity.
In conclusion, the main finding of this thesis is that CHOP and islet amyloid are important contributing factors to islet graft dysfunction and novel therapeutic targets by which to preserve islet graft mass and function. These factors, among others, point to important similarities between the dysfunctional beta cell in both islet transplantation and type 2 diabetes and provide therapeutic relevance not only to improving the success and availability of islet transplantation but also to treating type 2 diabetes in the face of a North American epidemic.
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