The Roles Of ATF3, An Adaptive-response Gene, In Pancreatic Islet β-cell Stress Response And Function

Dissertation

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By

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ABSTRACT

Currently, 23.6 million people (7.8%) in the United States and 171 million people (5.4%) worldwide have diabetes. Each year in America alone, approximately 1.6 million new cases are diagnosed, and estimates are that 366 million people worldwide will have diabetes by 2030. There are two forms of diabetes; type 1 and type 2. Type 1 diabetes mellitus (T1DM) is generally accepted to be a multifactorial disease of autoimmunity that affects individuals predisposed by genetic susceptibilities. In these individuals, environmental triggers are believed to instigate autoimmune-mediated destruction of the insulin secreting pancreatic $\beta$-cells. This $\beta$-cell deficit gives rise to a dependence on exogenous insulin for maintaining metabolic homeostasis. Hence, T1DM is often called insulin-dependent diabetes mellitus (IDDM). Type 2 diabetes mellitus (T2DM) is a chronic disease characterized by insulin resistance, increased hepatic glucose production, and impaired insulin secretion. Of the two types of diabetes mellitus, type 2 is by far the most prevalent and accounts for approximately 90% of diabetic cases. The etiology of T2DM is also complex, nevertheless it is generally believed to a disease of insulin resistance, brought on by obesity and obesity-induced systemic inflammation, which gives rise to defects in glucose homeostasis. However, individuals remain relatively asymptomatic until, in a genetically predisposed subset of the obese population, the pancreatic $\beta$-cells become stressed and fail. Therefore, $\beta$-cell stress response is of central
importance to the pathogenesis of diabetes. The focus of the research performed in this
dissertation is on ATF3, a stress inducible transcription factor that plays a central role in
the biology of β-cells. The hypothesis of this dissertation is that ATF3 functions by
contributing to the expression of target genes that strongly influence β-cell survival and
function.

Previously, studies in Dr. Hai’s lab demonstrated that activating transcription
factor 3 (ATF3) is a stress-inducible gene and pro-apoptotic in various cell types,
including β-cells. These results support a model that ATF3 plays a role in the
pathogenesis of diabetes, partly by its apoptotic effect in β-cells. ATF3 is a member of
the larger ATF/CREB family of transcription factors, which is comprised of a group of
basic region-leucine zipper (bZip) proteins related by their ability to bind the consensus
ATF/CRE site ‘TGACGTCA’. As an early response gene, steady state levels of ATF3
remain low until induced by stimulus. The ATF3 protein primarily functions as a
transcription factor, capable of regulating a diverse set of genes through selective
interaction with other bZip proteins. Due to the broad range of extra and intra-cellular
stimuli capable of inducing ATF3 and the fact that its transcriptional activity is
influenced by selective dimerization, ATF3 is capable of pleiotropic effects that influence
cellular survival, growth, metabolism and malignant progression. Based on this
information, I set out to investigate the potential functional significance of ATF3
expression in β-cells exposed to two physiologically relevant stress paradigms; islet
transplantation and high fat diet induced type 2 diabetes.
In chapter 2, I tested whether ATF3 plays a role in the β-cell stress response to islet transplantation, with an eye towards identifying the mechanism by which ATF3 may function. In the course of this study, I compared wild type (WT) and ATF3 knockout (KO) islets in vitro using stress paradigms relevant to islet transplantation: isolation, inflammation, and hypoxia. I also compared the WT and KO islets in vivo using a syngeneic mouse transplantation model. As a result, I found that ATF3 was induced in all three stress paradigms and played a deleterious role in islet survival, as evidenced by the lower viability of WT islets than KO islets. ATF3 regulated various downstream target genes in a stress-dependent manner. These target genes can be classified into two functional groups: (a) apoptosis (NOXA, bNIP3), and (b) immuno-modulation (TNFα, IL1β, IL6, and CCL2/MCP-1). In vivo, ATF3 KO islets performed better than WT islets after transplantation, as evidenced by better glucose homeostasis in recipients, and reduced caspase 3 activation and macrophage infiltration in the KO grafts. These results identified a role for ATF3 in islet graft rejection by contributing to islet apoptosis and inflammatory responses at the graft sites. Therefore, we feel that silencing ATF3 may provide therapeutic benefits in islet transplantation.

One way of accomplishing this silencing of ATF3 is to use gene targeting to deliver an ATF3 knock down construct to a large fraction of the cells within the islet. This approach requires the use of a viral vehicle that achieves very high islet infection efficiency with minimal side effects. Currently, the adenovirus based vector is the main gene targeting vehicle used in experimental islet transplantation studies. Adenovirus is an attractive reagent for these studies because it is capable of achieving a high infection
efficiency in islets. However, their use is not ideal because the concentrated titers of virus needed to achieve maximal infection induce a stress response that compromises islet function. Furthermore, when adenovirus infected cells are encountered in vivo, a robust immune response ensues resulting in the clearance of the infected cells. Given that retention of functional mass is one of the limiting factors for the success of islet transplantation, the consequence of adenoviral infection - reduced functional mass – is a major impediment to its utility. In contrast to adenovirus, several properties of the adeno-associated virus (AAV) make it ideally suited as a gene therapy vector. These properties include its lack of pathogenicity, low immunogenicity, negligible random integration into host DNA, and ability to infect both dividing and non-dividing cells. However, the AAV islet infection efficiency is fairly low and ranges from 15% to 20% of the cells from each islet being infected. Therefore, any efforts directed towards improving the islet infection efficiency of AAV would strengthen its value as a gene therapy vector for islet transplantation.

With this in mind, in chapter 3 I tested whether the insertion of the Arg-Gly-Asp (RGD) amino acid sequence, a potent cell adhesion motif, into a capsid protein of the non-immunogenic AAV was capable of improving its islet infection efficiency. This hypothesis was based on the literature supported idea that increasing AAV adhesion to the islet cell surface would increase the frequency with which infection would occur. To examine this possibility, I compared a panel of recombinant AAV pseudotypes as well as a RGD-modified AAV vector for their potential to infect islets in vitro. In addition, I tested the functional consequence of RGD/AAV infection in vivo using a syngeneic
mouse transplantation model. Work presented in chapter 3 demonstrates that the RGD modification improves AAV infection efficiency of murine, non-human primate and human islets in vitro. Furthermore, infecting islets with the AAV1/RGD vector did not compromise their ability to function in vivo, as transplant recipients of both non-infected and AAV1/RGD infected islets demonstrated similar ability to control blood glucose levels. These findings are significant in the context of islet transplantation as they identify AAV1/RGD as an improved non-immunogenic viral vector and leaves open the possibility for its use in many therapeutic applications.

In chapter 4, I tested whether ATF3 plays a role in the development of type 2 diabetes by comparing wild type (WT) and ATF3 knockout (KO) mice in high fat diet (HFD)-induced model of diabetes. To our surprise, I found that ATF3 played a protective role in the development of T2DM, as evidenced by reduced glycemic control and serum insulin levels in KO mice compared to WT. No differences in HFD induced insulin resistance, inflammation, and compensation in pancreatic islet mass were identified between WT and KO mice, thus suggesting a role for ATF3 in islet function. Subsequent in vitro studies using gain and loss of function approaches to examine steady-state mRNA levels, reporter activity, in vivo DNA binding, and in vivo transcription, strongly indicated that insulin is a target gene directly up-regulated by ATF. Thus, ATF3 plays a role in both β-cell apoptosis (from previous work) and function (from this work). Results described in chapter 4 are consistent with a diabetes model that, before β-cell apoptosis becomes an issue, expression of ATF3 is beneficial and helps β cells to cope with higher metabolic demand.
In conclusion, work presented in this dissertation offers insight into the significance of ATF3 expression during two distinct and separate stress contexts, islet transplantation and high fat diet induced diabetes. In each case, potential mechanisms by which ATF3 may function are identified. Furthermore, this dissertation identifies the RGD modified AAV1 viral vector as a highly infectious and non-immunogenic gene therapy tool that may contribute towards efforts aimed at improving outcomes from clinical islet transplantation. In light of the opposing functional consequences of ATF3 expression reported in chapters 2 and 4, work presented in this thesis has contributed to a more complex understanding of the biological significance of ATF3 expression. In this context, the activity of ATF3 may depend not only upon its induction by stress, but also upon other factors in the cell that are selectively regulated by the specific context of the stress. Therefore, ATF3 may operate as an “adaptive response” gene capable of promoting apoptosis or enhancing cellular function as dictated by the stress environment.
DEDICATION

This work is dedicated to my family:
Elizabeth, Ryan, Emily & Jonathan
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While conducting the research presented in this dissertation, several individuals have influenced my scientific and personal development. Through their selfless sharing of time, intellect and reagents, these individuals have demonstrated a genuine commitment to the development of those around them. For this, I am eternally grateful.

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I would like to thank the many collaborators here at Ohio State University and elsewhere for their assistance. Numerous faculty members contributed to this research and the details of their contribution are listed in the acknowledgement section of the indicated chapter. I would like to give special thanks to two core facilities for their contribution to my research: The Center for Molecular Neurobiology Imaging core for providing access and technical consultations for the imaging done in this dissertation and the Ohio State University College of Veterinary Medicine Histology Core for consistent and high quality assistance in sectioning of islet grafts.

Finally, I would like to thank my wife Elizabeth for her support and love. She has sacrificed much while I invested in my education. Through it all she has remained my best friend and I look forward to the next chapter in our lives together.
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<td>ATF3</td>
<td>Activating Transcription Factor 3</td>
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<td>AAV</td>
<td>Adeno-associated virus</td>
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<tr>
<td>bZIP</td>
<td>basic region leucine zipper</td>
</tr>
<tr>
<td>CRE</td>
<td>cyclic AMP response element</td>
</tr>
<tr>
<td>CREB</td>
<td>cyclic AMP response element binding protein</td>
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<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
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<td>TSS</td>
<td>transcriptional start site</td>
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CHAPTER 1

INTRODUCTION

Research presented in this dissertation provides insight into the mechanism by which Activating Transcription Factor 3 (ATF3) plays a role in β-cell adaption to stress. Additionally, this work includes the characterization of a novel modification to the Adeno-Associated Virus (AAV) that improves its potential as a gene therapy vehicle for delivering pro-survival agents to β-cells exposed to the stresses of clinical islet transplantation. In this chapter, three sections provide relevant background information and an overview of the research conducted during the course of this dissertation. Section A provides an overview of ATF3 that includes information about the ATF/CREB family of transcription factors, the identification and cloning of ATF3, the forces regulating ATF3 expression, and the functional significance of ATF3 expression. Section B reviews our current understanding of the development and treatment of Type 1 and Type 2 diabetes, with an emphasis on the role of β-cell stress response. Lastly, section C presents overview of the work conducted in this dissertation.

A.1 The ATF/CREB family of transcription factors

ATF3 is a member of the larger ATF/CREB family of transcription factors. This family is comprised of a group of basic region-leucine zipper (bZip) proteins related by
their ability to bind the consensus ATF/CRE site ‘TGACGTCA’. In the late 1980’s, the first ATF proteins were identified by their ability to bind and activate the adenovirus early promoters E2, E3, and E4 through what was defined as the common core ATF sequence ‘CGTCA’ [1]. This core sequence was later expanded to include ‘TGACGT(C/A)(G/A)’, a sequence identical to the mammalian cyclic AMP response element (CRE) [2, 3]. Around the same time, the first mammalian cyclic AMP response element binding protein (CREB) was identified to bind the somatostatin promoter in a CRE dependent manner [4]. Despite some initial confusion over the discovery of identical consensus sequences on viral and cellular promoters, the ATF and CRE response elements were considered to be one in the same, hence the ATF/CRE site nomenclature. Subsequently, ATF and CREB prefixes have been used to name bZIP proteins given that they meet two criteria: (1) they bind to the ATF/CRE site in vitro, and (2) they form homo- or heterodimers. Currently, there are more than 10 different mammalian cDNA clones that encode proteins with ATF/CREB properties [5].

Functionally, the ATF/CREB proteins contribute to a diverse range of biological processes, most of which involve transcriptional responses to extracellular signals. Some of these cellular processes include differentiation, synaptic remodeling, survival, and homeostasis [5-10]. The specific function of individual ATF/CREB proteins can be influenced by their dimerization partner. Given that dimerization of bZIP proteins mainly occurs through leucine zipper domains, the ATF/CREB proteins selectively heterodimerize among themselves and with members of the other bZIP protein families, such as the C/EBP and AP-1 proteins, which have their own sequence specific DNA response elements [11-15]. The DNA binding diversity achieved through these
interactions affords the ATF/CREB proteins great functional diversity in responding to extra cellular signals.

Since the early 1990’s, research in our laboratory has focused on the ATF3 transcription factor. Work presented in this dissertation provides insight into the mechanism by which ATF3 plays a role in β-cell adaption to stress. Therefore, this introduction will next briefly review the background relevant to ATF3, including its identification and characterization, regulation, transcriptional activity, and functional significance.

A.2 Identification and characterization of the ATF3 gene

One of the first observations that transcription factors can function in a sequence specific manner was reported by Robert Tjian in 1986, who at the time was working on the Sp1 transcription factor [16, 17]. In the years to follow, many investigators sought to identify and purify the sequence specific factor regulating their gene of interest. One of these investigators was Michael Green at Harvard University. His group was focused on identifying the mechanism used by the adenoviral E1A protein to regulate a set of E1A responsive viral early genes. At the time it was known that E1A was not a sequence specific transcription factor and that other viral and cellular factors within the cell functioned in a sequence specific manner to enhance the activity of E1A responsive promoters. Dr. Green’s group believed that they had identified one of these cellular factors through in vitro gel shift binding assays and named it Activating Transcription Factor (ATF) [1]. However, a graduate student in the lab, Tsong-Yung (Tsonwin) Hai, subsequently found through DNA affinity chromatography and screening human λgt11
expression libraries that ATF actually represented a series of 8 polypeptides related by their DNA binding activity and immuno-reactivity [18, 19]. These peptides were named ATF1 thru ATF8.

Subsequent characterization of human ATF3 genomic and cDNA sequences indicated that the 15 kilobase ATF3 gene contains 5 main exons, designated as exons A, B, C, D, and E [20, 21] (Fig. 1.1A). Exon A is 167bp in size and contains the 5’ untranslated region. Exon B spans 244bp and encodes the N-terminal 80 amino acids, including the AUG initiation codon. Exon C measures 108 bp in length and codes for the 36 amino acids primarily responsible for the basic region of ATF3. Exon D is 145bp in length and contains an in-frame termination codon. When incorporated into the mRNA transcript, Exon D gives rise to a truncated protein that lacks the leucine zipper [21]. Exon E measures 1395 bp in length and encodes the remaining 65 amino acids of ATF3, most of which is the leucine zipper domain. Exon E also contains the 3’-untranslated region. Genes that share approximately 95% amino acid homology to human ATF3 have been identified in other species and are referred to as LRF-1 (Rat) and TI-241 (mouse) [22, 23].

Seven splice variants and two promoters of ATF3 have been identified in a range of cell types that collectively give rise to 6 slightly different ATF3 proteins; full-length, ΔZip, ΔZip2a,b, ΔZip2c, ΔZip3, and ATF3b (Fig. 1.1B). The full length ATF3 protein is 181 amino acids and produced from a transcript that contain exons A, B, C and E [20]. The full length isoform primarily localizes to the nucleus where it functions to activate or repress transcription in a context dependent manner [9, 21, 22, 24-30]. Alternatively, the ATF3ΔZip protein is 118 amino acids and produced from a transcript that includes all
Figure 1.1: Schematic representation of the ATF3 gene, the mRNA species produced, the resulting proteins, and the known alternative promoters. (A) The genomic arrangement of ATF as well as all known splice isoforms of ATF3. Exons are indicated with boxes and labeled accordingly. Translational start (ATG) and stop sites (TAA, TAG, TGA) are indicated accordingly. (B) The predicted protein structures for each of the ATF3 mRNAs are shown with the activation (Act), repression (Rep), basic, and leucine zipper regions designated. (C) The alternative exons A-1 and A produced through differential promoter usage are shown. Figure reprinted with permission from The American Society for Biochemistry and Molecular Biology and Oxford University Press [31, 32].
As mentioned previously, inclusion of exon D introduces a stop codon that results in the production of a protein which lacks the leucine zipper domain [21]. Due to the loss of the leucine zipper domain, ATF3ΔZip cannot dimerize and form a functional DNA binding domain and therefore does not bind to DNA. However despite the inability to bind DNA, ATF3ΔZip was observed to stimulate transcription in an ATF/CRE independent manner, presumably by sequestering co-inhibitory factors away from the promoter [21]. Consistently, each of the ATF3 isoforms lacking a leucine zipper domain function in a similar manner and in many cases have been observed to counteract the activity of full length ATF3 [31, 33]. The ATF3ΔZip2a,b protein is 135 amino acids and produced from 2 different transcripts that utilize all five exons[33]. These transcripts differ from the ATF3ΔZip transcript in their use of splice acceptor sites, wherein both
ΔZip2a and 2b utilize a novel 3’-acceptor site 76 bases upstream of exon D. This 76 bp sequence is considered exon D’. Where ATF3ΔZip2a and 2b differ is in splicing between exon D and E, notably after the stop codon introduced by exon D. ATF3ΔZip2b splices exons D and E as in ATF3ΔZip, however this splicing does not occur in ATF3ΔZip2a and results in a transcript that is 91 base pairs longer. The ATF3ΔZip2c protein is 106 amino acids and produced from a transcript that utilizes exons A, B, C, D’, and D [31]. In this transcript, an alternative splicing event within exon B removes 87 base pairs giving rise to two exons designated B1 and B2. The ATF3ΔZip3 protein is 120 amino acids and is produced from a transcript containing exons A, B, C, D and D’ [31]. Splicing of this transcript does not occur between exons C and D which results in the addition of approximately 1000 base pairs that encodes a premature stop codon [31]. Finally, the ATF3b isoform is a 124 amino acid protein encoded by a transcript that contains exons A, B, C and E [34]. ATF3b lacks the N-terminal 57 amino acids due to an alternative splicing event within exon B that removes a 106 bp region containing the ATG start codon. This alternative splicing gives rise to two exons designated B1 and B2. The truncated protein is produced through translational initiation at an alternative downstream start codon and appears to function similarly to full length ATF3 [34].

In addition to the proximal promoter directly upstream from exon A, an alternative promoter approximately 50kb upstream has recently been identified and characterized [32, 35] (Fig. 1.1C). This promoter was found to be highly conserved between human and mouse and contains multiple transcriptional start sites that encode an alternate exon A labeled as A-1. No additional translational start sites were identified in exon A-1 therefore any transcripts resulting from the use of the alternative promoter are
predicted to result in a similar protein to that produced from transcripts generated by the proximal promoter. However, the different regulatory elements in the two promoters, as well as the different 5’ UTRs do appear to convey stress specific transcriptional and translational properties. This additional regulatory control may provide the cells with a greater capacity to selectively induce ATF3 expression in response to varying stresses.

A.3 Regulation of ATF3 expression

Overwhelming evidence indicates that ATF3 mRNA and protein levels are relatively low in most cell types, but dramatically increase following exposure to a wide range of extracellular stimuli [12]. The finding that ATF3 induction is neither tissue nor stimulus specific supports the notion that ATF3 is an early response gene and its expression may be regulated by a number of factors. In recent years, experimental approaches have lead to a broader understanding of the forces regulating ATF3 expression (Summarized in Fig. 1.2). One strategy has been to combine bioinformatic and biochemistry approaches to identify and test potential transcription factor response elements in ATF3’s promoter. Additional work has been done to interrogate specific signaling pathways for their involvement in ATF3’s induction. Sequence analysis of the approximately 2000 base pairs flanking the 5’ end of the ATF3 gene revealed several consensus elements for factors that may contribute to the expression of ATF3 [20]. Detailed analysis of the alternative proximal promoter has yet to be completed [32]. Notably, the proximal promoter contains a TATA box and an ATF/CRE site respectively located around -30 and -90 base pairs relative to the transcriptional start site (TSS).
Additional transcription factor response elements were identified within the -1850 base pairs relative to the TSS and these can be classified into two functional groups: response elements regulated by stress (NFκB, p53, HIF, AP1, and Smad) and elements regulated in a cell cycle-dependent manner (E2F and Myc/Max). Empirical evidence indicates that ATF3 is induced, at least in part, at the transcriptional level by these stress responsive factors [36-38].
As an example, recent studies have reported data that supports both the correlative and direct regulation of ATF3 by p53 in a variety of cell types. In several of these reports, microarray analysis of cancer cell lines (melanocyte, breast, and myeloid) exposed to p53 inducing stress (UV irradiation, ionizing irradiation, and gemcitabine) found that ATF3 induction correlated with the expression of functional p53 [39-41]. In other studies, p53 recruitment to response elements in ATF3’s promoter was reported to be required for both UV and TPA induced ATF3 expression [36, 42]. Interestingly, in response to treatment with TPA, p53 also contributed to the expression of MEF2D, a factor known to be responsible for TPA induced activation of ATF3 [42].

Additionally, microarray analysis of genes expressed during different phases of the cell cycle indicate that ATF3 is regulated in a cell cycle-dependent manner with peak expression achieved during mid S-phase, suggesting that cell cycle-dependent regulatory elements found in ATF3’s promoter may be functionally significant [43]. Collectively, these findings suggest that cis elements located in ATF3’s promoter may convey responsiveness to a diverse spectrum of extracellular signals, in part through the activation of sequence specific transcription factors.

Given the wide range of extracellular stimuli capable of inducing ATF3, the current opinion is that ATF3 is generally induced following alterations in cellular homeostasis. Once expressed, ATF3 functions to facilitate cellular adaptation to the disturbance, thus allowing restoration of homeostasis. A partial list of specific extracellular stimuli known to induce ATF3 expression are listed in table 1.1 [12]. In general, these stimuli fall into 3 broad categories: cellular injury, growth signals, and changes in metabolic demand. Many signaling pathways activated in response to these
stimuli have been linked to the induction of ATF3, including MAPK pathways (JNK, p38), ER stress kinases, NF-κB, Smad, calcium, and hypoxia (Fig. 1.2). Evidence for the role of these pathways in the induction of ATF3 will be discussed below.

<table>
<thead>
<tr>
<th>Animal Models</th>
<th>Cultured Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tissue</strong></td>
<td><strong>Cell type</strong></td>
</tr>
<tr>
<td>Liver</td>
<td>Hepatocytes</td>
</tr>
<tr>
<td>Heart</td>
<td>Fibroblasts</td>
</tr>
<tr>
<td>Kidney</td>
<td>&amp; other cells</td>
</tr>
<tr>
<td>Skin</td>
<td>Myeloid cells</td>
</tr>
<tr>
<td>CNS</td>
<td>Macrophages</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Neuroblastoma</td>
</tr>
<tr>
<td>PNS</td>
<td>Islet Cells</td>
</tr>
<tr>
<td>Thymus</td>
<td>CHX, HGF</td>
</tr>
<tr>
<td></td>
<td>UV, IR, MMS</td>
</tr>
<tr>
<td></td>
<td>CHX, Aniso.</td>
</tr>
<tr>
<td></td>
<td>Hypoxia</td>
</tr>
<tr>
<td></td>
<td>Fas antibody</td>
</tr>
<tr>
<td></td>
<td>Cytokines, LPS</td>
</tr>
<tr>
<td></td>
<td>Forskolin</td>
</tr>
<tr>
<td></td>
<td>STZ, Cytokines,</td>
</tr>
<tr>
<td></td>
<td>Glucose, FFA</td>
</tr>
</tbody>
</table>

Table 1.1: A partial list of treatments known to induce ATF3 expression. All treatments resulted in increased steady-state levels of ATF3 mRNA.

**MAPKs:** Numerous reports have established a link between the stress response MAPK signaling pathways and ATF3 induction. The findings from many of these reports are summarized in table 1.2. From these results, two main conclusions can be drawn; (1) JNK and p38 contribute to ATF3 induction in a cell type and stimulus dependent manner and (2) ATF2/c-Jun, two down stream targets of MAPKs, play a role in converting the MAPK phosphorylation cascade into a transcriptional response.
<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Kinase</th>
<th>Result</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotoxic carcinogen</td>
<td>JNK</td>
<td>Correlatively induced ATF3 &amp; c-jun in epithelial &amp; T-cells.</td>
<td>[44, 45]</td>
</tr>
<tr>
<td>Ischemia/Reperfusion</td>
<td>JNK &amp; p38</td>
<td>Correlatively induced ATF3 in heart and kidney.</td>
<td>[46, 47]</td>
</tr>
<tr>
<td>Homocysteine (oxidation)</td>
<td>JNK</td>
<td>ATF2/c-Jun induced ATF3 in a MKK4/7 dependent manner in endothelial cells.</td>
<td>[37]</td>
</tr>
<tr>
<td>Ionizing radiation</td>
<td>JNK &amp; p38</td>
<td>Pathway inhibitors blocked ATF2 mediated ATF3 induction in fibroblasts.</td>
<td>[48]</td>
</tr>
<tr>
<td>Wound response</td>
<td>p38</td>
<td>Pathway inhibitor blocked ATF2 mediated ATF3 induction in keratinocytes.</td>
<td>[49]</td>
</tr>
<tr>
<td>TNFα</td>
<td>JNK &amp; p38</td>
<td>Pathway inhibitors blocked JNK induction and ERK repression of ATF3 in endothelial cells.</td>
<td>[50]</td>
</tr>
<tr>
<td>Anisomyosin</td>
<td>JNK, p38 &amp; ERK</td>
<td>p38 pathway inhibitor blocked ATF3 induction while constitutively active MKK6 induced ATF3 in HeLa, MEF, and Cos-1. JNK &amp; ERK were neither necessary nor sufficient to induce ATF3.</td>
<td>[51]</td>
</tr>
<tr>
<td>IL-1β</td>
<td>JNK</td>
<td>JNKi peptide blocked ATF3 induction in the INS832/13 β-cell line.</td>
<td>[7]</td>
</tr>
<tr>
<td>Anisomyosin</td>
<td>p38</td>
<td>p38 inhibitor blocked ATF3 induction in epithelial cells.</td>
<td>[52]</td>
</tr>
<tr>
<td>UV irradiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ricin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deoxynivalenol</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.2: A summary of the reports linking MAPK activation to ATF3 induction.

**ER Stress Kinases:** Professional secretory cells, such as the pancreatic β-cells, plasma cells, hepatocytes, and osteoblasts, utilize a highly evolved mechanism to properly fold and process secretory proteins passing through the endoplasmic reticulum (ER). In these cells, protein concentrations within the ER lumen are estimated to be extremely high, approximately 100mg/ml [53]. Under these conditions, ER homeostasis and function is maintained with the assistance of multifunctional ER chaperones, such as GRP78/Bip, which are capable of detecting the accumulation of unfolded proteins. Once activated, these proteins function to promote protein folding and to initiate the ER stress response.
Several ATF/CREB family members, including ATF3, are known to play a role in the cellular response to ER stress [56-59]. The induction of ATF3 by ER stress occurs through multiple pathways that separately involve phosphorylation of eIF2α, IRE1 activation, and possibly ERK activation. The accumulation of unfolded proteins in the ER lumen induces the release of the inhibitory interaction between GRP78 and initiators of the ER stress response, namely pancreatic eIF2 kinase (PEK) and inositol-requiring enzyme 1 (IRE1). In the absence of GRP78, PEK and IRE1 form homooligomers and become catalytically active. PEK activation results in a general translational block by phosphorylating the eukaryotic initiation factor 2α (eIF2α). Despite the general block in translation, eIF2α phosphorylation does permit selective translation of proteins that facilitate restoration of ER homeostasis. ATF4 is one of these proteins, and it functions in part through the induction of ATF3 [56]. In addition to PEK, three other eIF2α kinases have been reported. One of these kinases, GCN2, is activated by limitations in amino acid availability and leads to the ATF4 dependent induction of ATF3 [56, 60]. IRE1 activation results in a frame shift splice variant of XBP1 that encodes a transcription factor responsible for the induction of critical ER stress response proteins [61]. In addition to modulating its enzymatic function, IRE1 oligomerization also promotes the recruitment of the JNK activating adaptor protein TRAF2 to the cytoplasmic domain of IRE1 [54]. JNK activation through this IRE1/TRAF2 pathway contributed to the induction of ATF3 in vascular endothelial and pancreatic β-cells [57, 62]. ERK activation was recently implicated in ER stress response by microarray analysis of cells treated with farnesol, an isoprenoid alcohol known to induced apoptosis [58]. The microarray results revealed a large number of ER stress response genes,
including ATF3, were rapidly induced following farnesol exposure. Chemical inhibitors and siRNA targeted at the MAPK kinases revealed that activation of ERK kinase MEK1/2 is an upstream event in the induction of ER stress by farnesol. Collectively, these data indicate that ATF3 can be induced by multiple pathways tied to ER stress response.

**NF-κB:** Many of the extra cellular stimuli capable of initiating the MAPK signaling cascade are also known to induce the NF-κB pathway. For example, the β-cell relevant pro-inflammatory molecules IL-1β, TNFα and IFNγ all activate MAPK and NF-κB signaling. The intracellular integration of extracellular signals through multiple signaling pathways is generally accepted to be a critical cell fate determinant. While JNK and p38 typically favor the induction of pro-apoptotic molecules, NF-κB signaling traditionally promotes cellular survival by initiating transcription of anti-apoptotic molecules. However, in β-cells, NF-κB appears to play a biphasic role in promoting both survival and apoptosis [63-65]. While the specific mechanisms employed by NF-κB to mediate its pleiotropic functions are still being characterized, recent correlative findings indicate that ATF3 expression may be one of the many down stream consequences of NF-κB signaling [7, 50]. As mentioned previously, several NF-κB response elements have been identified in the promoter of ATF3. Two lines of evidence suggest that these response elements may be functionally relevant: (1) ATF3 mRNA abundance correlated with NF-κB activity in HUVECs treated with TNFα, and (2) modulation of NF-κB activity by genetic and chemical inhibitors in the INS832/13 β-cell line dampened the induction of ATF3 by IL-1β. However, in this latter report, JNK inhibition almost entirely blocked
ATF3 induction by IL-1β. Collectively these results indicate that while signaling through both NF-kB and MAPK contribute to ATF3 expression, MAPK signaling may play a dominant role. The observation that ATF3 expression is regulated by both pro-survival and pro-apoptotic signaling pathways leaves open the interesting possibility that ATF3 may play a role in the determination of cell fate following extracellular stimulation.

**Smad:** Five recent reports, as well as unpublished findings from our lab, have provided evidence that signaling through the TGFβ pathway is capable of inducing ATF3. From these studies, ATF3 was found to be induced in epithelial cells either by TGFβ or TGFβ inducing treatments [38, 66-69]. TGFβ was observed to directly target the transcription of ATF3 through (1) in vivo binding of Smad2/3 and Smad4 proteins to the TGFβ responsive region of the ATF3 promoter, and (2) the inability of cyclohexamide to block TGFβ induction of ATF3 mRNA [38]. Cyclohexamide is frequently used as a general inhibitor of translation. Therefore, the induction of ATF3 mRNA by TGFβ in the presence cyclohexamide suggests that TGFβ can regulate the transcription of ATF3 independent of de novo protein synthesis. This emphasizes a direct role for the activation of Smad proteins in TGFβ induction of ATF3. Consistent with these results, over expression of constitutively active Smad3 was sufficient to induce ATF3 protein levels [68]. However, in addition to activating the Smad pathway, TGFβ treatment is also known to induce signaling through the MAPK pathway. Unpublished findings from our lab show that specific MAPK pathway inhibitors do not block the induction of ATF3 by TGFβ. These findings further support the notion that TGFβ signaling through Smad proteins is capable of regulating ATF3 expression.
**Calcium:** Calcium is an essential element for living organisms. As a major component to skeletal bones, calcium is the most abundant mineral by mass in humans. In addition to its structural function, calcium plays an essential role in cell physiology where movement of calcium across cellular membranes initiates signaling that regulates diverse cellular processes. Calcium influx results in the activation of adenylate cyclase, thus giving rise to the second messenger cAMP, which binds to and activates Protein Kinase A (PKA). The catalytic subunit of PKA then goes on to phosphorylate numerous downstream targets which ultimately mediate the transcriptional response of calcium signaling. Several recent reports have shown that ATF3 is induced by calcium signaling, primarily through cAMP and MAPK activation. In the first, phosphorylation of CREB by PKA was found to induce the expression of ATF3 in adipocytes treated with forskolin, an activator of adenylate cyclase [24]. In this report, CREB was demonstrated to directly regulate ATF3 expression by (1) the observation that it interact with the ATF/CRE in ATF3’s promoter in ChIP and (2) the use of a dominant negative CREB, A-CREB, which blocked forskolin induction of ATF3. Similarly, a second report found that activation of PKA in cardiac myocytes by forskolin or adenylate cyclase type VI resulted in the induction of ATF3 [70]. In addition to the conventional calcium signaling pathway that stimulating cAMP production, calcium signaling can also activate MAPK signaling [71, 72]. The calcium dependent activation of JNK, p38 and ERK signaling has been observed to induce ATF3 in pituitary gonadotroph and cancerous breast epithelial cell lines [73, 74]. Interestingly, the induction of ATF3 in pituitary gonadotroph cells by ERK and JNK was blocked by the expression of dominant negative Egr-1, CREB, or ATF2. This result suggests the possibility that diverse upstream calcium signaling events
may converge to activate common downstream transcription factors. Collectively these findings indicate that ATF3 can be induced through multiple calcium regulated signaling pathways.

**Hypoxia:** The induction of ATF3 following hypoxia or ischemia has been demonstrated to occur in numerous types of cells and tissues. [12, 46, 47, 75, 76] Several signaling pathways are initiated by the cells adaptive reaction to hypoxia, most notably NF-κB and HIF-1α [77-79]. Hypoxia was first observed to activate NF-κB signaling in 1996 where it was found that phosphorylation of IκBα tyrosine 42, rather than the traditional serine 32/36 residues, induced the release of this inhibitory subunit from the p65/p50 heterodimer [77, 80]. Interestingly, IκBα tyrosine 42 phosphorylation only disrupted the interaction between IκBα and p65/p50 and did not induce its traditional ubiquitin mediated proteosomal degradation. Src/Ras/Raf were implicated in the hypoxic response leading to IκBα phosphorylation. Once activated by hypoxia, NF-κB contributes to the production of pro-angiogenic factors as well as the nitric oxide (NO) producing enzyme iNOS [81-83]. In a separate study, ATF3 was shown to be induced by NO in a JNK dependent manner, suggesting that ATF3 induction by hypoxia may be downstream from NF-κB activation [84, 85]. Interestingly, while the ATF3 promoter contains at least one HIF1α response element (TCAGGT, ~ -1700bp from TSS), activation of the conventional hypoxic response through HIF1α stabilization does not appear to contribute to ATF3 induction, as ATF3 protein was found to be induced by hypoxia in wild type and HIF1α knock out MEFs to an equal degree [85]. Overall, these findings support the
notion that ATF3 expression can be induced by the adaptive response to hypoxia and in large part is regulated by the production of nitric oxide.

A.4 Transcriptional activity of ATF3

The DNA binding and transcriptional activity of ATF3 is dramatically influenced by the protein with which it forms a dimer. As a homodimer, ATF3 does not function as an activator of transcription as implied by its name, but rather represses transcription [9, 21]. This discrepancy between nomenclature and function arose from the original assumption that ATF represented only one protein [1]. The subsequent realization that ATF actually represented a large family of genes, some of which were activators while others were repressors, made the nomenclature somewhat confusing. However, despite its intrinsic potential to repress transcription, selective heterodimerization with other bZip proteins allows ATF3 to function as a repressor or activator of transcription. Specifically, ATF3 dimerization with ATF2, c-Jun, JunB, JunD or the HTLV-1 Tax proteins has been found to promote both transcriptional activation or repression depending on the promoter context [11, 19, 22, 25, 28, 86-88]. In addition to this mechanism of DNA dependent transcriptional regulation, ATF3 has also been found to non-specifically promote transcriptional activation by sequestering co-repressors away from promoters [21].

While it is likely that the majority of ATF3 target genes have yet to be identified, several are known and they are summarized in table 1.3.
### Induced by ATF3

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Cell Type</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proenkephalin</td>
<td>SK-N-MC</td>
<td>[25, 28]</td>
</tr>
<tr>
<td>Hif2α</td>
<td>HaCaT/Hela</td>
<td>[89]</td>
</tr>
<tr>
<td>TNFR-SF12a</td>
<td>TFNSF7 TFNSF10</td>
<td></td>
</tr>
<tr>
<td>Twist1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibronectin1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAI1 uPA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caveolin1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Slug</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insulin</td>
<td>β-cells</td>
<td>Presented in this dissertation</td>
</tr>
<tr>
<td>NOXA bNIP3</td>
<td>β-cells</td>
<td>Presented in this dissertation</td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td>IL-6</td>
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<tr>
<td>IL-12b</td>
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<td>IL-6</td>
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<tr>
<td>CCL2</td>
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<td>Tissue Factor</td>
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<tr>
<td>NOXA</td>
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<tr>
<td>multiple myeloma &amp; mantle cell lymphoma</td>
<td>[94]</td>
<td></td>
</tr>
<tr>
<td>TGFβ</td>
<td>MCF10CA1a</td>
<td>Hai Lab Unpublished</td>
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</tbody>
</table>

### Repressed by ATF3

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Cell Type</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>gadd153/chop10</td>
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<td>[9]</td>
</tr>
<tr>
<td>IRS2</td>
<td>β-cells</td>
<td>[26]</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>3T3-L1</td>
<td>[90]</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>GLUT-4</td>
<td>Adipose Tissue [24]</td>
</tr>
<tr>
<td>TNFα</td>
<td>IL-12b IL-6</td>
<td>Macrophages [30]</td>
</tr>
<tr>
<td>NOXA bNIP3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNFα</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
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</tr>
<tr>
<td>CCL2</td>
<td></td>
<td></td>
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<tr>
<td>E-selectin</td>
<td>Endothelial</td>
<td>[95]</td>
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<tr>
<td>CCL4</td>
<td>Macrophages</td>
<td>[96]</td>
</tr>
<tr>
<td>Collagenase</td>
<td>MMP-2</td>
<td>Prostate, Endothelial &amp; Saos-2 [97-99]</td>
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<td>Epithelial</td>
<td>[38]</td>
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<tr>
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<td>HUVECs</td>
<td>[100]</td>
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<tr>
<td>Cyclin D1</td>
<td>Chondrocytes</td>
<td>[101]</td>
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<td>Cyclin A</td>
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<tr>
<td>PEPCK</td>
<td>Liver Tissue</td>
<td>[102]</td>
</tr>
</tbody>
</table>

**Table 1.3:** Summary of known ATF3 target genes. Genes are grouped in two main categories based on whether ATF3 activates or represses their expression.
Collectively, these results suggest that ATF3 contributes to a diverse range of cellular processes; including inflammation, metabolism, apoptosis, cell cycle regulation, differentiation, and tumor metastasis.

Beyond the transcriptional regulation of ATF3 and the dimerization requirement before binding DNA, little is known about other mechanisms that may regulate ATF3 function. Preliminary data from our lab suggests that ATF3 is a substrate for JNK phosphorylation, however the functional significance of this post-translational modification has yet to be elucidated.

In conclusion, a wide range of stimuli are capable of inducing ATF3 through a variety of cellular pathways. Once expressed, ATF3 may promote or repress transcription depending on the promoter context and ATF3’s dimerization partner. Functionally, ATF3 contributes to cellular adaptive response by regulating the expression of genes involved in diverse cellular processes.

A.5 ATF3, an adaptive response gene

The traditional view of ATF3 has been one of a stress response or immediate early gene. This is due to the fact that the original stimuli found to induce ATF3 expression all resulted in cellular damage and were therefore viewed as stress signals [12]. However in recent years, the conventional view of ATF3 is giving way to a more complex understanding of the biological significance of ATF3 expression. Emerging evidence suggests that depending on the cellular context, ATF3 can function dichotomously by contributing to opposite biological processes within the cell. Two
examples of this dichotomy of ATF3 include its role in apoptosis and breast cancer tumor development.

Considerable evidence exists that supports both the pro- and anti-apoptotic effects of ATF3. The pro-apoptotic role of ATF3 has been established through both gain- and loss-of function studies. When ectopically expressed in vitro, ATF3 was found to enhance the apoptotic effects of etoposide, camptothecin and curcumin in HeLa cells and serum deprivation in MCF10A cells [27, 103, 104]. Furthermore, transgenic expression of ATF3 in the heart, liver and pancreatic ductal epithelium of mice led to functional and developmental defects in those tissues [76, 105]. Consistent with these findings, blocking the expression of ATF3 has been shown to attenuate stress induced death. Knocking down ATF3 expression protected both the MIN6 and INS832/13 β-cell lines from cytokine and glucolipotoxicity induced apoptosis [26]. Endothelial cells were also protected from cytokine and lipid induced death when ATF3 expression was reduced [8]. Likewise, pancreatic islets isolated from ATF3 knock out mice showed reduced cell death when exposed to a wide range of stresses, including isolation stress, hypoxia, cytokine treatment, and transplantation ([7] and work presented in this dissertation). However, ATF3 has also been reported to mediate a protective response to stress stimuli. In neuronal cells, ectopic expression of ATF3 reduced cell death induced in vitro by neuronal growth factor (NGF) withdrawal and in vivo by cerebral injection of Kainic acid [106, 107]. In endothelial cells and cardiac myocytes, two cell types in which ATF3 was shown to promote apoptosis, ATF3 was also found to promote survival. For example, ectopic expression of ATF3 reduced apoptosis in HUVECs treated with TNFα and cardiac myocytes treated with doxorubicin [100, 108]. Similarly, a protective role for
ATF3 was observed in ATF3 knock out mice challenged with lipopolysaccharide (LPS) or the ovalbumin allergen. In these studies, ATF3 was found to dampen the inflammatory response of immune cells. By dampening the macrophage toll-like receptor 4 (TLR4) dependent inflammatory response to acute LPS treatment, ATF3 protected the mice from a lethal systemic hyper-inflammatory reaction [30]. In CD4(+) lymphocytes, ATF3 functioned to attenuate ovalbumin-induced airway inflammation and hyperresponsiveness, thus affording protection to mice in a model of human allergic airway disease [29]. Therefore, based on the protective and deleterious function described by these findings, ATF3 plays a dichotomous role in cellular and organismal survival. Presumably, the dichotomy of ATF3 is in part possible given the context-dependent environments established within the cell.

This dichotomy of ATF3 has also been observed in breast cancer development. Several lines of evidence support a dichotomous function of ATF3 in cancer. First, ATF3 is known to play a role in two cellular processes critical for the development of cancer, apoptosis and proliferation. Importantly, ATF3 was found to both promote and suppress these processes [104, 108-110]. Secondly, in a xenograft cancer model, ATF3 was observed to function either as a tumor suppressor or an oncogene in various cell lines [111-114]. Additionally, ATF3 was found to play a role in mediating the effects of TGFβ, a molecule previously identified to have a dichotomous function in cancer progression [38]. Finally, to account for the variables introduced by the use of different cell lines and models in the previous studies, Dr. Xin Yin, a recent graduate student from this lab, sought to test the dichotomy of ATF3 using an isogenic breast cancer cell system. Findings from Dr. Yin’s work supported a dichotomous role of ATF3, where
ATF3 was pro-apoptotic in untransformed breast epithelial cells, and yet promoted cell cycle progression, motility and invasiveness in aggressive epithelial breast cancer [27].

Recent evidence, including the above mentioned work supporting a dichotomous function of ATF3, suggests that the traditional view of ATF3 as a stress response gene is overly simplistic. Rather, the evolving view is that ATF3 represents an adaptive response gene used by cells to remedy an underlying cellular disturbance or alternatively to induce apoptosis. This change in paradigm is also driven in part by the observation that some of the ATF3-inducing signals can not be classified as stress signals. Examples of ATF3 inducing stimuli that are not traditional stress signals include adipokines, S-phase transition, serum, and glucose [7, 43, 115-117]. Furthermore, the broad spectrum of signaling pathways that connect ATF3 induction to extra- and intra-cellular disturbances in homeostasis suggests that ATF3 may represent a signaling “hub”. Therefore, through the induction of ATF3, multiple signaling pathways may be integrated to yield greater functional diversity in cellular response.

A.6 Conclusion

ATF3 is an early response gene that gives rise to a protein that has primarily been described to function as a transcription factor. Through selective interaction with other bZip proteins, ATF3 regulates the expression of a diverse set of genes. Due to the broad range of extra and intra-cellular stimuli capable of inducing ATF3 and the fact that its transcriptional activity is influenced by selective dimerization, ATF3 is capable of pleiotropic effects that have influence on cellular survival, growth, metabolism and malignant progression. Work presented in this dissertation characterizes the functional
significance of ATF3 expression in β-cells exposed to two physiologically relevant stress paradigms; islet transplantation and high fat diet induced type 2 diabetes. β-cells are integral to the maintenance of glucose homeostasis due to their ability to produce and secrete insulin. Given that β-cell stress induced death plays a central role in the development and treatment of diabetes, the following section provides a summary of our current knowledge of type 1 and type 2 diabetes.

B.1: Epidemiology of diabetes

The Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO) have reported that 23.6 million people (7.8%) in the United States and 171 million people (5.4%) world wide have diabetes [118, 119]. Each year in America alone, approximately 1.6 million new cases are diagnosed. The WHO estimates that 366 million people world wide will have diabetes by 2030. In 2005, diabetes was the 6th leading cause of death in the United States and was responsible for $174 billion dollars in direct and indirect medical costs [120]. While diabetics have twice the risk of death as non-diabetics, they also suffer from complications such as high blood pressure, heart disease, stroke, blindness, kidney disease, amputation, biochemical imbalances, and complications of pregnancy. Due to the cost, both financially and in human life, and the rate at which diabetes is spreading qualifies it as a serious public health issue and justifies continued research into its treatment and prevention. In the remaining part of this section I will summarize our understanding of the two forms of diabetes, type 1 (insulin dependent) and type 2 (insulin independent), with an emphasis on etiology, the role of β-cell stress response, and current therapeutic options.
B.2: **Type 1 diabetes**

Type 1 diabetes (T1DM) is generally accepted to be a multifactorial disease of autoimmunity that affects individuals predisposed by genetic susceptibilities. In these individuals, environmental triggers are believed to instigate autoimmune mediated destruction of the insulin secreting pancreatic β-cells. This β-cell deficit gives rise to a dependence on exogenous insulin for maintaining metabolic homeostasis. Currently, there are 3 million Americans afflicted with this type of diabetes, and each year an additional 15,000 citizens under the age of 20 are newly diagnosed [118]. T1DM can occur at any age, however it is most commonly diagnosed in individuals under the age of 30. While a small number of genetic markers are known to predict 90% of all T1DM cases, the utility of this information is limited because all clinical trials testing potential preventative treatments have had little or no effect on diabetic onset [121]. Several therapeutic strategies employed after the onset of diabetes have produced encouraging results; however there currently are no known treatments that cure the underlying immune disorder. As a result, β-cell replacement therapy has emerged in recent years as a viable treatment option for T1DM. Research presented in this dissertation, in part, characterizes a role for ATF3 in the biology of β-cell replacement therapy and illustrates the therapeutic potential of blocking ATF3 expression.

B.2.1: **Disease of autoimmunity**

Type 1 diabetes is believed to be an autoimmune disease due to the presence of islet-infiltrating T cells, detectible circulating levels of anti-islet antibodies, and a strong genetic association with loci of the major histocompatibility complex (MHC). This view
of T1DM as an autoimmune disorder has been supported by the identification of several β-cell specific auto-antigens in diabetic humans and rodents. While at least 15 distinct β-cell derived peptides have been identified as auto-antigens, the three major antigens are believed to be insulin, glutamic acid decarboxylase isoform 65 (GAD65), and protein tyrosine phosphatase (ICA512) [122]. Both the corresponding CD4+ T cells and antibody producing B cells for insulin and GAD65 have been extensively studied. While most data indicate that T cells are the primary mediators of β-cell death, B cells are also implicated in the pathogenesis of the disease. The role for B cells is evidenced by the presence of antibodies to insulin and GAD65 preceding overt diabetes in 70% of all cases [123, 124]. However, data generated using both gain of function (adoptive transfer of insulin-specific T cells) and loss of function (orally induced tolerance to insulin) approaches, indicate that T cells are sufficient and necessary for the induction of diabetes in a mouse model of T1DM, the non-obese diabetic (NOD) mouse [125, 126]. Consistent with these findings, real-time PCR studies indicate that insulin expression in the thymus of NOD mice, is approximately half of that seen in the thymus of non-diabetic strains [127]. These findings implicate defects in the negative selection of self-reactive T cells in the development of T1DM and underscore the importance of T cells in mediating the autoimmune disorder.

Limited information is available regarding the auto-antigens recognized by the immune system in T1DM. Insulin and GAD65 contain the epitopes most often recognized by diabetic auto-reactive T cells. To date, four epitopes on insulin have been observed to activate self-reactive T cells [128]. One frequently recognized epitope maps to amino acid residues 9-23 on the B chain of insulin. The relevance of insulin as an
auto-antigen was recently emphasized by the finding that a peptide derived from the B9-23 sequence could be used to induce tolerance in a colony of NOD mice and prevent the disease entirely [126, 129]. Furthermore, examination of insulin reactive T cells has identified a specific T cell receptor (TCR) β-chain rearrangement that leads to the expression of the Vα 13.3 locus [130]. This locus is believed to have enhanced binding affinity for the B9-23 peptide. Efforts aimed at identifying antigenic epitopes within GAD65 have been less successful. While initial reports suggested that several fragments of GAD65 were capable of activating T cells early in the disease process, subsequent studies found that GAD65 reactive T cells isolated from peripheral lymph organs in diabetic mice were unresponsive when co-cultured with isolated islets [131-133]. Furthermore, adoptive transfer of GAD65 reactive T cells was non-pathogenic [133]. Despite these conflicting findings, a potential role for GAD65 as an auto-antigen was ultimately supported by the finding that tolerance induced with a GAD65 peptide was capable of reducing the incidence of diabetes in NOD mice [132, 134]. In addition to insulin and GAD65, several other auto-antigens have been identified in both mouse and man and these antigens are summarized in table 1.4.

Prior to the onset of T1DM, several types of effector cells contribute to the initiation of the immune response by processing and recognizing auto-antigens produced by the islets. Pancreatic biopsies from patients recently diagnosed with T1DM indicate that islets encounter aggressive infiltration by macrophages, B cells, and T cells (CD4+ and CD8+) [135]. It is generally accepted that macrophages and B cells play a key role in initiating the immune response that leads to T cell activation and islet death. The specific functions of each effector cell type are discussed below.
Candidate auto-antigens in Type 1 Diabetes Mellitus

Insulin
Glutamic Acid Decarboxylase Isoform 65kD (GAD65)
protein tyrosine phosphatase (ICA512)
Phogrin (IA-2β)
Retroviral Envelope Protein (EAD1)
Insulinoma Cell Membrane Protein (IA-2)
Bovine Serum Albumin (BSA)
Glucose Transporter 2 (GLUT2)
Heat Shock Proteins 62 and 65 (HSP62/65)
Gangliosides
Carboxypeptidase H
Islet Amyloid polypeptide (IAPP)

Table 1.4: Auto-antigens associated with Type 1 Diabetes. Several self-antigens have been identified in mouse and man to be associated with T1D. These islet produced auto-antigens are the targets or T and B cell mediated immune responses. While as many as 15 auto-antigens have been identified, the three most common self-antigens are insulin, GAD65, and ICA512.

Macrophages: Macrophages play an essential role in the development of diabetes. Histological analysis of diabetic pancreata indicate that macrophages are among the first cell types to infiltrate the islets in BB rats and NOD mice [136, 137]. The observation
that diabetes can be prevented by depleting macrophages in NOD mice further emphasizes the importance of macrophages to the onset of diabetes [138, 139]. Upon infiltration of the pancreas, macrophages are known to induce β-cell death through local secretion of pro-apoptotic factors such as IL-1β, TNFα, and Nitric oxide. Macrophages also promote β-cell death by contributing to T cell activation in two ways. First, as macrophages encounter and phagocytize β-cell antigens, the antigens are presented to T cells in draining regional lymph nodes and the spleen [139]. Second, the T cells capable of responding to the antigen presentation are further activated by IL-12 secreted from the macrophages. IL-12 specifically stimulates the activation of Th1 type CD4+ T cells.

**T Cells:** T cells are generally accepted to be the main effectors of β-cell death in T1DM. Once activated by macrophages and other antigen presenting cells, the CD4+ T cells promote a broader inflammatory response by secreting IFNγ and IL-2 [140-142]. IFNγ functions to enhance macrophage β-cell toxicity by inducing the secretion of IL-1β, TNFα, and free radicals from local resting macrophages. At the same time, IL-2 secretion from the CD4+ T cells promotes the recruitment of CD8+ pre-cytotoxic T cells to the site of the inflamed islets [143]. Once recruited, the CD8+ T cells recognize β-cell specific auto-antigens presented by macrophages and CD4+ T cells and differentiate into mature cytotoxic effector T cells. The cytotoxic T cells inflict widespread β-cell damage through the initiation of Fas-mediated apoptosis and the secretion of perforin and granzyme molecules.

**B Cells:** B cells are known to play an important role in the development of T1DM; however the mechanism of their contribution has yet to clearly be defined. It is known
that NOD mice deficient in B cells rarely become diabetic [144]. However, the adoptive transfer of auto-reactive T cells from diabetic NOD mice are still pathogenic when delivered to B cell deficient NOD recipients [145]. This finding suggests that B cells most likely play a role in the early stages of the disease and are not required once T cell activation has occurred. The known functions of B cells suggest two possible mechanisms for their involvement in the development of diabetes. First, given that B cells are the main immunoglobulin secreting cells of the body and that the presence of auto-antibodies serves as a strong predictor of disease development, B cells may contribute to the disease through the production of auto-antibodies. This notion is supported by the observation that depletion of maternally transmitted antibodies prevented diabetes in NOD mice [146]. Auto-antibodies are believed to promote the disease by enhancing dendritic cell presentation of antigens, both through improved uptake of specific auto-antigens and by increasing the availability of islet cell antigens by promoting tissue damage [147]. The second mechanism by which B cells may contribute to the development of diabetes is through their antigen presenting capability. While B cells do not present antigens as efficiently as many other dendritic cells, their ability to bind to specific auto-antigens with high affinity results in selective activation of complementary auto-reactive T cells [148]. Currently, the relative contribution of these two B cell functions towards the development of diabetes has not been clearly described. However, in one study the repopulation of B cell deficient NOD mice with transgenic B cells incapable of secreting antibody did increase the frequency of T1DM, albeit not to the same extent seen in NOD mice with wild type B cells [147]. This finding suggests
that both the immunoglobulin secreting and antigen presenting function of B cells play a role in the development of diabetes.

**B.2.2: Genetic predispositions**

T1DM is generally accepted to be a polygenic disease, in that affected individuals are predisposed by the inheritance of a “phenotype” that varies in degree based on the interaction of 2 or more quantitative trait loci (QTL) and the environment. QTLs are not necessarily individual genes, but are rather stretches of DNA closely linked to the genes that underlie the inherited phenotype. In this case, the inherited phenotype is the autoimmune condition of diabetes. Table 1.5 summarizes the QTLs identified through various genetic screens to be linked to insulin dependent diabetes mellitus (IDDM) [149].

While no single gene from this table is either necessary or sufficient for diabetic development, the genes located within the IDDM1 locus play a major role in conferring T1DM susceptibility. The region is proposed to account for up to 40-50% of inherited risk, due in large part to specific allelic variants of the human leukocyte antigens (HLA) [150, 151]. HLA gene products give rise to the proteins of the antigen presenting major histocompatibility complexes found on the surface of most cells in the body. One allelic variant in particular, HLA-DQB1*0302, is recognized as the most prevalent risk conferring allele as it is detected in 74% of Caucasian T1DM patients [152]. Consistent with the polygenic classification of T1DM, additional genes are known to modify the risk associated with this DQB1*0302 allele. Of note, the HLA-DR3 and/or HLA-DR4 genes are associated with increased risk while HLA-DR2 appears to protect against T1DM [153].
<table>
<thead>
<tr>
<th>Locus</th>
<th>Chromosome</th>
<th>Candidate Genes/Microsatellites</th>
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</thead>
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<tr>
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<td>HLA-DQ/DR</td>
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<tr>
<td>IDDM2</td>
<td>11p15*</td>
<td>INS VNTR</td>
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<tr>
<td>IDDM3</td>
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<td>IDDM4</td>
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<td>IDDM5</td>
<td>6q24-27</td>
<td>ESR, MnSOD</td>
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<tr>
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<td>18q12-q21</td>
<td>D18s487, D18s64, JK (Kidd locus)</td>
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<td>IDDM7</td>
<td>2q31</td>
<td>D2s152, IL-1, NEUROD, GALNT3</td>
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<td>D3s1303</td>
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<td>D10s193, D10s208, D10s588</td>
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<td>14q24.3-q31</td>
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</tr>
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<td>D2s137, D2s164, IGFBP2, IGFBP5</td>
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<tr>
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</table>

**Table 1.5: Quantitative Trait Loci associated with IDDM.** This table summarizes the known loci and candidate genes associated with type 1 diabetes. No single locus is necessary or sufficient for the development of diabetes. However, the two most frequently associated loci map to chromosomal regions that encode major histocompatibility molecules and insulin.

The exact mechanism by which HLA variants modify T1DM susceptibility is not known; however structure function analyses have revealed some insight into the process.

As mentioned above, the HLA gene products most frequently associated with T1DM...
susceptibility are class II antigen presenting MHC molecules. This suggests that professional antigen presenting cells (APC) may influence the pathogenesis of the disease through both the inappropriate selection of self-reactive T cells in the thymus and their subsequent activation in the periphery. Gerald Nepom and William Kwok have proposed a four step pathway in which APCs play a role in the pathogenesis of autoimmune diabetes [154]. In the first step, sequence variations in the MHC molecules of susceptible individuals give rise to reduced communication between APCs and T cells. At the time Nepom and Kwok proposed their pathway, peptide binding affinity assays had suggested that specific DQ polymorphisms could play a critical role in establishing the nature of bound antigen [155]. In the case of the diabetes susceptibility allele, HLA-DQB1*0302, an aspartic acid to alanine variation at residue 57 was observed to alter its peptide binding affinity via reduced charge interaction with the peptide’s anchor residue 9. The altered affinity for peptide binding weakened the stability of the peptide-MHC complex and led to a reduced interaction with peptide specific T cell receptors (TCRs). This would allow potentially self reactive T cells to escape the negative selection process within the thymus, as only strong interactions between self-antigen presenting MHC complexes and TCRs will elicit T cell death. In the second step, environmental or viral stimuli lead to the amplification of self reactive T cells. As a consequence of this amplification, the number of self-reactive T cells increases above a threshold required for subsequent pathological events. This step highlights a potential role for antigen mimicry in contributing to the onset of diabetes. Antigens such as bovine serum albumin or proteins from the coxsackie virus are known to cross react with islet antigens and therefore may play a role in amplifying the self-reactive T cell repertoire [156]. The third step involves
the recognition of self antigens and subsequent activation of T cells in the islet compartment. Consistent with interactions between the peptide-MHC and TCR in the thymus, the affinity of the antigenic peptide for the MHC binding pocket influences T cell activation in the periphery. However, additional factors contribute to T cell fate in the periphery. Tissue damage and inflammatory stimuli lead to local secretion of cytokines and chemokines that recruit additional T cells and enhance antigen presentation on the targeted tissue. These additional stimuli in the periphery are believed to contribute towards the progression of diabetes in predisposed individuals. Finally, in the fourth step, an as of yet unidentified regulatory mechanism fails and affected individuals present clinically with the disease. While the specific regulatory mechanism suggested by Nepom and Kwok to function by repressing the development of autoimmune disease has not yet been identified, they speculate that one exists because relatives of diabetic individuals who themselves are autoantibody positive and have some compromised insulin secretory function ultimately do not progress into diabetes. Recently, it was suggested that regulatory T cells may play a role in slowing or halting the progression of the autoimmune response [157].

In addition to HLA risk factors, other non-MHC alleles are associated with diabetic susceptibility, including insulin (IDDM2), MnSOD (IDDM5), and CTLA4 (IDDM12) (Table 1.5). The IDDM2 locus is of particular interest because it contains a gene only expressed in the pancreatic β-cells; insulin. Since T1DM involves an autoimmune reaction that specifically targets the pancreatic β-cells, it is believed that the self antigen recognized by the immune system must have β-cell restricted expression. While point mutations of the INS gene are rarely found in diabetics, polymorphisms in
the variable number tandem repeat (VNTR) located 596bp upstream from the translational start site appear to influence diabetic risk [158]. The INS-VNTR is a non-coding region with tandem repeats of the sequence “ACAGGGGTCTGGGG” that plays an important role in regulating insulin expression in the thymus and pancreas. Individuals with a larger number of repeats (Class III, 140-200 repeats) demonstrate increased insulin expression and protection from diabetes while individuals with a smaller number of repeats (Class I, 26-63 repeats) are more susceptible to the disease [159, 160]. It has been proposed that the higher level of thymic insulin expression observed from class III alleles allows for enhanced negative selection of insulin reactive T cells. Therefore, individuals with the class I insulin allele may be more susceptible to the development of insulin reactive T cells, in part, due to reduced opportunities for negative selection to occur. The IDDM5 locus contains a region that encodes the gene for manganese superoxide dismutase (MnSOD). Recent evidence supports a protective role for MnSOD in β-cell stress response, where MnSOD activity dampened the apoptotic response to inflammation and injury, presumably due to reduced levels of free radicals [161, 162]. The variant alleles of MnSOD associated with T1DM are known to have reduced activity and therefore may contribute to diabetic susceptibility by enhancing stress induced β-cell apoptosis [163]. The IDDM12 locus encodes several genes of relevance to T1DM as they function as T cell costimulatory receptors and control the T cell proliferative response. One notable gene in this region is the cytotoxic T lymphocyte antigen 4 receptor (CTLA-4). CTLA-4 is a glycosylated transmembrane protein expressed on activated T cells and functions through binding the B7 co-receptor to limit the T cell proliferative response. In type 1 diabetics, several polymorphisms
within the promoter and coding region of CTLA-4 have been identified to reduce gene expression levels [164]. Therefore, through reduced expression of CTLA-4 in susceptible individuals, constitutive T cell activation may result in proliferation of self-reactive T cells and further increase the risk of diabetic onset.

In conclusion, investigations into candidate genes associated with IDDM loci have revealed insight into the disease, making its polygenic nature more tangible. While much remains to be learned, our current understanding of the genetic basis of diabetes illustrates a clear relationship between genetic and environmental factors that are capable of promoting both the selection of self-reactive T cells and sensitizing β-cells to stress induced death.

**B.2.3: Role of β-cell stress response**

In the progression towards overt autoimmune diabetes, the pancreatic β-cells are not bystanders passive to their own demise. Rather, β-cells are active participants responding to the inflamed environment within the pancreas. With the onset of insulitis, the macrophages, neutrophils, and T cells that have infiltrated the pancreas provoke a β-cell response through both humoral and cell mediated mechanisms. The remainder of this section will review our current understanding of the molecules used by immune cells to effect the humoral and cellular response, the signaling pathways that play a role in the β-cell response to these molecules, and finally the transcriptional response that ultimately determines β-cell fate.

**Humoral and cellular responses:** In the classical sense, humoral immunity is defined as the secretion of pro-inflammatory cytokines or antibodies from immune cells
that promote target cell death or neutralize antigens in the absence of direct cell to cell contact. While this classical humoral response ensues with the onset of T1DM, recent reports indicate that the islet are also producing inflammatory mediators [165]. Therefore, the evolving view of the humoral response in diabetes is that local secretion of pro-inflammatory cytokines, including IL-1β, TNFα, and IFNγ, induces a response in the islets that promotes diabetes via three mechanisms: (a) enhancing the local inflammatory response, (b) directly promoting islet cell apoptosis, and (c) sensitizing β-cells to cell mediated apoptosis. With respect to the first mechanism, as discussed in section B.2.1 above, the positive feedback loop between immune cells leading to robust local inflammatory responses has been well characterized. Macrophage and neutrophil secretion of IL-2, IL-8 and IFNγ each have been demonstrated to promote the local inflammatory response. More recently, islets have been implicated in this process as well. In response to exposure to IL-1β, TNFα, and IFNγ, islets secret a number of chemotactic factors that promote the recruitment and activation of additional immune cells. Some of these factors include IP-10, MCP-1, MIP-3a, CXCL9, CXCL10, and IL-15 [165-169]. In addition, it has also been observed that insulitis induces the expression of FasL by the islets [170]. While this response may in part be a survival effort by the islets to induce apoptosis in the infiltrating T cells, the mechanism by which will be discussed below, it also has the undesirable effect of promoting local inflammation. Matrix metalloproteinases cleave FasL from the surface of the islets to generate soluble FasL, a potent neutrophil chemo attractant [171]. The recruitment of more neutrophils by soluble FasL leads to increased local IL-8 secretion, further enhancing the recruitment of neutrophils and macrophages in what has been termed a “vicious cycle” of inflammation.
In support of this notion, the ectopic expression of FasL in islets by the rat insulin promoter was sufficient to induce diabetes in mice due to robust neutrophil-mediated inflammation [171]. In the second mechanism used by the humoral response, IL-1β, TNFα, and IFNγ directly induce β-cell apoptosis through both transcription dependent and independent pathways. Ultimately, the induction of apoptosis through these pathways requires activation of effector caspases secondary to mitochondrial release of cytochrome C and activation of endoplasmic reticulum (ER) stress response [172, 173]. The transcription dependent mechanism will be discussed in a subsequent section (page 44). The transcription independent pathway involves activation of TNF death receptor signaling where the TRADD/TRAF-2/FADD complex facilitates the conversion of pro-caspase8 to cleaved caspase8 [174]. Activated caspase8 cleaves the cytosolic Bid protein to give rise to mitochondrial localized tBid, a potent activator of the mitochondrial death pathway. The important role for TNFα signaling in diabetes has been demonstrated in TNF-R1 knockout NOD mice, which are protected from spontaneous development of diabetes [175]. The third and possibly most robust mechanism by which the humoral response promotes diabetes is by sensitizing β-cells to apoptosis induced thru direct cell-cell contact with T cells. “Naïve” β-cells have a notable resistance to T cell induced apoptosis. However, β-cells become “sensitized” to the effects of T cells in the presence of proinflammatory cytokines due to the induction of the cell surface receptors Fas and MHC I [176-178]. The mechanism by which Fas expression sensitizes β-cell to T cell induced apoptosis will be discussed below. The enhanced expression of MHC class I molecules is suspected to facilitate the activation of cytotoxic CD8+ T cells during the late stages of insulitis as overt diabetes develops [179]. In line with this, chemical or
genetic inactivation of MHC I signaling in animal models of diabetes severely delayed the onset of disease [180].

In addition to production of the humoral mediators mentioned above, T cells in particular also have the capacity to induce β-cell death through direct cell-cell contact. T cells carry out this “cellular response” through Fas- and perforin/granzyme-dependent mechanisms [181]. Fas is a pro-apoptotic transmembrane protein that belongs to the TNF receptor family. It is predominantly expressed within the thymus, liver, heart, and kidney; however proinflammatory cytokines (IL1β, TNFα, IFNγ) are known to induce its expression in β-cells [182, 183]. The natural ligand for Fas, FasL, has been identified and is primarily expressed by activated T cells. Therefore, when activated T cells expressing FasL encounter cells expressing the Fas receptor, such as cytokine “sensitized” β-cells, the engagement of FasL with the Fas receptor induces apoptosis in the receptor bearing cell. The relative importance of this cellular response to the development of diabetes has been emphasized by studies involving Fas deficient NOD mice, which are resistant to both spontaneous and adoptively transferred diabetes [184, 185]. Perforin/granzyme dependent cellular response involves T cell release of pre-stored granules that contain perforin and granzyme enzymes [181]. Once released, the tubular perforin molecules fuse with the plasma membrane of target cells to create a pore through which the pro-apoptotic granzymes enter the cell. Granzymes are serine proteases that induce apoptosis in two ways: caspase activation and reactive oxygen species production. Granzyme deficient NOD mice develop insulitis to the same extent as normal NOD mice, however they have significantly delayed onset of diabetes [186].
Signaling pathways of β-cell stress response: As discussed above, exposing islets to the humoral mediator IL-1β alone or in combination with TNFα and/or IFNγ induces significant β-cell death and dysfunction. It is generally accepted that IL-1β and TNFα primarily mediate their effect on islets by signaling through JNK/p38 and NFκB while IFNγ signals through the Jak/Stat pathway. The individual signaling events mediated by each of these cytokines are reviewed below and summarized in figure 1.3.

Figure 1.3: Signal transduction and cross talk of β-cell stress response. IL-1β, TNFα and IFNγ primarily mediate β-cell response to diabetic inflammation. Shown are the signaling events that lead to β-cell death following receptor activation. Reproduced with kind permission from Springer Science+Business Media: Diabetologia, A choice of death – the signal-transduction of immune-mediated beta-cell apoptosis, 44, 2001, p2122, Eizirik and Mandrup-Poulsen, Figure 2.
**IL-1β:** The IL-1 protein family is comprised of two IL-1 receptor agonists (IL-1α and IL-1β) and one antagonist (IL-1Ra). β-cells are known to express both types of IL-1 receptor (IL-1R1/IL-1R2), however signaling only initiates when IL-1 binds to the low affinity IL-1R1 receptor. The high affinity receptor, IL-1R2, does not mediate intracellular signaling and is proposed to be a decoy receptor. Binding of IL-1 to IL-1R1 induces a conformational change in the receptor that allows an accessory protein (IL-1AcP) to interact with IL-1R1, a necessary step in receptor activation. Once this occurs, MyD88, IL-1R1 activated kinase (IRAK) and TNF-receptor associated factor 6 are recruited to the receptor and 4 major signaling pathways are activated; NFκB, MAPK, PKC, and PI3K. Of these pathways, NFκB and MAPKs are believed to play a predominant role in mediating β-cell death and dysfunction as chemical and genetic inhibitors of their activation attenuate IL-1β induced apoptosis [64, 187-189].

**TNFα:** The tumor necrosis factor (TNF) protein superfamily consists of 18 ligand members with 26 known cognate receptors. In general, TNF family ligands are type II transmembrane proteins that become biological active upon homotrimerization. In some cases, as with TNFα, ligands are also active in their soluble form following cleavage by extracellular metalloproteinases. TNF family receptors also function in trimeric form and are structurally characterized by extracellular cysteine-rich domains (CRDs). Based on intracellular sequence, TNF receptors can be classified into three categories; with “death domain”, without “death domain” and no intracellular sequence. In response to receptor activation by ligand, the intracellular domain dictates the activation of specific signaling pathways through the recruitment of adaptor proteins. Receptors lacking intracellular
domains do not initiate a signaling cascade and therefore likely function as decoy receptors. TNFα selectively activates both the ubiquitously expressed death domain containing TNFR1 and the death domain lacking TNFR2 found only on immune and endothelial cells. In β-cells and others, TNFα activation of TNFR1 induces the recruitment of TNF receptor associated death domain (TRADD), Fas-associated death domain (FADD), TNF receptor associated factors (TRAF), and the death domain kinase receptor interacting protein (RIP). Activation of these death domain associated proteins by TNFR1 results in apoptosis in part, through the activation of NIK, PKC, JNK, and p38 signaling as well as conversion of pro-caspase8 into caspase8 [190]. Kinase activation leads to a pro-apoptotic transcriptional response while the activation of caspase8 promotes apoptosis by converting pro-caspase3 into caspase3, both directly and indirectly through Bax/Bak mediated mitochondrial cytochrome C release [191]. Interestingly, activated caspase3 is known to promote the cleavage of caspase8, giving rise to a potent positive feed back loop accelerating the apoptotic response to TNFα [192].

Consistent with activation of the pro-apoptotic pathways described above, TNFα has been demonstrated to play a role in β-cell death in diabetic mice using both gain and loss of function approaches. A study by Holstad et al. found that subcutaneous injection of mice with a selective inhibitor of TNFα transcription prevented diabetes induced by multiple low-dose streptozotocin injections [193]. Furthermore, conditional expression of TNFα in the islets of transgenic mice has been reported to accelerate diabetic onset by inducing β-cell death and promoting insulitis [194]. Interestingly in this study, the timing of TNFα expression played an important role in modulating the development of overt
diabetes. TNFα expressed early in the development of diabetes exacerbated the disease while prolonged expression was protective, which may have been due, in part, to reduced accumulation of self reactive T cells in the pancreas [194]. Collectively these findings support a complex cell type specific role for TNFα in the development of diabetes.

**IFNγ:** The interferon family of proteins consists of five members classified into two groups based on sequence homology and receptor specificity. Type 1 interferons selectively bind to the INFAR1/2 heterodimeric receptor and are comprised of IFNα, IFNβ, IFNω, and IFNτ proteins. There is only one type 2 interferon, IFNγ, and it is known to activate the IFNGR1/2 heterodimeric receptor. IFN receptors lack intrinsic kinase activity and therefore rely on constitutive association with the accessory molecules Janus tyrosine kinases (JAK) 1 and 2 to elicit a cellular response to IFN binding. As IFN binds the R1 molecule, a functional heterodimeric receptor is formed by the recruitment of the R2 molecule. When the R1 and R2 subunits dimerize, the associated JAK1/2 molecules become activated by auto-phosphorylation and phosphorylate Tyrosine residues on the receptor. Receptor phosphorylation leads to the recruitment and docking of two signal transducers and activators of transcription 1 (STAT1) molecules. JAK2 then phosphorylates the STAT1 molecules, leading to their homodimerization and nuclear translocation. Once in the nucleus, STAT1 is known to bind to DNA through gamma activated response elements and regulate the expression of more than a hundred target genes, including several caspases [195]. Therefore, IFNγ may promote an apoptotic cellular response in part by increasing steady state protein levels of precursors used in the programmed death response. Furthermore, JAKs can also activate MAPK,
PI3K and Phospholipase A2 signaling leading to synergistic cross-talk with the IL-1 receptor signaling pathway [196].

Similar to IL-1β and TNFα signaling, the specific role of IFNγ in the pathogenesis of diabetes has been deduced through gain and loss of function approaches in mice. Specifically, transgenic expression of IFNγ under the control of the insulin promoter induced acute inflammatory islet destruction [197]. Likewise, the administration of neutralizing antibodies against IFNγ blocked both chemically and diabetogenic T cell transfer induced diabetes [198, 199]. The importance of IFNγ was further supported by the finding that IFNγ receptor knock out (Ifngr -/-) mice crossed into the NOD background were completely protected from the development of diabetes [200]. Importantly, data in this study suggested that IFNγ signaling in the islets was of particular importance since adoptive transfer of diabetogenic T cells into Ifngr -/- NOD mice was not sufficient to induce diabetes.

**Transcriptional regulation of β-cell stress response:** Despite the immediate signal transduction response that occurs following cytokine dependent receptor activation, β-cell apoptosis can remain undetectable in isolated human, rat or mouse islets for up to 9 days [187, 201, 202]. This suggests that β-cells are basally equipped to sense and respond to changes in their extracellular environment; however the mechanism controlling an apoptotic response to cytokines requires de novo gene expression. The prevailing hypothesis for the induction of β-cell apoptosis is one of a complex biological phenomenon regulated by parallel and sequential change in expression of large groups or
biological modules of genes [203]. Consistent with this hypothesis is the observation that the two main transcription factors targeted for activation by IL-1β, TNFα and IFNγ are NF-κB and Stat1, factors known for their capacity to induce modular changes in gene expression. Microarray and proteomic analysis of β-cell response to cytokine treatment has identified changes in at least six groups of gene modules; pro-apoptotic, pro-survival, β-cell function, ER stress, transcription factor, and immunogenicity [204]. A large number of the genes known to be regulated following cytokine exposure are shown in figure 1.4 [205].

Figure 1.4: β-cell transcriptional response to proinflammatory cytokines. Shown are examples of genes regulated as part of the modular transcriptional response to the inflammatory cytokines IL-1β and IFNγ. Copyright © 2005 American Diabetes AssociationDiabetes®, Vol. 54; 2005, S97-S107 Reprinted with permission from The American Diabetes Association.□
In addition to these genes, others known to respond to cytokine treatment include; upregulated pro-apoptotic genes (Fas, caspase1, TNFα receptor and GADD153), downregulated pro-survival gene (BCL2), and upregulated transcription factors (c-jun, c-fos, ATF2, and ATF3) [7, 206]. Overall, a wealth of evidence indicates that β-cell apoptosis following cytokine exposure, and by deduction during T1DM, occurs through a complex process whereby extracellular stimuli initiate intracellular signaling cascades that result in an integrated pro-apoptotic and pro-inflammatory transcriptional response.

**B.2.4: Therapeutic interventions**

Despite the accumulation of data offering mechanistic insight into the aetiology of T1DM, no clinical trial has successfully blocked diabetic onset or permanently halted progression of the disease once diagnosed (Table 1.6) [121]. Therefore, to maintain normal glucose levels, affected individuals are managed clinically through insulin replacement therapy. Insulin replacement therapy typically involves the systemic administration of both long and short acting recombinant insulin molecules. Long acting insulin injections are performed once a day and maintain the basal level of insulin action necessary to avert the development of a starved state. Short acting insulin injections are typically performed at intervals and doses that correspond to the consumption of meals. In order to avoid toxic hyper- or hypo-glycemic episodes, patients must calculate the necessary dose of short acting insulin based on pre-meal glucose levels and the carbohydrate content of the meal in grams. While the process of monitoring and maintaining normal glucose levels is cumbersome, it is the most effective measure for reducing the incidence of diabetic complications. In a 10 year study performed by The
Diabetes Control and Complications Trial (DCCT), it was found that intensive insulin therapy effectively achieved long term normoglycemia while slowing the onset and progression of eye, kidney, nerve and cardiovascular complications of the disease [207-209].

<table>
<thead>
<tr>
<th>Administration</th>
<th>Trial</th>
<th>Compound</th>
<th>Efficacy</th>
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<tbody>
<tr>
<td>Before onset diabetes (prevention trials)</td>
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<td>Nicotinamide</td>
<td>No effect</td>
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<td></td>
<td>Deutsche Nicotinamide Intervention Study</td>
<td>Nicotinamide</td>
<td>No effect</td>
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<td>Diabetes Prevention Trial-Type 1</td>
<td>Parenteral Insulin</td>
<td>No effect</td>
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<td>Type 1 Diabetes Prediction and Prevention Study</td>
<td>Nasal insulin</td>
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<td>Australian phase I nasal insulin trial</td>
<td>Nasal insulin</td>
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<td></td>
<td>Canadian/European CsA trial</td>
<td>Cyclosporin A</td>
<td>Preservation of C-peptide only during drug administration</td>
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<td></td>
<td>IMDIAB VII</td>
<td>Oral insulin</td>
<td>No effect</td>
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<tr>
<td></td>
<td>GAD65 (DIAMYD)</td>
<td>Human GAD65</td>
<td>24-weeks positive effect in LADA patients</td>
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<td></td>
<td>DiaPep277</td>
<td>Hsp60 immunodominant peptide</td>
<td>1-year positive effect (preservation of C-peptide concentration)</td>
</tr>
<tr>
<td></td>
<td>Non-Fc binding anti-CD3 monoclonal antibody</td>
<td>hOKT3γ1 – mutated human anti-CD3</td>
<td>24-months positive effect with transient remission</td>
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<td></td>
<td>Non-Fc binding anti-CD3 monoclonal antibody (European trial)</td>
<td>ChAglyCD3 – aglycosylated human anti-CD3</td>
<td>18-months positive effect seen in patients with the highest C-peptide levels at entry (≥50th percentile)</td>
</tr>
<tr>
<td></td>
<td>NBI-6024 (Neurocrine)</td>
<td>Altered peptide ligand insulin B9-29</td>
<td>Ongoing</td>
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**Table 1.6: Type 1 diabetes clinical trials.** This chart summarizes the result from several independent clinical trials aimed at preventing or treating T1DM. While some modest benefit has been observed with treatments initiated after disease onset, no treatment has permanently cured the underlying auto-immune disorder.
While the advantages of intensive insulin therapy are clear, many diabetics are unable to achieve its beneficial effect due to the complexity of insulin dosing and administration. The advent of the insulin pump has offered a better method for insulin therapy by allowing for continuous subcutaneous insulin infusion. However, current insulin pumps are functionally limited by the absence of real time blood glucose monitoring technology and their susceptibility to pump failure or blockage. Therefore, as an alternative to exogenous insulin administration, the restoration of endogenous insulin production by total pancreas or islet cell transplantation is currently being investigated. However, these approaches have been slow to reach widespread clinical use due to marginal long term efficacy and a shortage of human donor pancreases. Due to the shortfall of available pancreas donors, additional methods are being pursued for restoration of endogenous insulin production. These alternative approaches include transplantation of pancreatic xenografts, differentiated β-cell precursors, and cells engineered with glucose responsive insulin expression constructs. Each of these alternative therapeutic options have shown great promise in small animal studies, however few if any have been validated in pre-clinical or clinical settings.

Recently, the development of the “Edmonton” and “University of Illinois at Chicago” (UIC) protocols for human islet transplantation have increased the therapeutic potential of this treatment option [210, 211]. The use of the Edmonton protocol raised the one year success rate of transplantation from 8% to 44%. More recently, the modified Edmonton protocol developed at UIC reduced by half the number of islets required to achieve the same result. However, the success of human islet transplantation is still limited by the need for recipients to receive repeated transplantations in order to
maintain long term insulin independence. Currently, it is believed that multiple transplants are needed because a significant number of insulin secreting cells are lost due to stress induced apoptosis. Therefore, efforts aimed at protecting islets from stress induced death will greatly enhance the long term therapeutic potential of islet transplantation.

Work presented in this dissertation tests the role of ATF3 in the stress induced death of islets following transplantation and presents a potential mechanism by which ATF3 promotes apoptosis. In support of these findings, the use of ATF3 knock out islets for transplantation in mice increased the rate of success by 80% when compared to wild type islets. This identifies ATF3 as a potential therapeutic target in islet transplantation and suggests that inhibiting ATF3 expression in human islets prior to transplantation may improve the clinical outcome. In pursuit of this application, work presented in this dissertation also reports my characterization of a novel modification to the adeno-associated virus (AAV) that dramatically improves its islet infection efficiency. The use of this highly infectious and non-immunogenic virus as a gene therapy vehicle has the broad potential to improve islet transplant outcome through the delivery of any gene targeting agent, an ATF3 knock down construct being only one of those options.

B.3: Type 2 diabetes

Type 2 diabetes mellitus (T2DM) is a chronic disease characterized by insulin resistance, increased hepatic glucose production, and impaired insulin secretion. Of the two types of diabetes mellitus, type 2 is by far the most prevalent and accounts for approximately 90% of diabetic cases [118]. While T2DM can occur at almost any age
and in any ethnicity, it is more common in African Americans, Latinos, Native Americans, Asian Americans/Pacific Islanders, and the elderly [119]. In the western hemisphere, there is a strong correlation between body mass and T2DM. Despite this, several other factors are known to influence the development of T2DM, including physical activity level, diet, alcohol consumption, and cigarette smoke inhalation [212]. However, it is generally accepted that T2DM can be prevented in most individuals by maintaining a healthy body weight and an active lifestyle.

For the remainder of section B.3, I will review our current understanding of T2DM. This review is organized into five sections that focus on the topics of glucose homeostasis, obesity & inflammation, insulin resistance, β-cell stress response, and therapeutic treatments. The logic for this organization is based on the inter-relationship between each of these aspects of biology and the development of diabetes. In short, insulin resistance, brought on by obesity and obesity induced systemic inflammation, is the underlying force giving rise to defects in glucose homeostasis. However, individuals remain relatively asymptomatic until, in a genetically predisposed subset of the obese population, the pancreatic β-cells become stressed and fail. Therefore, current therapeutic strategies for treating T2DM are generally aimed at promoting β-cell function and reducing insulin resistance.

**B.3.1: Physiology of glucose homeostasis**

In a healthy individual, insulin and glucagon play integral roles in maintaining glucose homeostasis during both fasted and fed states. During periods of extended fast, typically greater than 10-12 hours, the body is considered to be in a postabsorptive state.
(no longer absorbing nutrients from the alimentary canal). In these periods, individuals with normal glucose homeostasis maintain a rate of glucose utilization that exactly matches the rate of glucose production, averaging approximately 2.0mg/kg/min [213-216]. To achieve this homeostasis, the liver (85%) and kidneys (15%) produce glucose through glycogenolysis and gluconeogenesis, while the brain, liver, and muscle account for almost 100% of total body glucose utilization. [214, 217]. Under these conditions, approximately 50% of hepatic glucose production is dependent upon glucagon, a hormone secreted by pancreatic α-cells [218]. Following consumption of a meal, the balance between endogenous glucose production and tissue glucose uptake is disrupted. The resulting elevation in plasma glucose concentration is sensed by the pancreatic β-cells and triggers their release of insulin. Circulating insulin serves three main functions; (a) to stimulate glucose uptake by splanchnic (liver and gut) and peripheral (primarily muscle) tissue, (b) to suppress endogenous glucose production directly and indirectly by inhibiting glucagon secretion, and (c) to lower plasma Free Fatty Acids (FFA) by reducing adipocyte lipolysis. [213, 216, 219-221]. While adipocytes account for only 5% of peripheral insulin induced glucose uptake, their ability to modulate plasma FFA levels in response to insulin makes them an essential tissue in maintaining glucose homeostasis. Free fatty acids reduce the insulin sensitivity of muscle and liver [222], and since small increases in plasma insulin levels are capable of decreasing plasma FFA levels dramatically, adipocytes act indirectly to significantly enhance glucose uptake in muscle and further inhibit glucose production by the liver [218, 223-225]. Overall, the functions of insulin are directed towards one objective, to reduce plasma glucose levels to the desired 4-7mM (80-90mg/100ml) range. Plasma glucose levels can be maintained in this
range since falling glucose levels attenuate secretion of insulin, leading to reduced plasma glucose clearance by muscle and increased pancreatic secretion of glucagon, which in turn stimulates the liver’s production of glucose.

While not directly related to the research conducted in this dissertation, the increasingly complex picture of glucose and nutritional homeostasis that has emerged in recent years merits acknowledgment. Two discoveries in particular have added to our understanding of the hormonal communication that occurs across a multi-organ network to achieve glucose homeostasis. The first discovery arose from the observation that obesity increases bone mineral density (BMD), suggesting to some that a feedback loop may exist between bone and energy homeostasis. However, at the time, the functional significance of increased BMD in response to obesity was unclear. In pursuit of an explanation for this observation, Lee et al. identified osteocalcin as a bone derived molecule secreted specifically from osteoblasts [226]. Osteocalcin was found to function systemically by increasing peripheral insulin sensitivity as well as pancreatic β-cell number and secretory capacity. This finding was consistent with the existence of a feedback loop in that it connected signals of elevated nutritional supply from the pancreas and expanded adipose depots to a functional response in bone, making normoglycemia possible. In this feedback loop, hypernutrition initiates the signaling by increasing serum insulin levels and lipid accumulation within adipocytes. Insulin, in addition to its many other functions, promotes the proliferation and eventual differentiation of osteoblast progenitor cells [227]. As osteoblasts are the cells that secrete bone matrix, an increase in their number shifts the homeostasis of bone remodeling in a way that gives rise to
increased BMD. Consistently, the elevation in serum insulin level therefore favors osteocalcin secretion by increasing osteoblast abundance.

Adipocytes also contribute to this feedback loop. The accumulation of lipid within adipocytes alters the secretion of two well known adipokines, adiponectin and leptin. Adiponectin secretion into the blood stream is inversely proportional to body fat percentage. Therefore, as individuals become more obese, adiponectin levels decrease. While adiponectin is known to regulate several metabolic processes, it was more recently identified as a negative regulator of BMD [228]. Therefore, the decrease in adiponectin levels observed with obesity may favor osteocalcin production as a byproduct of increased BMD. Serum levels of leptin are opposite from adiponectin in that they are proportional to body fat percentage. Leptin functions in part through a hypothalamic circuit, acting as an appetite suppressant by triggering a “sense” of fullness. Leptin signaling has also been found to both directly promote and indirectly repress osteoblast proliferation and function [229, 230]. However, the net effect as body fat percentage and leptin levels rise is an increase in osteoblast activity. Interestingly, this may in part be possible due to the influence of serum free fatty acids on leptin transport across the blood brain barrier. The indirect repressive effect of leptin on osteoblast function is dependent on hypothalamic signaling. However, for leptin to initiate this signaling it must first cross the blood brain barrier. It was recently reported that serum triglycerides induce central lepin resistance by blocking leptin transport across the blood brain barrier [231]. Therefore, given that body fat percentage and serum triglyceride levels are proportionally correlated, the net effect of leptin signaling on osteoblasts in obese individuals may favor activation since the repressive signaling is blocked by triglycerides. Collectively, these
findings support a novel endocrine function of bone, where osteocalcin secretion in response to increased nutritional supply facilitates the cellular uptake and utilization of glucose by stimulating insulin sensitivity and secretion.

A second major discovery that added to our understanding of hormonal control over glucose homeostasis was the realization that a hypercaloric diet induces central insulin resistance prior to the onset of obesity and peripheral insulin resistance. Zhang et al. found that even brief periods of hypernutrition induced ER stress and NF-κB activation in the hypothalamic neurons responsible for stimulating appetite [232]. The stress response that ensued induced a resistance in those cells to the inhibitory effects of insulin signaling (central insulin resistance). Therefore, as individuals begin to eat more than their body needs, the appetite suppressing effect of insulin is blunted and chronic overnutrition ensues. Ultimately, the hypercaloric diet leads to greater central insulin resistance, obesity and peripheral insulin resistance. In addition, a hypercaloric diet promotes the accumulation of serum triglycerides, which as mentioned previously blocks the transport of leptin across the blood brain barrier. This contributes to the hypercaloric cycle by further dampening the function of leptin, a second potent appetite suppressing hormone. Given the developing obesity epidemic in industrialized societies, particularly in the United States, this finding offers significant insight into the epidemics etiology. Our access to calorically dense nutrients facilitates short term overnutrition since hormonal regulation of appetite and satiation can not respond quickly enough to a calorically dense meal. As a consequence, consumption continues even after caloric needs have been met. Therefore, by the time you “feel full” from a calorically dense meal, you have already consumed a hypercaloric quantity that has the potential to induce
central insulin resistance. After several hypercaloric feedings, the set point at which the hypothalamus attempts to cease consumption has already been changed and it becomes easier and easier to chronically over nourish. This spiral of overnutrition brought on by calorically dense nutrients may be playing a large role in the obesity epidemic plaguing the United States.

B.3.2: Obesity and inflammation

Driven by forces of evolution, mammals have developed and retained mechanisms for energy storage during periods of nutritional abundance. Excess nutrients are converted to triglycerides and stored primarily in the adipose tissue. While this storage mechanism offers the benefit of increased survival during periods of famine, prolonged periods of weight gain result in obesity and have serious negative impacts on an individual’s health and life-span. Due to the abundant supply of calorically dense and carbohydrate laden nutrition, particularly in developed Western societies, obesity has reached epidemic proportions. In fact, the WHO has estimated that greater than 1 billion adults worldwide are overweight [233]. Interestingly, as the incidence of obesity has increased, so has the prevalence of obesity associated diseases, including T2DM, cardiovascular disease, hypertension, nonalcoholic fatty liver disease, arthritis, asthma, and cancer. Many of these obesity associated diseases are characterized by aberrant inflammatory responses. One of the first clues that connected inflammation to the negative effects of obesity occurred in the 1870’s when high doses of sodium salicylate were observed to reduce glycosuria in diabetic patients [234]. Sodium salicylate, otherwise known as aspirin, is a non-steroidal anti-inflammatory compound that
functions in part through irreversibly inhibiting the rate limiting cyclooxygenase enzymes known to produce pro-inflammatory prostaglandins [235]. Additional studies performed in the 1950s confirmed the beneficial effect of aspirin in treating diabetes as obese insulin-dependent diabetic patients did not require daily insulin injections when given high doses of aspirin [236]. At the time, mechanistic studies into this observation focused on aspirin induced insulin secretion rather than on insulin resistance and little headway was made on how aspirin reduced diabetic symptoms. Only recently was it found that the hypoglycemic and insulin sensitizing effect of aspirin on obese individuals was tied to the inhibition of NF-κB dependent pro-inflammatory gene transcription [237-239]. This finding combined with the observation that TNFα, a cytokine overproduced in obese adipose tissue, was able to induce insulin resistance solidified the connection between obesity, inflammation and insulin resistance [240, 241]. Since then, numerous additional cytokines, chemokines and adipokines have been identified to play a role in promoting insulin resistance by mediating the inflammatory effects of obesity.

**B.3.3: Insulin resistance**

Type 2 diabetes is a progressive disorder linked to increasing degrees of insulin resistance, giving rise to defective glucose homeostasis and ultimately hyperglycemia [214, 242]. Early in the development of diabetes, compensatory increases in circulating insulin adequately maintain glucose homeostasis in the face of increased metabolic demand. However, as the disorder progresses, genetic predispositions in a subset of the population leads to insufficient insulin secretion, and fasting plasma glucose levels begin to rise [243]. Once fasting plasma glucose levels rise above 300mg/dl, β-cells begin to
“fail” and secretion of insulin declines dramatically. [244, 245]. This represents a critical event in the development of overt T2DM, since it is at this point that hepatic glucose production (the primary determinant of fasting plasma glucose concentration) begins to rise.

Primary to all other defects in T2DM is a multi-tissue resistance to the functions of insulin [246-248]. The mechanisms that initiate insulin resistance are complex and likely haven’t been fully identified. However, based on current information, insulin resistance appears mainly to be a result of defects with (or down stream from) the insulin receptor. These defects can specifically be associated with decreases in (a) the overall number of insulin receptors present on the surface of a cell, (b) the tyrosine kinase activity of the receptor itself, or (c) signaling down stream from the receptor through insulin receptor substrate (IRS) -1 or -2 and phosphatidylinositol 3-kinase (PI3-kinase) [249-254].

A general overview of insulin signaling pathway is illustrated in figure 1.5 [255]. Briefly, as insulin binds to the alpha subunit of the insulin receptor, it stimulates beta subunit tyrosine kinase activity, resulting in phosphorylation of its own tyrosine residues. The activated receptor can then phosphorylate specific intracellular proteins (9 identified as of 1998), initiating a cascade of phosphorylation events involved in regulating glucose transport and metabolism, cell growth and differentiation, and general gene expression. Four of these intracellular proteins belong to the family of insulin-receptor substrate proteins (IRS-1, -2, -3, -4) and they are integral to insulin’s control over glucose metabolism. Tyrosine phosphorylation of IRS promotes their association with SH2 domains in the regulatory subunit (p85) of PI3-kinase, allowing activation of the catalytic
Figure 1.5: **Insulin receptor signaling overview.** The ability of cells to take up and metabolize glucose in response to insulin depends upon signals transduced through the insulin receptor. “Insulin resistance” occurs when signaling is blocked at any number of points in the pathway. Figure reproduced with permission from Nature Publishing Group: Saltiel and Kahn, Nature, 2001, v414, p801.

Subunit (p110). p110 then catalyzes the 3-prime phosphorylation of phosphatidylinosol (PI) 4 phosphate and PI-4,5-diphosphate, generating a signaling intermediate PI-3,4,5-triphasphate that activates downstream pathways regulating glucose and lipid metabolism.
In T2DM, two predominant mechanisms for developing defective insulin signaling involve serine phosphorylation of the IRS proteins and over activation of mitogen-activated protein (MAP) kinases. Serine phosphorylation blocks tyrosine phosphorylation of IRS proteins by the insulin receptor and consequently inhibits the IRS-p85 interaction, effectively blocking insulin signal transduction [256, 257]. Insulin receptor activation of the MAP kinase pathway is also illustrated in figure 1.5. In this pathway, ERKs have the ability to serine phosphorylate IRS proteins [258]. Since MAP kinase activation through the insulin receptor is independent of IRS activity, as the cells become progressively more resistant to the metabolic effects of insulin signaling, MAP kinase activity retains its responsiveness to insulin. Therefore, early in the development of T2DM, during the compensatory increase in plasma insulin levels, the incidental over activation of ERK actually contributes to the progression of the disease.

Prior to the involvement of ERK in propagating insulin resistance, elevations in two other factors prompt the initial serine phosphorylation of IRS; plasma free fatty acids (FFAs) and circulating inflammatory cytokines. Elevated plasma FFAs give rise to an accumulation of intramuscular and intrahepatic lipid pools that are made up primarily by triglyceride, diacylglycerol (DAG), and long chain fatty acyl CoAs (FA-CoA) [259]. These lipid pools promote insulin resistance by activating Protein Kinase C theta and zeta, two kinases known to serine phosphorylate IRS-1 [256, 260]. Circulating inflammatory cytokines similarly promote insulin resistance by inducing IRS-1 serine phosphorylation, however they function in part through a JNK dependent mechanism [261]. In addition, cytokine stimulation initiates a feed-forward mechanism whereby a
NF-κB transcriptional response increases production of inflammatory cytokines and chemokines, giving rise to more JNK and NF-κB activation.

**B.3.4: Role of β-cell stress response**

Similar to type 1 diabetes, type 2 diabetes ultimately is a disease of relative insulin deficiency. While the etiologies behind the insulin deficit are quite different, the diseases only present when β-cells fail to secrete enough insulin to meet systemic demand. In type 1 diabetes, a primary deficit in insulin arises from the immune attack against the insulin secreting cells. In type 2 diabetes, the insulin deficit is secondary to a failure in β-cell adaption to increased insulin demand. Recent evidence suggests that in all individuals, the initial increases in metabolic demand brought on by obesity are met with increased β-cell mass and insulin secretion. However, in a subset of obese individuals, this adaptive response by the β-cells ultimately fails and diabetes ensues.

Currently the literature supports the notion that, of the 4 parameters regulating steady state levels of β-cell mass (neogenesis, replication, hypertrophy, and apoptosis), stress induced β-cell apoptosis is the primary factor responsible for reduced β-cell mass in human and mouse type 2 diabetics [262-265]. Several of the stresses, as well as the conferred molecular responses that mediate β-cell apoptosis in diabetics have been identified and they will be reviewed below.

**Sources of β-cell stress:** Obesity induces systemic changes that create several β-cell stresses, most predominantly are elevations in serum glucose and lipid, inflammatory cytokines and local Islet Amyloid Associated Peptide (IAPP) deposition. Chronic
exposure to elevated serum glucose induces a response in β-cells known as glucotoxicity, which ultimately leads to irreversible deterioration of β-cell function and the induction of apoptosis. Glucotoxicity is mediated, in part, by chronic oxidative stress and ER stress. Both stresses result as a byproduct of the increased insulin demand that β-cells must accommodate. Part of the mechanism by which β-cells secrete insulin in response to glucose involves the production of ATP through glycolysis. The increased demand for insulin secretion therefore increases glucose metabolism which generates harmful reactive oxygen species (ROS) as protons are transferred from NADH to Complex I of the electron transport chain [266]. β-cells are particularly sensitive to ROS because they have low levels of the major anti-oxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase [267]. Therefore, when glycolytic activity is sustained, the elevated ROS levels harm β-cells by inducing DNA damage and reducing insulin transcription. In addition to promoting insulin secretion, glucose exposure also induces the transcription and translation of insulin and other secretory granule proteins. Consequently, the increased translation of these secretory granule proteins has the potential to induce apoptosis via ER stress response [268].

Similar to the negative effects of hyperglycemia, prolonged elevations in serum lipid levels induce a response in β-cells known as lipotoxicity [269]. Lipotoxicity occurs as triglycerides accumulate within β-cells and enhance the rate of fatty acid oxidation. The accumulation of cytosolic metabolites from fatty acid oxidation are known to impair glucose induced insulin secretion and transcription. Furthermore, the generation of ROS by fatty acid oxidation leads to ER stress and apoptosis [62].
Local and systemic inflammation acts as a third source of β-cell stress in diabetes. Chronic hyperglycemia leads to cytokine gene expression in both islets (IL-1β) and adipocytes (leptin, TNFα and IL-6) [270, 271]. The increased circulating levels of adipocyte derived cytokines have the potential to both directly induce β-cell death and sensitize β-cells to IL-1β induced apoptosis [272].

Lastly, deposition of islet amyloid polypeptide (IAPP) on the surface of islets is believed to play a role in the diabetic reduction of β-cell mass. IAPP is a 37-amino acid molecule that is co-stored and co-secreted with insulin in a molar ratio of 1:100 (IAPP:Insulin) [273]. IAPP is known to play a physiological role in glucose metabolism by suppressing glucose uptake in muscle and by blocking insulin secretion from β-cells. However, the pathological accumulation of IAPP on the surface of islets leads to the generation of nonselective ion channels and cellular toxicity [274]. Several studies have reported the accumulation of IAPP deposits on the pancreatic islets from diabetic patients [275, 276].

**Molecular response to β-cell stress:** Each of the stresses discussed above are capable of inducing a diverse array of molecular responses. In addition to inducing ER stress, these responses mainly involve the activation of kinase signaling pathways that include IKKβ, JNK/p38, PKC, SOCS and mTOR [271]. While diverse in their origin, the pathways ultimately converge on IRS-2, a molecule that is critically important for maintaining β-cell mass (Figure 1.6). IRS2 is a member of the IRS protein family and is a powerful mediator of the anti-apoptotic effects of insulin and insulin like growth factors [26, 277, 278]. The importance of IRS2 in β-cell survival is emphasized by experimental data that
found increased β-cell neogenesis, replication and survival with over expression of IRS2 and spontaneous apoptosis following IRS2 repression [277-280]. Activation of the stress kinases in β-cells from diabetic individuals leads to Ser/Thr phosphorylation of IRS2

**Figure 1.6: Mechanisms for IRS-2 degradation in type 2 diabetes.** IRS-2 is a potent mediator of growth factor survival signaling in many cells, including the β-cell. The stresses of obesity initiate several signaling pathways that ultimately converge on IRS-2 and target it for proteosomal degradation. With reduced levels of IRS-2 to propagate the pro-survival signals from the extracellular environment, pro-apoptotic factors are left unregulated and β-cells undergo apoptosis. Reproduced with permission from The American Association for the Advancement of Science: Rhodes, Science, 2005 Jan 21;307(5708):380-4.

that, as mentioned previously, blocks the capacity of IRS2 to propagate insulin signaling through tyrosine phosphorylation. Furthermore, Ser/Thr phosphorylation reduces steady state levels of IRS2 protein within the cell by promoting ubiquitination and proteosomal
degradation of IRS2. As a consequence, loss of functional IRS2 protein in islets favors β-cell apoptosis and contributes to reduced β-cell mass observed in the diabetic pancreas [280].

3.5: Therapeutic interventions

The algorithm recommended by the UK Prospective Diabetes Study (UKPDS) and the American Diabetes Association (ADA) for treating T2DM is illustrated in figure 1.7 [281]. Initiation of treatment follows the diagnosis of T2DM as defined by patient presentation of any of the following; (a) symptoms of diabetes and non-fasting plasma glucose of 200mg/100ml (classic symptoms of diabetes include polyuria, polydipsia, and unexpected weight loss), (b) fasting plasma glucose of 126mg/100ml or (c) two hour plasma glucose of 200mg/100ml during an oral glucose tolerance test [282]. This approach recommends pharmacological intervention if changes in diet and exercise fail to meet treatment goals. The treatment goals defined by the ADA call for plasma glucose values between 90-130 mg/100ml (fasting) and <180 mg/100ml (2 hours post prandial) with hemoglobin A1C values below 7%. A1C values reflect the percentage of glycosylated red blood cells and is a measure of mean plasma glucose levels. For example, a 7% A1C value correlates with a mean plasma glucose value of 170 mg/100ml [282].

The pharmacological agents used to treat T2DM generally function either by augmenting the supply of insulin or by supplementing endogenous insulin production. A number of agents have been approved for the treatment of diabetes and, as illustrated in
Figure 1.7: ADA recommended algorithm for treating type 2 diabetes. While the first approach in the treatment of T2DM is address issues with diet and exercise, this approach alone is often ineffective. Therefore additional pharmacological interventions have been recommended for improving glucose homeostasis in diabetic individuals. Figure reproduced from the American Diabetes Association website.

In addition to oral medications, the injection of insulin can be used to supplement endogenous insulin production. In the United States between the years 2001 and 2003, 57% of all diagnosed diabetics were taking oral medications only, 12% combined insulin therapy with oral agents, 15% used insulin therapy only, and 16% relied solely on diet and exercise [284]. In spite of the treatment options available, reports suggest that between 50% and 85% of diagnosed diabetics have sub-optimally controlled plasma glucose levels after 6 years [285-288]. This indicates a serious shortcoming in current treatment options since tight
control of plasma glucose levels is known to dramatically reduce the incidence of costly and life threatening diabetic complications [281, 284, 289, 290]. While mean plasma

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<td>Gastrointestinal tract</td>
<td>Delay of gastric emptying</td>
<td>Pramlintide</td>
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<td></td>
<td>Inhibition of glucagon release</td>
<td>α-glucosidase inhibitors</td>
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<td>Stimulation of GLP-1 release</td>
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<td>Pancreatic β cell</td>
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<td>Stimulation of insulin biosynthesis</td>
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<td>Inhibition of β-cell apoptosis</td>
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<td>Stimulation of β-cell differentiation</td>
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<td>Liver</td>
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<td>Suppression of NEFA release</td>
<td>Thiazolidinediones</td>
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<td>Modulation of adipokine release</td>
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**Figure 1.8: Pharmaceutical agents used to treat type 2 diabetes and their mechanism of action.** One of the goals of pharmaceutical intervention in the treatment of T2DM is to reduce serum glucose levels. This figure summarizes the different strategies for improving serum glucose concentration. Reprinted from The Lancet, Vol. 365, Stumvoll et. al., Type 2 diabetes: principles of pathogenesis and therapy, p1338.2005, with permission from Elsevier.
glucose and A1C levels decrease following the initiation of treatment, their levels steadily rise and β-cell function decreases (between 28 and 52%) during the 6 years following diagnosis, irrespective of lifestyle change and treatment plan [291]. Thus, suggesting the reason for the shortcoming of current treatment options lies in their failure to halt the progression of insulin resistance and subsequent β-cell failure.

Until recently, no pharmacological agent for the treatment of diabetes had been shown to definitively influence insulin receptor sensitivity. While metformin, often a first choice therapy for T2DM, has been the topic of considerable debate regarding it's influence on insulin sensitivity, the thiazolidinedione (TZD) class of drugs are the first to result in reduced plasma glucose levels by increasing insulin responsiveness [281, 292-297]. TZDs are synthetic ligands for the nuclear hormone receptor (NHR) peroxisome proliferator receptor gamma (PPARγ), a protein with abundant expression in adipose tissue and moderate expression in skeletal and cardiac muscle, liver, renal collecting ducts, colon, small and large intestines, pancreas, and spleen [298, 299]. Several lines of evidence suggest that TZDs act primarily in adipose tissue and skeletal muscle by inducing a subset of PPARγ responsive genes involved in glucose and fatty acid metabolism [300]. The expression of these genes ultimately results in decreased peripheral insulin resistance and reduced circulating free fatty acids. However, in addition to these desirable effects, TZD treatment also induces genes that promote the differentiation of preadipocytes, thus contributing to obesity [291, 301-303]. Furthermore, TZDs agonize PPARγ in the inner medulary collecting duct of the kidney, mediating the induction of genes that increase sodium retention and consequently,
produces edema [304]. These transcriptional side effects ultimately reduce the efficacy of treatment since it causes many diabetics to discontinue their use. Currently, there are no treatments known to cure T2DM. While difficult to achieve, it is generally accepted that intervention with diet and exercise before β-cell failure is the only way to eliminate or reduce the underlying stresses that promote insulin resistance.

**B.4: Conclusion**

Nearly 8% of the men, women, and children living in the United States have diabetes. While the etiology of type 1 and type 2 diabetes are very different, both diseases ultimately result in abnormally high serum glucose levels. The observation that hyperglycemia doesn’t occur in either disease while β-cells are alive and functioning suggest a central role for β-cell biology in the pathogenesis of diabetes. Therefore, a greater understanding of β-cell response to diabetic stresses may lead to the development of therapies capable of treating both forms of the disease. Work presented in this dissertation identifies a molecular mechanism that supports the role for ATF3 in both the survival and function of β-cells.

**C: Overview of thesis work**

The focus of the research performed in this dissertation is on ATF3, a stress inducible transcription factor that plays a central role in the biology of β-cells. The hypothesis of this dissertation is that ATF3 functions by contributing to the expression of target genes that strongly influence β-cell survival and function. Work presented in this dissertation provides mechanistic insight into the previously established proapoptotic
effect of ATF3 in β-cells. In addition, this work has identified a novel and surprisingly beneficial role for ATF3 in promoting β-cell function by increasing insulin gene expression. Chapter 2 utilizes islet transplantation as a clinically relevant stress model to examine the mechanism by which ATF3 contributes to stress induced β-cell apoptosis. In this chapter, *in vitro* stress models of transplantation and *in vivo* transplantation stress were utilized to compare the transcriptional response of wild type and ATF3 knock out islets following stress. This approach identified several previously unknown pro-apoptotic and pro-inflammatory target genes of ATF3. Work presented in chapter 3 characterizes the gene therapy utility of a novel recombinant adeno-associated virus (AAV) that we identified to have significantly enhanced islet infection efficiency. Utilization of this non-immunogenic virus, that expresses the classical cellular adhesion motif Arg-Gly-Asp (RGD), to deliver an ATF3 knock down construct prior to islet transplantation may have the potential to improve the clinical outcome from human islet transplantation. Finally, chapter 4 uses high fat diet induced obesity in mice to examine the effect of ATF3 expression in the context of type 2 diabetes. This work led to the surprising finding that expression of ATF3 in the islets of obese and insulin resistant mice did not lead to β-cell apoptosis, but rather to an increase in insulin gene expression. Given the associated increased demand for insulin with type 2 diabetes, the observation that ATF3 contributes to the compensatory increase in insulin expression suggests that ATF3 is promoting β-cell function and is therefore playing a beneficial role in β-cell response to the stresses of type 2 diabetes.

In light of the opposing functional consequences of ATF3 expression reported in chapters 2 and 4, work presented in this thesis has contributed to a more complex
understanding of the biological significance of ATF3 expression. In this context, the activity of ATF3 may depend not only upon its induction by stress, but also upon other factors in the cell that are selectively regulated by the specific context of the stress. Therefore, this work further supports the notion that ATF3 may operate as an “adaptive response” gene, capable of promoting apoptosis or enhancing cellular function as dictated by the stress environment. Additional insight into the mechanism controlling the pleiotropic effects of ATF3 may enhance our basic understanding of how cells adapt to stress.
CHAPTER 2

THE ROLES OF ATF3, AN ADAPTIVE-RESPONSE GENE, IN ISLET TRANSPLANTATION

SUMMARY

Islet transplantation is a potential therapeutic option for type 1 diabetes. However, the need for multiple donors per patient and heavy immunosuppression of the recipients currently limit its use. ATF3 has previously been characterized as a pro-apoptotic transcription factor induced in β-cells by stresses relevant to type 1 and type 2 diabetes. Therefore, the goal of research conducted in this chapter was to test whether ATF3 plays a role in the β-cell stress response to islet transplantation, and if so, identify the mechanism by which it functions. In the course of this study, I compared wild type (WT) and ATF3 knockout (KO) islets in vitro using stress paradigms relevant to islet transplantation: isolation, inflammation, and hypoxia. I also compared the WT and KO islets in vivo using a syngeneic mouse transplantation model. As a result, I found that ATF3 was induced in all three stress paradigms and played a deleterious role in islet survival, as evidenced by the lower viability of WT islets than KO islets. ATF3 regulated various downstream target genes in a stress-dependent manner. These target genes can be classified into two
functional groups: (a) apoptosis (NOXA, bNIP3), and (b) immuno-modulation (TNFα, IL1β, IL6, and CCL2/MCP-1). In vivo, ATF3 KO islets performed better than WT islets after transplantation, as evidenced by better glucose homeostasis in recipients, and reduced caspase 3 activation and macrophage infiltration in the KO grafts. In conclusion, work presented in this chapter identifies a role for ATF3 in islet graft rejection by contributing to islet apoptosis and inflammatory responses at the graft sites. Therefore, silencing ATF3 may provide therapeutic benefits in islet transplantation.

INTRODUCTION

Since the early pioneering work demonstrating that transplantation of islets of Langerhans into diabetic rodents could normalize their blood glucose levels, islet transplantation has been proposed to be a potential therapeutic option to treat type 1 diabetes [305]. The success of the “Edmonton Protocol” [210] further strengthened this view. However, major limitations prevent islet transplantation from being a widespread clinical reality (a review, [201]): (1) the requirement for large numbers of islets per patient, which severely reduces the number of potential recipients, and (2) the need for heavy immunosuppression, which significantly affects the pediatric population of patients due to their vulnerability to long-term immunosuppression. Strategies that can overcome these limitations will greatly enhance the therapeutic potential of islet transplantation.

A major reason for islet loss during transplantation is the apoptotic death of islet cells, an event caused by multiple factors. These factors can be classified into two main groups. The first consists of those leading to “primary non-function,” a term referring to islet dysfunction and loss due to reasons other than specific immune rejection by the
recipients. Factors causing primary non-function include deleterious conditions that
islets face during isolation and the hostile environment that islets encounter in the
recipients. During isolation, islets are subjected to stresses such as enzyme digestion,
shearing, hypoxia, and temperature fluctuations. Upon transplantation, islets are under
attack by the non-specific inflammatory reactions from the host innate-immunity, which
involves primarily macrophages and dendritic cells. In addition, islets are under hypoxia
and nutrient deprivation stresses before graft vascularization occurs, and are exposed to
the hostile hyperglycemic milieu of the diabetic hosts [306-314]. The second group of
factors causing islet apoptosis in transplantation is the specific immune attacks by the
host adaptive-immunity, which involves T lymphocytes, B lymphocytes, and other
immune cells.

Although both primary non-function and specific immune attack contribute to
graft failure, primary non-function is thought to be the main reason for islet failure in the
early transplantation stage and for the requirement of large number of islets (≥2
pancreata/patient) to achieve euglycemia (see [315] for references). Despite its
importance, primary non-function is not well understood at the molecular level. In this
study, I investigated the potential roles of ATF3, a stress-inducible pro-apoptotic gene, in
primary non-function. ATF3 encodes a member of the ATF/CREB family of
transcription factors that share the basic region/leucine zipper DNA binding motif and
bind to the CRE/ATF consensus sequence TGACGTCA (some reviews, [5, 12]).
Overwhelming evidence indicates that the expression of ATF3 is up-regulated by a
variety of signals, including some seemingly unrelated signals such as cytokines, nutrient
deprivation, serum stimulation, and calcium signaling (a review, [12]). The list of the
stimuli that can induce ATF3 is considerably long and it appears that ATF3 is an “adaptive-response” gene for the cells to adapt to extra- and/or intra-cellular changes. Borrowing a concept from the network theory, we suggest that ATF3 can be viewed as a hub in the biological networks to respond to signals perturbing homeostasis. Functionally, ATF3 is pro-apoptotic in pancreatic β cells. Previously, we demonstrated that knockout or knockdown of ATF3 protects islets or β cells from stress-induced death ([7], [316]). One mechanism by which ATF3 promotes β cell death is by reducing the express of the pro-survival gene IRS2 [316].

The deleterious effect of ATF3 in β cells, combined with its general inducibility, prompted us to hypothesize that ATF3 is induced by signals encountered by the islets during transplantation, and that deletion of ATF3 would protect islets in a syngeneic transplantation model. In this model, donor mice have the same genetic backgrounds as recipient mice; thus the islets would not invoke the adaptive-immune response, allowing us to investigate the roles of ATF3 in primary non-function. In this chapter, I present evidence supporting this hypothesis. I also present evidence that ATF3 up-regulates the expression of several pro-inflammatory cytokines and CCL2 (MCP-1), which encodes a potent macrophage recruitment factor [317]. These results suggest that ATF3 contributes to not only apoptosis but also an inflamed state of the islet grafts. Considering the deleterious effects of apoptosis and inflammation, these findings have significant implications for islet transplantation.
MATERIALS AND METHODS

Cell culture and treatments. INS-1 and INS-r3 immortalized β-cell lines used in this study were grown in a 37 °C humidified tissue culture incubator in the presence of 5% CO₂ as previously described [318] using RPMI1640 supplemented with 10% heat inactivated FBS, 100 units Pen/Strep, 1mM Sodium Pyruvate, 10mM HEPES pH 7.4, and 50μM β-mercaptoethanol. Both cell lines were used for chromatin immunoprecipitation (ChIP) and yielded consistent results. Cytokine treatments were the following: IL-1β (5ng/ml), TNFα (30ng/ml), and IFNγ (125ng/ml). Hypoxia was established by continuous flow of a specialty mixed gas comprised of 1% O₂, 5% CO₂, and 94% N (Praxair) fed into a humidified custom hypoxia chamber (American Plastics).

Animals and adenoviruses. ATF3 knockout mice were generated in the 129SVJ background as described previously [7]. The knockout allele was distinguished from the wild-type allele by Southern blot or PCR. Congenic knockout mice in the background of C57BL/6NTac were generated by 10 backcrosses. Wild type and ATF3 KO mice were then maintained through homozygous breeding in a colony house in a University Laboratory Animal Resources facility at the Ohio State University. Age and weight matched male mice were used for all experiments. Adenoviruses were constructed and purified as previously [316]. Briefly, adenoviruses were generated using the Invitrogen Gateway technology and purified by cesium chloride ultracentrifugation.

Quantitative RT-PCR (qRT-PCR) analyses, ChIP, immunoblot and immunohistochemistry. RNA isolation, qRT-PCR, and ChIP analyses were carried out
as previously [114, 316]. All PCR primers are listed in Table 2.2. Immunoblot and immunohistochemistry were carried out as previously [7, 114], using the following antibodies: anti-ATF3 (Santa Cruz, Atlas Antibodies), anti-actin (Sigma), anti-caspase 3 (Cell Signaling), anti-HIF-1α (Abcam), anti-insulin (Dako), and anti-F4/80 (Caltag Laboratories).

**Cell viability analysis.** Islets were stained with propidium iodide (1μg/ml) and Hoechst 33258 (2μg/ml) and imaged by epifluorescence. Image analysis was performed using the NIH ImageJ Software (version 1.41a) as detailed in Appendix B.

**Islet isolation and transplantation.** Islets were isolated from adult male wild type and ATF3 knock out C57BL/6 mice as previously described [316] and as detailed in Appendix A. Islet transplants were carried out as previously [319, 320] with minor modifications. Briefly, male C57BL/6 mice between 8-12 weeks of age were rendered diabetic by a single injection (180 mg/kg of body weight) of streptozotocin (STZ) (Sigma). Onset of diabetes was confirmed by 3 consecutive days of hyperglycemia (>300mg/dL). Diabetic mice were transplanted with 200, 250, 400 or 500 freshly isolated WT or ATF3 KO islets beneath the kidney capsule. See Appendix D for additional details.

**Glucose tolerance tests and insulin ELISA.** Mice were fasted for 16 hours and subjected to glucose tolerance test as described previously [316]. Fasting serum insulin levels were measured by radioimmunoassay (Linco) using retro-orbital blood samples.
**Statistics.** All quantitative data in this chapter are expressed as means ±SEM, and comparisons made by the Student’s *t* test, unless otherwise indicated.

**RESULTS**

**The roles of ATF3 in isolation stress.** I examined whether ATF3 is expressed in the islets after isolation by immunoblot. Fig. 2.1A shows that the level of ATF3 mRNA level was the highest immediately after isolation and declined at 12-24 hours, consistent with the idea that ATF3 is induced by the isolation stress. Fig. 2.1B is a representative immunoblot, confirming the induction of ATF3 in the WT islets but the lack of ATF3 in the KO islets. To test the potential significance of ATF3 expression, WT islets and islets derived from ATF3 KO mice were compared for their viability using propidium iodide (PI) coupled with Hoechst stain. Viable cells are not permeable to PI and will only be stained by Hoechst; however, dead cells will be stained by both PI and Hoechst. Fig. 2.1C shows a representative picture with less PI stain in the KO islets than in the WT islets, consistent with a pro-apoptotic role of ATF3. Data quantification using the NIH Image J program (detailed in Fig. 2.7) showed lower percentage of PI-positive pixels in the KO islets than in the WT islets (Fig. 2.1D). Interestingly, both the WT and KO islets showed decline in cell death over time, but the WT islets showed a slight increase at the 48-hour time point, paralleling an increase in ATF3 expression at this time point.
**FIG. 2.1. ATF3 KO islets are protected from isolation stress.**  
* A: Primary islets from WT and ATF3 KO mice were isolated and allowed to recover for the indicated times. ATF3 mRNA levels were determined by qRT-PCR and standardized against β-actin. The standardized signal at time point 0 was arbitrarily defined 1. Shown are mean±SEM from three experiments.  
* B: Same as A but immunoblot is shown (a representative of three experiments).  
* C: Islet cell death was evaluated by propidium iodide (red) and Hoechst (blue) stain. Groups of 100-150 islets from each group were analyzed and representative fields from each time point are shown. Bar = 400 μm.  
* D: Quantitation of images in C. Y axis is the percentage of Hoechst positive pixels that are also PI-positive (see Fig. 2.7 for detailed steps of image analysis). * p<0.001  
* E: Same as in A except the indicated mRNAs were analyzed. Shown are representative graphs from three experiments. * p<0.05, # p<0.08 versus KO.
Since ATF3 is a transcription factor, it must exert its action, at least in part, by regulating downstream genes. Thus I examined the expression of various candidate genes by quantitative RT-PCR (qRT-PCR), focusing on two categories of genes: pro-apoptotic genes and genes involved in immuno-modulation. The reason for testing immuno-modulating genes is that, upon transplantation, the grafts induce immune responses from the hosts. Their ability to modulate the host responses greatly impacts the survival of the grafts. Although limited in scope, this screen showed higher expression of the following genes in the WT islets than in the KO islets (Fig. 2.1E): NOXA, bNIP3 (pro-apoptotic genes), and IL-1β, TNFα, IL-6, and CCL2 (also known as MCP-1) (immuno-modulating genes). The differences are statistically significant (p <0.05, that is 95% confidence) except for bNIP3 and CCL2, which showed a consistent trend in three independent experiments but had a p value of > 0.05 due to variations. I also examined CSF-1 (M-CSF), a macrophage recruitment factor, and Tissue Factor (TF), a factor whose expression contributes to islet loss in transplantation ([321]). No difference in CSF-1 expression was observed between the WT and KO islets (Fig. 2.1E), and the difference for TF was subtle (data not shown). These results indicate that ATF3 contributes to the increased expression of certain pro-apoptotic and pro-inflammatory genes and would thus enhance islet apoptosis and inflammation. The notion of increased apoptosis is consistent with the higher cell death in the WT islets shown above (panel C) and the functional consequences of ATF3 in inflammation will be addressed below (see Fig. 2.6).
The roles of ATF3 in proinflammatory stress. Upon transplantation, grafts face a barrage of inflammatory attacks from the hosts. Therefore, I examined the induction of ATF3 by proinflammatory cytokines, either as a combination of two cytokines (IL1β+IFNγ) or three cytokines (IL1β+INFγ+TNFα). Islets at 72 hours after isolation were used in this experiment, so that the ATF3 levels in the WT islets before induction were relatively low. Fig. 2.2A shows that ATF3 is induced in the islets both at the mRNA (left panel) and protein levels (right panel). Previously, we reported that ATF3 KO islets are partially protected from two cytokine–induced apoptosis as evidenced by the decrease in the cell population with sub-2N DNA content ([7]). Consistent with the previous results, the ATF3 KO islets had reduced levels of activated caspase 3 than the WT islets upon cytokine treatments (Fig. 2.2B). I then examined the expression of the candidate target genes described above. Fig. 2.2C shows that the WT islets had higher expression of TNFα, IL1β, IL6, and CCL2 than the KO islets (p< 0.05, see figure legend), indicating that ATF3 also up-regulates these genes (directly or indirectly) in the islets under the proinflammatory paradigm. No differences were observed for bNIP3 or TF (Fig. 2.2B and data not shown). The data for NOXA were not reproducible in four repeated experiments (not shown). Since ATF3 is induced by the proinflammatory cytokines (TNFα, IL1β), its ability to induce the expression of TNFα and IL1β indicates a positive feedback loop.
FIG. 2.2. ATF3 KO islets are protected from inflammatory cytokines.  A: Primary islets from WT and ATF3 KO mice were isolated and allowed to recover for 72 hours prior to three cytokine treatment (IL-1β, TNFα, and IFNγ).  ATF3 mRNA levels were determined by qRT-PCR and analyzed as in Fig. 2.1A.  Shown are mean±SEM from three experiments.  B: Islets prepared as in A were treated with three cytokines or two cytokines (IL-1β and TNFα) and analyzed by immunoblot for the indicated proteins at 24 h after treatment.  Shown is a representative of three experiments.  C: Same as in A except the indicated mRNAs were analyzed.  Shown are representative graphs from four experiments.  * p<0.05, ** p<0.02 versus KO.
The roles of ATF3 in hypoxia stress. Upon removal from the donors, the islets are subjected to potential hypoxic stress until graft vascularization in the host. Therefore, I examined the induction of ATF3 by hypoxia (1% O2). Again, islets at 72 hours after isolation were used in this experiment to reduce basal ATF3 levels. Because the majority of islets start to disintegrate at four to six hours under hypoxia, it is not possible to keep them continuously under this condition. I found that cyclic exposures of islets to hypoxia (1%) and normoxia (20%) for one hour each allow them to maintain their overall integrity for eight hours. However, at the end of four cycles, the islets start to lose their smooth boarders and appear unhealthy so I terminated the experiments after four cycles of hypoxia and normoxia as diagramed in Fig. 2.3A. Immunoblot indicated that ATF3 was induced at the end of all cycles, and Fig. 2.3B shows the data for cycles two and four. I also examined the induction of HIF-1α as a positive control for the hypoxic conditions (Fig. 2.3B). PI coupled with Hoechst stain indicated reducing viability (more PI stain) over cycles of hypoxia and normoxia (Fig. 2.3C). As shown in the representative images, PI signals are always at the center of the islets, consistent with the notion that the center of the islets have least oxygen and are most vulnerable to hypoxic stress. This is in contrast to isolation stress, where PI signals started as scattered on the islet surface then focused in the center of the islets at 48 hours (Fig. 2.1C), presumably due to the development of hypoxic center over time in the culture. I also quantified the signals by counting PI-positive pixels. Under hypoxia, the size of the islet affects the size of the hypoxic center; the bigger the islets, the bigger the hypoxic center. Thus, I plotted the number of PI-positive pixels against islet size (as measured by the Hoechst-positive pixels). Fig. 2.3D shows a bar graph of the PI-positive signals after arbitrarily dividing the islets into four groups: from
FIG. 2.3. ATF3 KO islets are protected from hypoxic stress. A: A schematic of the intermittent hypoxia treatments: hypoxia (1% O2) and normoxia (20% O2) for 1 h each with assays at the end of each cycle (red arrows). B: Primary islets from WT and ATF3 KO mice were isolated and allowed to recover for 72 h prior to hypoxia treatments. At the indicated cycles of treatments, the islets were analyzed by immunoblot for the indicated proteins. Shown is a representative of three experiments. C: Islet cell death was evaluated by PI (red) and Hoechst (blue) stain as in Fig. 2.1C, except that 250-300 islets were used in each group. Shown are representative fields from the indicated cycles. Bar = 200 μm. D: Quantitation of data in C for islets treated with two (top) or four (bottom) cycles of hypoxia. Islets were arbitrarily divided into four size groups and the mean ±SEM of PI-positive pixels in each group are shown (* p<0.001 versus WT). E: Islets treated with two cycles of hypoxia were analyzed for the indicated mRNAs as in Fig. 2.1E. The respective mRNA level under the un-stressed condition was arbitrarily defined as 1. Shown are average values from three experiments.
small (<20,000 Hoechst-positive pixels) to big (>40,000 Hoechst-positive pixels). After two cycles of hypoxia treatment, the differences between WT and KO islets are statistically significant in the larger islet groups (top panel), and after four cycles, the differences are significant for all four groups (bottom panel). Thus, WT islets were more vulnerable to the hypoxia stress than the KO islets. I then examined the expression of various candidate genes in the islets after hypoxia stress. Surprisingly, none of the genes described above showed statistically significant differences between WT and KO islets (Fig. 2.3E). Thus, under the hypoxia stress, ATF3 contributes to the reduced viability of the islets via a mechanism independent of inducing the genes I have examined thus far.

**Recruitment of ATF3 to various target promoters in the pancreatic β cells.** Taken together, the above results indicate that ATF3 is induced by the stress signals and contributes to the lower viability of islets under these conditions. However, ATF3 regulates various target genes in a stress-dependent manner. To address whether ATF3 directly regulates the target genes reported here, I analyzed their corresponding promoters by several programs and found multiple ATF/CRE or ATF/CRE-like sites—potential ATF3 binding sites—on the promoters (Fig. 2.8). Significantly, the majority of these sites are conserved in mouse and rat. To check the ability of ATF3 to bind to these sites in vivo, I carried out chromatin immunoprecipitation (ChIP) assay using INS-1 and INS-r3 cells infected with adenovirus expressing ATF3 or βgal as a control. Comparable results were obtained from these two cell lines and the data from INS-1 cells are shown in Fig. 2.4. Panel 2.4A shows that ATF3 indeed binds to the NOXA, bNIP3, IL1, IL-6, and CCL2 promoters at multiple sites. Several controls were carried out for the ChIP assay. (a) The
binding of ATF3 to the α- or β-actin promoter, which lacks recognizable ATF/CRE sites, were examined. No binding was observed, suggesting (albeit not proving) that the signals we observed on the target genes were not due to non-specific binding. In the same samples, PolIII was found to bind to the promoter of β-actin (a β-cell gene) but not α-actin (a non-β-cell gene), validating the ChIP procedure in that experiment (data not shown). (b) For all experiments, the signals from ATF3-expressing cells were normalized against that from βgal-expressing control cells to give rise to the fold enrichment. If the fold enrichment was the same for the ATF3 antibody and IgG (such as that at the -4,800 site on the IL1β promoter and the -4,400 site of the IL-6 promoter), I interpreted it as background fold enrichment (thus no binding). Only if the fold enrichment using the ATF3 antibody is much larger than that using IgG, did I interpret it as ATF3 binding (detailed in Fig. 2.8 legend). In four independent experiments, the pattern of binding (relative ATF3 recruitment to different sites on a given promoter) was largely the same, although the absolute fold enrichment varied to some extent from one experiment to another. Fig. 2.4A shows a representative set of data. Fig. 2.4B shows the result from the ChIP PCR reactions stopped during the linear phase of amplification; the most proximal site for each promoter was examined except those indicated as distal (D) in the figure (see legend). Collectively, these results indicate that ATF3 has the capacity to bind to various sites on the promoters examined in the studies.
FIG. 2.4. ATF3 binds to the promoters of the pro-apoptotic and pro-inflammatory target genes. A. INS-1 cells were infected with adenovirus expressing ATF3 (Ad-ATF3) or βGal (Ad-βGal) for 24 h prior to ChIP analysis using primer pairs (schematics in Fig. 2.8) for the potential binding sites at the indicated distance from the transcriptional start site (TSS). For each primer pair, the ChIP signals were determined by qPCR and normalized to input chromatin. The normalized ChIP signals from the Ad-ATF3-infected cells were divided by that from the Ad-βGal-infected cells to obtain the “fold enrichment.” Fold enrichment from immunoprecipitation using either IgG (black bars) or ATF3 antibodies (white bars) is shown. B: ChIP signals were analyzed by agarose gel electrophoresis at the end of the linear phase of PCR reactions. All primer pairs were for the most proximal binding sites relative to the corresponding TSS, except those indicated as distal (D). TNFα(D): -4800bp; IL-6(D): -2400 bp. Shown is a representative of four experiments.
The roles of ATF3 in a syngeneic mouse islet transplantation model. To test whether ATF3 KO islets would perform better than WT islets in transplantation, I implanted the islets under the kidney capsules of syngeneic wild type mice. C57BL/6 mice were rendered diabetic by streptozotocin (STZ) one week prior to transplantation. At a saturating dose (400 islet equivalents), both WT and KO islets restored euglycemic (Fig. 2.9). At a marginal dose (250 islets equivalents), KO islets performed better than the WT islets as indicated by several criteria: (a) lower blood glucose levels from post-operation day (POD) 1 to POD28 (Fig. 2.5A), (b) higher percent of euglycemic mice from POD 1 to POD28 (Fig. 2.5B), (c) better glucose tolerance test on POD28 (Fig. 2.5C), (d) smaller “area under the curve” in the glucose tolerance test (Fig. 2.5D), and (e) higher serum insulin level on POD28 (Fig. 2.5E). Removing the grafts at POD28 rendered the mice hyperglycemic (Fig. 2.5A), confirming that the therapeutic effects were due to the transplanted islets. Since ATF3 is pro-apoptotic, I examined whether the KO islets had reduced apoptosis using activated caspase 3 as a marker by immunohistochemistry assay. The signals on POD5 were the highest among the days we examined (POD1, 3, 5, and 7). To semi-quantify the signals, I used the NIH Image J program and carried out the following steps: (a) delineate the region of interest (ROI)—islets, (b) threshold the image and convert to black and white binary signals, (c) count the positive pixels, and (d) calculate the ratio of the activated caspase 3-positive pixels to the total pixel counts in the ROI. This analysis showed that KO islets had a lower percentage of area stained positive for activated caspase 3 than the WT islets (4.51±1.07 % versus 15.69±3.12 % in WT, multiple sections from four mice per group, p< 0.05). Fig. 2.5F shows the representative images.
FIG. 2.5. ATF3 KO islets are protected from syngeneic transplantation stress.  

A. C57BL/6 mice were rendered diabetic by STZ prior to receiving a marginal dose of 250 WT or ATF3 KO islets as detailed in the method section. Non-fasted blood glucose levels were monitored from post-operative day (POD) 1 to POD28, when the grafts were removed (UNX). N=5 for WT and N=6 for KO islet recipients (* p<0.05 versus WT). A mock transplant recipient died at POD 14 as indicated (grey dotted line). 

B. Percent of mice maintaining euglycemia (blood glucose <200 mg/dL) through POD25 is shown (* p<0.05, Fisher’s exact test). 

C: The result from intraperitoneal glucose tolerance test on POD28 is shown (* p<0.05 versus WT). 

D: Area under curve (AUC) from panel C is shown (* p<0.005 versus WT). 

E: Fasting serum insulin on POD28 is shown (* p<0.05 versus WT). 

F: Islets grafts at the indicated PODs were analyzed by H&E stain or immunohistochemistry using the indicated antibodies. Bar = 100µm.
Parallel experiments using IgG as a negative control showed no signals. I also stained the
graft sections for ATF3 and confirmed its expression in the WT islets but not KO islets.
Insulin stain and hematoxylin plus eosin (H&E) stain were shown for comparison.

As shown above, ATF3 contributes to the up-regulation of CCL2 in the islets under
the isolation and proinflammatory stresses. Since CCL2 is a potent macrophage
recruitment factor ([317]), we asked whether the WT and KO islets differ in their abilities
to recruit macrophages. To address this question, I stained the grafts for F4/80, a
macrophage marker ([322]). Fig. 2.6A shows that macrophage infiltration became obvious
at POD2 and continued to increase over time to POD7, when the experiments were
terminated. To semi-quantify the signals, I used the NIH Image J program and the same
steps described above. In this case, the ROI is the areas within the kidney sub-capsule that
are not occupied by the islets. Fig. 2.10 shows an example of ROI. Fig. 2.6B shows that
macrophage infiltration was lower in the KO than in the WT grafts. Although the
difference were only 10-20%, they are statistically significant ($P < 0.05$), suggesting that
ATF3 expression in the WT islets contributes to their ability to recruit macrophages. To
further test this idea, I carried out an in vitro migration assay using the Boyden chamber. I
isolated bone marrow cells from the tibia and differentiated them into macrophages in
vitro. The migration of macrophages was then examined by placing them in the top half of
the Boyden chamber and with the conditioned media from islets in the bottom half. After a
12 hour incubation, the relative number of macrophages that migrated through to the
underside of the membrane was measured. Normal media was used as a control and
macrophage migration under this condition was arbitrarily defined as 1. Fig. 2.6C shows
that conditioned media from the WT islets induced macrophages to migrate

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FIG. 2.6. Transplanted ATF3 KO islets promote less macrophage recruitment. A: WT and ATF3 KO grafts at the indicated PODs were analyzed by immunohistochemistry for F4/80, a macrophage marker. Shown are representative fields from 4 mice each with 10-20 fields examined in each mouse. Bar = 100µm. B: Immunohistochemistry data in panel A were quantified using the methods detailed in the text and Fig. 2.10. * p<0.05 versus WT. C: Bone marrow derived macrophages were analyzed by the Boyden chamber migration assay using either media control (-) or conditioned media from the indicated islets in the bottom chambers. Migrated cells were stained by crystal violet and the signal from the media control was arbitrarily defined as 1. The signals from the islet conditioned media were then standardized against the total RNA contents in the islets. *p<0.05 versus WT.
more efficiently than that from the KO islets. Thus, the in vitro migration assay supported the notion that ATF3 facilitates macrophage recruitment by the islets.

DISCUSSION

In this chapter, I present evidence that ATF3 up-regulates pro-apoptotic genes and genes involved in immuno-modulation. Previously, our lab showed that ATF3 down-regulates IRS2 ([316]), a potent pro-survival factor in β cells, providing a mechanistic explanation for the pro-apoptotic action of ATF3 in pancreatic β cells ([316], [7], and this report). Results described in this chapter indicate that ATF3 up-regulates the expression of NOXA, providing additional mechanistic insight into the ability of ATF3 to promote β cell death. Since apoptosis is a major reason for islet loss during transplantation, it is not surprising that the ATF3 KO islets performed better than WT islets in transplantation. These results are consistent with previous reports that dampening apoptosis improves transplantation. However, the main difference here is that apoptosis is being dampened by deleting a pro-apoptotic gene, rather than expressing anti-apoptotic genes, such as A20, hepatocyte growth factor (HGF), Bcl-2, Akt, IRS-2, and XIAP, in the previous reports ([277, 319, 320, 323-326]).

The up-regulation of CCL2 (MCP-1) by ATF3 has significant implications. CCL2 is a chemokine and recruits macrophages ([317]). Thus, its up-regulation by ATF3 provides an explanation for the reduced macrophage recruitment in the KO grafts. Importantly, macrophages depletion in the recipients has been demonstrated to improve islet graft survival ([313, 327]). These results complement and further strengthen the idea that ATF3 deficiency is beneficial to the islet grafts. The up-regulation of TNFα,
IL1β, and IL-6 by ATF3 also has significant implications. These cytokines have been demonstrated to have deleterious effects on β cells ([174], [205]) and contribute to primary non-function in islet transplantation ([168, 315]). Intriguingly, TNFα receptor antagonist, in conjunction with a glucagon-like peptide-1 agonist, was recently shown to improve the Edmonton Protocol in a clinical trial ([211]). Importantly, ATF3 itself is induced in the islets by TNFα and IL1β. Thus, ATF3 forms a positive-feedback loop to amplify the signals. In this context, it is notable that islets from mice deficient in macrophage migration inhibitory factor (MIF), a proinflammatory cytokine secreted in a large quantity by islets after isolation, had an 8% increase in cure rate than the WT islets ([328]). Since MIF is considered a stress mediator, it would be interesting to find out whether ATF3 regulates its expression. It is also important to note that ATF3 has been shown to down-regulate the expression of IL6, IL12a, and TNFα in macrophages upon lipopolysaccharide (LPS) treatment ([30, 96], [51]). Thus, ATF3 functions in macrophages to dampen, rather than enhance, the inflammatory responses (as shown here in islets). Explanations for this apparent discrepancy include the differences in cell types (macrophages versus islet epithelia) and stress signals (LPS versus cytokines or isolation stress).

Taken together, my results suggest a critical role for ATF3 in primary non-function. To our knowledge, this is the first report to demonstrate the protective effects of ATF3 knockout in islet transplantation, and the first to implicate ATF3 in the ability of islet grafts to induce inflammatory reactions. In light of the emerging views that inflammation plays an important role in islet graft rejection ([329], [330]), my results suggest that ATF3 may be a therapeutic target to improve islet transplantation. The
benefits of silencing ATF3 include not only the reduction of intracellular decision to undergo apoptosis, but also the reduction of the ability of islets to provoke host inflammatory response.

ACKNOWLEDGMENTS

I would like to thank several people for their contribution to the work presented in this chapter. Several islet transplant experiments conducted in the course of this study were performed by our collaborators Dr. Shane Grey and Dr. Adolfo Garcia-Ocana. Their efforts to initially characterize the phenotype of mice receiving ATF3 KO islets, as well as their intellectual contributions, dramatically accelerated the development of this project. I thank Dr. Lei Zhang for the helpful tips he provided as I learned how to perform islet transplantation. I would also like to thank Dr. Mariano Viapiano as well as Dr. Anthony Brown for the intellectual assistance they provided in regards to the image analysis performed in this chapter. My special thanks to Dr. Viapiano for writing the custom ImageJ macro that greatly facilitated the quantitation of islet viability in vitro. Finally, I thank the Ohio State University College of Veterinary Medicine Histology Core for consistent and high quality assistance in sectioning of islet grafts.
FIG. 2.7. Quantitation of the propidium iodide and Hoechst fluorescent signals. All fluorescent signals were imaged with identical settings. The red (propidium iodide) and blue (Hoechst) channels were separated and a threshold for each channel was applied. The threshold was pre-determined in a preliminary analysis of all images to exclude background signals. The islets—the region of interest (ROI)—were automatically delineated by a custom-written ImageJ macro program (described in the Appendix B). The blue and red pixels within the ROIs (islets) were then counted and the ratio calculated to obtain the percent of the Hoechst-positive pixels that are also PI-positive (Y-axis in Fig. 2.1D).
FIG. 2.8. Schematic representations of the potential ATF3 target promoters. The indicated rat promoter sequences up to 5,000bp from the transcriptional start site (TSS) were obtained from the Human Genome Browser Gateway, followed by the analyses using the Vector NTI v10.3.0 and the MotifScanner v3.1.1 programs. The results were compared to the Salk Institute CREB Target Gene Database. Any sites on the Salk Database that were missed by our analyses were added to the promoters. The ATF/CRE or ATF/CRE-like sites and the amplicon produced by the primer pairs used in the ChIP analyses were indicated. The alignments with the corresponding mouse promoters were carried out using Vector NTI and the homology is represented as a Boolean homology plot (threshold value 0.85). ATF3 binding was arbitrarily classified as below. First, the fold enrichment was calculated: the ratio of ChIP signals using the ATF3 antibody from the ATF3-expressing cells to that from the βgal-expressing cells. Second, the fold enrichment of ChIP signals using IgG was similarly determined and subtracted from the ATF3 antibody signal to obtain the “delta fold enrichment.” The delta number is then used to classify binding: None = ≤1; Low= 1 to <8; Medium= 8-40; High= >40.
FIG. 2.9. The WT and ATF3 KO islets performed similarly at the dose of 400 islets in a syngeneic transplantation. C57BL/6 mice were rendered diabetic by STZ prior to receiving WT or ATF3 KO islets as detailed in the method section. Non-fasted blood glucose levels were monitored from post-operative day (POD) 1 to POD7. n=5 for WT islet recipients; n=5 for KO recipients.
FIG. 2.10. Quantitation of the F4/80-positive immunohistochemistry signals. Islet grafts after immunohistochemistry for F4/80 were imaged under identical bright field settings. The region of interest (ROI) was defined as the area within the kidney subcapsule region that was not occupied by the islet graft. This was manually delineated using the ImageJ polygon selection tool by tracing around the islets within the boarders of the kidney capsule and the kidney parenchyma. The immunohistochemistry data were converted to grey scale images and the pixel intensity above a predefined threshold within the ROI were counted. The percent F4/80-positive area (Y-axis in Fig. 2.6B) was calculated as the ratio of black pixel counts to the total pixel counts in the ROI.
<table>
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<th>Gene</th>
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<th>Percent Sequence Homology</th>
<th>ATF3 Binding Score</th>
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Table 2.1. **Sequences of the potential ATF3 response elements within the putative target promoters.** Potential ATF3 binding sites were identified based on rat promoter sequence and aligned to mouse as indicated in Fig 2.8. Shown are the positions of the potential binding sites, their sequence, and the ATF3 binding classifications (defined in Fig. 2.8 legend).
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**Table 2.2. Sequences of the primers used in this study.** Rat and mouse primer sequences were designed for each gene using PerlPrimer (version 1.1.14).
Table 2.2 continued

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CHAPTER 3

THE USE OF A RGD-MODIFIED AAV VECTOR AS A STRATEGY TO IMPROVE ISLET GENE TARGETING

SUMMARY

The utilization of gene therapy to express pro-survival genes in islets \textit{ex vivo} has been proposed as a potential method to improve the clinical outcome from islet transplantation. In recent years, the adeno-associated virus (AAV) has received much attention as a potential gene therapy vector because of its relatively low immunogenic profile. However, its application towards islet transplantation has been hindered by poor islet infection efficiency. Therefore, the goal of this study was to test whether the insertion of the Arg-Gly-Asp (RGD) amino acid sequence, a potent cell adhesion motif, into a capsid protein of AAV was capable of improving its islet infection efficiency. This hypothesis was based on the literature supported idea that increasing AAV adhesion to the islet cell surface would increase the frequency with which infection would occur. To address this question, I compared a panel of recombinant AAV pseudotypes as well as a RGD-modified AAV vector for their potential to infect islets in vitro. I also tested the functional consequence of RGD/AAV infection in vivo using a syngeneic mouse
transplantation model. Work presented in this chapter demonstrates that the RGD modification improves AAV infection efficiency of murine, non-human primate and human islets in vitro. Furthermore, infecting islets with the AAV1/RGD vector did not compromise their ability to function in vivo, as transplant recipients of both non-infected and AAV1/RGD infected islets demonstrated similar ability to control blood glucose levels. In conclusion, work presented in this chapter demonstrates that the RGD-modified AAV gene therapy vector efficiently infect islets without compromising their function in vivo. This finding is significant in the context of islet transplantation as it identifies AAV1/RGD as an improved non-immunogenic viral vector and leaves open its use for many therapeutic applications.

INTRODUCTION

As mentioned in chapter 2, pancreatic islet transplantation represents a potential therapeutic option for the treatment of type 1 diabetes. Subsequent to initial successes with islet transplantation in rodent models of diabetes, the ―Edmonton Protocol‖ for human islet transplantation was developed and reported to convey insulin independence to recipients for a median of 15 months [210, 331]. However, the widespread clinical use of islet transplantation has been limited by a necessity for multiple rounds of transplantation and life-long systemic immunosuppression in recipients. In 2008, a modified ―Edmonton Protocol‖ from the University of Illinois at Chicago (UIC) was reported to reduce the number of islets needed for recipients to achieve insulin independence. In this modified protocol, GLP-1 and TNF-α decoy receptor were added to the systemic immunosuppressive regimen to enhance islet function and reduce
systemic inflammation [211]. While the long term benefits from the UIC modification are yet to be known, their initial results suggest that the development of new strategies geared towards improving functional islet mass and reducing inflammation may increase the efficacy and clinical utility of islet transplantation.

To this end, the use of gene therapy to ectopically express pro-survival or immunomodulatory genes in transplanted grafts has shown great promise in recent years. Several adenoviral based approaches have improved outcomes in animal models and identified effective therapeutic candidate genes. However, while adenoviral based gene therapy vectors have the advantage of broad tropism and a large capacity for DNA inserts, they are known to elicit an adenovirus-specific immune response when the infected cells are exposed to the innate and adaptive immune response. The immune response directed against adenovirus infected cells has been proposed to reduce the duration of transgene expression as well as directly compromise islet function [332-335]. Thus, adenoviral based gene therapy vectors are not ideally suited for pancreatic islet transplantation.

In contrast to adenovirus, several properties of the adeno-associated virus (AAV) make it ideally suited as a gene therapy vector. These properties include its lack of pathogenicity, low immunogenicity, negligible random integration into host DNA, and ability to infect both dividing and non-dividing cells. In fact, several promising phase I and II clinical trials using AAV based gene therapy vectors have found AAV to be well tolerated in subjects [336]. However, in one clinical trial, the genomic integration of AAV near a potential oncogene was associated with the development of leukemia in 4 out of 10 patients [337, 338]. As a result, the tumor inducing potential of AAV is
currently a topic of debate and is considered by some to be a disadvantage to its systemic administration. Fortunately, the use of AAV as a gene therapy vector in islet transplantation does not involve systemic administration of virus, but rather ex vivo infection of islets immediately following isolation from a donor. Therefore viral particles not taken up by the islets can be washed away prior to transplantation, thus reducing the opportunity for adverse events to occur in the recipient.

There have been numerous reports of AAV being used to infect isolated islets from rodents, pigs, non-human primates and humans. Early studies focused on the AAV2 serotype, while more recently improved but varying results have been obtained through the use of different serotypes, with either AAV1 or AAV6 generally outperforming others in isolated rodent islets [339-343]. These islets can be transplanted into syngeneic animals with no apparent loss of function. In contrast to rodent islets, transduction patterns in human islets show little difference in efficiency between 2, 6, or 8, but less transduction from AAV1 vectors. However, in these reports, the infection efficiency ranged from 15% to 20% of the cells from each islet being infected. Therefore, any efforts directed at improving the islet infection efficiency of AAV will further strengthen its utility as a gene therapy vector for islet transplantation.

Modifications to viral coat proteins or the addition of linker agents that improve the affinity of viral particles for the cell surface, such as bispecific antibodies or viral receptor/cellular receptor ligand scaffolds, have been reported to improve viral tropism and infection efficiency [344]. For example, the addition of the prototypical cell adhesion motif Arg-Gly-Asp (RGD) to viral coat proteins of both adenovirus and AAV improved their ability to infect a range of cell types [345-348]. While the modification of
an adenovirus vector with RGD has been found to dramatically improve viral infection of pancreatic islet cells *in vitro*, the effect of RGD modification on the islet infection efficiency of AAV has not been reported [345]. Therefore, we sought to test the hypothesis that the RGD modification of an AAV vector would increase its capacity to infect islets *in vitro* and strengthen the utility of AAV as a gene therapy vector for islet transplantation.

**MATERIALS AND METHODS**

**Animals, islet isolation, and peptides.** Wild type C57BL/6 mice were housed under conventional conditions and cared for according to Ohio State University Laboratory Animal Resources guidelines. Islets were isolated from adult mice as previously described [319, 349]. Briefly, 0.25mg/ml of rat liberase (Roche) was perfused to the pancreas through the common bile duct. Pancreata were removed and incubated at 37 °C for 13-16 minutes prior to filtration through a 419µm wire mesh. Islets were then isolated using Histopaque (Sigma) gradient, capture on 100µm nylon filter, and hand-picked. After isolation, islets were incubated at 37 °C in a humidified cell culture incubator. For additional details see Appendix A. Human islet isolations were performed at the Ohio State University’s Cell Therapy Lab. Briefly, the pancreatic duct was cannulated, and the pancreas was perfused with a 0.0125% solution of Liberase™ HI (Roche, Indianapolis, IN). Mechanical and enzymatic digestion was performed in the Ricordi digestion chamber (Biorep Technologies, Miami, FL) until 50% free islets were observed by dithizone staining (FisherChemical, Fair Lawn, NJ). Digestion was stopped by cold dilution. Pooled and washed islets were purified as needed to reduce the tissue pellet to less than 5mL.
Purification was performed using a continuous density gradient of 1.100-1.077 g/mL ficoll (Cedar Lane Laboratories, Hornby, ON) on the COBE® 2991™ Cell Processor (Gambro BCT, Lakewood, CO). The final islet product was resuspended in Miami Media (Mediatek, CT) supplemented with an additional 0.5% human serum albumin. Islets were then hand-picked for experiments. When indicated, islets were incubated with a control (R-G-E-S) and blocking (G-R-G-D-S-P-K) peptide (Sigma).

**Adeno-associated viruses and adenoviruses.** Adenoviruses were constructed using the Invitrogen Gateway technology and purified by cesium chloride ultracentrifugation [350]. Recombinant self-complementary AAV (scAAV) vectors containing the enhanced green fluorescent protein gene (eGFP) driven by the U1A promoter/enhancer region were produced by triple plasmid transfection, as previously described [351]. Briefly, 293 cells were co-transfected with the adenoviral helper plasmid pXX-680, the scAAV-U1a-GFP construct and the corresponding AAV serotype specific packaging plasmid, using the calcium phosphate transfection system [352]. Forty-eight hours after transfection, cells were harvested and pelleted by low-speed centrifugation. Cells were resuspended in lysis buffer, freeze-thawed several times, and treated with Benzonase nuclease (Sigma Life Science) at 50U/ml for 30 min at 37°C. Cell debris was spun down at 2700g for 15 min at 4°C. Virus was purified from supernatant by two rounds of cesium chloride gradient ultracentrifugation. After the second centrifugation, the peak fractions, as determined by dot blot hybridization, were dialyzed for 2 rounds against 10mM Tris-buffer containing 200mM NaCl for removal of cesium chloride. Final viral preparations were kept at -80°C.
Final viral titers were quantified by dot-blot assay in comparison to a standard plasmid range, as previously described [353].

**Image analysis and statistics.** Confocal GFP image and fluorescent immunohistochemistry analysis was done using the NIH ImageJ Software (version 1.41a). To identify the area of GFP colocalizing with insulin, the following steps were taken. Red and Green channel images were converted to 8bit and the “smooth” function under the process menu was executed. Next, the channels brightness and contrast were adjusted using the “autobright” function. Regional holes were then filled using the binary “fill holes” function under the process menu. Images were thresholded and the separate channel areas measured. The red channel area above the threshold was selected as a mask using the copy function and pasted onto the green channel with the paste control function set to “and”. The result is an image that only shows the green pixels co-localizing with red. Measuring the area of this image is the quantitative measure of co-localization and the fraction obtained by dividing this number from the area measurements obtained from the red and green channels separately is the percent of co-localization. For our purposes, we were interested in identifying the β-cell area infected by AAV, therefore, the co-localization area was divided by the total red area. All quantitative data are expressed as means ±S.E.M., and comparisons are made by the Student’s t test.

**Islet transplantation and glucose tolerance test.** Islet transplants were carried out as previously ([319], [320]) with minor modifications. Briefly, adult mice were rendered diabetic by a single injection (190 mg/kg of body weight) of streptozotocin (STZ) (Sigma).
Onset of diabetes was confirmed by 3 consecutive days of hyperglycemia (>300mg/dL). Diabetic mice were transplanted with 150 or 300 islets beneath the kidney capsule. For additional details see Appendix D. Glucose tolerance tests were performed on 16 hour fasted mice by intraperitoneal injection with 1.5 grams of glucose/kg of body weight as previously reported [354]. Blood samples were obtained from the snipped tail and analyzed using an Ascensia Contour portable glucometer at 15, 30, 60, 120 and 180 minutes after injection.

RESULTS

The role of RGD in promoting AAV infection of isolated mouse islets. Due to somewhat varying reports on the infection efficiency of different AAV pseudotypes for murine islets, I first examined the relative performance of our GFP expressing AAV vectors using GFP as a marker for cellular infection. Fig. 3.1A shows representative GFP images from confocal microscopy on islets during the 6 days of infection. In three independent experiments, I consistently observed AAV1 infection to produce the greatest area of GFP positivity per islet when compared to the other vectors. AAV2 and AAV6 infections yielded comparable GFP positive area to each other, but they did not perform as well as AAV1. Very few GFP positive cells were detected in islets infected with AAV5 or AAV8. These results indicated that AAV1 had the highest murine islet infection efficiency of the AAV pseudotypes tested. In support of this conclusion, Fig. 3.5 shows a representative result from dot-blot analysis for the titers of the different AAV pseudotypes. This result confirms that the AAV viral titer determinations were accurate.
FIG. 3.1. Addition of a RGD motif to AAV improves mouse islet infection efficiency. Mouse islets were infected with the indicated GFP expressing AAV pseudotypes immediately following isolation and imaged daily by confocal microscopy for GFP expression. Shown are representative images. (A) Survey of the relative islet infection efficiencies of the non-RGD modified AAV pseudotypes. (B) Comparison of AAV1 and AAV1/RGD for islet infection efficiency. (C) The contribution of the RGD motif towards islet infection efficiency was evaluated by adding a soluble competitor RGD peptide at the indicated concentrations one hour prior to and during a 12 hour infection. Imaging for GFP expression was performed 4 days after infection. (D) Quantitation of GFP positive area was performed on images taken in C. Data represented as means ±S.E.M. * p<0.0001 AAV vs. AAV/RGD. # p< 0.003 AAV/RGD 0µM competitor vs. indicated competitor concentration.
and that islets were being incubated with a comparable number of DNA packaged viral particles for each pseudotype.

Previously, AAV1 and AAV2 vectors modified by the insertion of a RGD peptide into a viral coat protein were shown to have enhanced infection efficiency for cells expressing low levels of the viruses natural and primary attachment receptor [348, 355].

Given the superior performance of AAV1 with our murine islets, I tested the RGD modified AAV1 (AAV1/RGD) vector to see if it had enhanced islet infection efficiency. Fig. 3.1B shows representative images from confocal microscopy on islets infected with GFP expressing AAV1 or AAV1/RGD. AAV1/RGD infection consistently resulted in an earlier appearance of GFP signal and an overall increase in the area of GFP positivity per islet, indicating that the addition of the RGD motif to AAV1 markedly improved its islet infection efficiency. To test the specificity by which RGD insertion functioned, infections were conducted in the presence of increasing amounts of free RGD peptide. Representative images in Fig. 3.1C and quantitation in Fig. 3.1D show a dose dependent inhibition of AAV1/RGD mediated gene expression upon the addition of free RGD peptide. These data demonstrate that the improved infection efficiency of AAV1/RGD is conveyed by the presence of the RGD motif.

**The AAV1/RGD vector infects insulin positive β-cells within murine islets.**

The application of gene therapy to islet transplantation requires the targeting of a pro-survival expression construct to the insulin producing β-cells of the islet, as their survival is imperative to achieve maximal efficacy from transplantation. To evaluate the ability of AAV1/RGD to infect β-cells within the islet and provide persistent gene expression
FIG. 3.2. AAV1/RGD infects insulin positive β-cells within islets. To determine the AAV1/RGD β-cell infection efficiency, as well as persistence of ectopic gene expression after 14 days in vivo, mouse islets were picked into groups immediately following isolation for mock infection or infection with GFP expressing AAV1/RGD. Six days after infection, islets were washed free of virus and transplanted under the kidney capsule of syngeneic STZ-induced diabetic mice. Fourteen days after transplantation, grafts were recovered from the mice and processed for histological analysis. Shown are representative images from immunofluorescence assay on non-infected and GFP expressing AAV1/RGD infected grafts for insulin and GFP. Signals for insulin, GFP and Toto-3 (DNA) are shown merged. GFP positive insulin area was quantitated.
subsequent to transplantation, I transplanted non-infected and AAV1/RGD infected syngeneic islets to the kidney subcapsule of C57BL/6 mice. Fourteen days after transplantation, grafts were recovered and examined immunohistochemically for insulin and GFP expression (Fig. 3.2). Quantitation of the insulin positive pixel area that overlapped with GFP indicated a 52.41% +/- 0.06% co-localization, suggesting that the AAV1/RGD vector can be used ex vivo to mediate persistent gene expression in β-cells transplanted into diabetic recipients.

**The AAV1/RGD vector does not compromise islet graft function.**

Previous work done in our lab as well as others has suggested that isolated islets are exceptionally sensitive to stress induced death [7, 319]. Therefore, an ideal gene targeting vehicle for islet transplantation is one that has the least intrinsic capacity to induce β-cell death. An overwhelming majority of reports using gene therapy to improve islet transplant outcome have relied upon adenovirus infection to mediate gene expression. Despite the known drawbacks of adenoviral delivery vehicles, many of these reports still demonstrated therapeutic benefit. However, the potency of their gene therapy efforts may have been limited by adverse consequences from adenoviral infection. In fact, Cheng et. al. recently characterized the negative effect of adenovirus on islets during transplantation and found that adenovirus infection, when compared to no infection, increased the time that it took to cure diabetes, induced transient but robust infiltration of inflammatory cells, and reduced short term graft performance during intraperitoneal glucose tolerance test [356]. These findings suggest that the utilization of an alternative vector may improve the efficacy of gene therapy efforts in islet transplantation.
Therefore, I compared the performance of islets in a syngeneic model of transplantation that had either been mock infected, adenovirus infected or AAV1/RGD infected. In preliminary experiments, I found that the expression of GFP negatively affected the performance of transplanted islets (Fig. 3.6), therefore in this experiment I used ‘control’ viruses that did not drive ectopic gene expression. In Fig. 3.3A, non-fasted blood glucose measurements are shown for the three infection groups as well as diabetic and non-diabetic control mice. No differences were observed in non-fasting blood glucose levels, suggesting that all groups received and maintained at least the minimal functional islet mass necessary for maintaining euglycemia. However, upon intra-peritoneal glucose tolerance test (IPGTT), mice that received adenovirus infected islets failed to perform as well as the other groups (Fig. 3.3B). Area under the glucose tolerance curve is shown in Fig. 3.3C. These results indicate that AAV infection does not compromise transplanted islet function while adenovirus infection does. To further evaluate the functional consequence of AAV1/RGD infection on islets, I repeated the transplantation experiment using both a curative (300) and marginal (150) islet dose (Fig. 3.3D). Even with a marginal dose of islets, similar non-fasting glucose levels in non-infected and AAV1/RGD infected grafts were observed. This result further suggested that AAV1/RGD infection does not compromise islet function.

The role of RGD in promoting AAV infection of isolated human and non-human primate islets.

To evaluate the potential utility of AAV1/RGD as a gene targeting vector for human islets, I repeated the AAV pseudotype survey on freshly isolated human islets. Fig. 3.4A
FIG. 3.3. AAV1/RGD does not compromise islet function in vivo. Mouse islets were picked into groups immediately following isolation for mock, adenovirus, or AAV1/RGD infection. Six days after infection, islets were washed free of virus and transplanted under the kidney capsule of syngeneic STZ-induced diabetic mice and monitored for blood glucose levels. (A) Non-fasted blood glucose levels of mice transplanted with 300 non-infected islets or islets infected as indicated. (B) Intraperitoneal glucose tolerance test performed on POD 28. (C) Area under the glucose tolerance curves in B. (D) Non-fasted blood glucose of mice transplanted with either a curative (300) or marginal (150) number of non-infected or AAV1/RGD infected islets. Data represent means ±S.E.M. * p< 0.03 Adenovirus vs. AAV1/RGD infection.
Addition of a RGD motif to AAV improves human islet infection efficiency.

Human islets were infected with the indicated GFP expressing AAV pseudotypes immediately following isolation and imaged daily by confocal microscopy for GFP expression. Representative images are shown. (A) Survey of the relative islet infection efficiency of AAV1/RGD and the non-RGD modified AAV pseudotypes. (B) The contribution of the RGD motif towards human islet infection efficiency was evaluated by adding a soluble control RGE peptide or competitor RGD peptide at the indicated concentrations one hour prior to and during a 12 hour infection. Imaging for GFP expression was performed 4 days after infection. (C) Quantitation of GFP positive area was performed on images taken in B. Data represented as means ±S.E.M. # p< 0.0001 AAV vs. AAV/RGD at the indicated control peptide concentration. *p< 0.0006 AAV/RGD1 for 0μM RGD competitor vs. indicated competitor concentration.
shows representative GFP images from confocal microscopy on these islets during the 6 days of infection. While all AAV infections resulted in some detectible GFP positive signal, the AAV1/RGD infection produced the greatest area of GFP positivity per islet. Consistent with reports in the literature, AAV2 infection resulted in more GFP signal than any other non-engineered pseudotype, however it did not infect as well as the AAV1/RGD virus. Infection with AAV5, AAV6 or AAV8 resulted in similar GFP positivity that was slightly reduced when compared to AAV2. Interestingly, AAV1 infection yielded the least GFP positive signal. Thus, the engineering of AAV1 with a RGD motif dramatically increases its ability to infect human islets. Consistent with this finding, Fig. 3.7 shows the result from an AAV pseudotype survey on non-human primate islets where AAV1/RGD was again observed to have the highest islet infection efficiency.

To test the specificity by which RGD improved AAV1 infection of human islets, infections were conducted in the presence of increasing amounts of free RGE control peptide and RGD blocking peptide. Representative images in Fig. 3.4B and quantitation in Fig. 3.4C show a dose dependent inhibition of AAV1/RGD mediated gene expression upon the addition of free RGD peptide but not control RGE peptide. These data demonstrate that the improved infection efficiency of AAV1/RGD is conveyed by the presence of the RGD motif.

DISCUSSION

Pancreatic islet transplantation has been utilized as a treatment for type 1 diabetes, however its widespread use has been limited by a substantial loss of functional islet mass
in the months following transplantation. Several factors are believed to contribute to this reduction in graft function and survival, some of which include the allo and autoimmune responses, the local non-specific inflammatory response, and the ischemic graft microenvironment. Even the immunosuppressive regime, which is intended to promote graft survival, has been reported to be toxic to the islets and directly promote their death [357]. Therefore, strategies aimed at improving islet cell survival and engraftment in recipients with minimal immunosuppressive therapy are currently being pursued.

Several groups, including our own, have reported on different strategies to promote islet graft survival in animal models of type 1 diabetes. Table 3.1 summarizes a recent survey of these strategies. The criteria for inclusion in this survey were three fold and allowed for a direct comparison of the relative efficacy of each approach. First, the work had to involve primary islets. Second, “no intervention” control data had to be included as a reference point for the therapeutic effect. Third, therapeutic interventions had to be performed directly on the islets and not the recipient host. From this survey, treatment strategies were generally found to fall into four main categories; (1) genetic modification to express a protective gene, repress a deleterious gene or alter immunogenicity, (2) in vitro treatment with a chemical or biological agent, (3) islet encapsulation and (4) combination treatments. The effects from the interventions summarized in this survey are represented by changes in one of three measures; cure rate, non-fasting blood glucose concentration, or time to reject. In each case, the intervention effect has been normalized to the “no treatment” control group and is sorted in table 3.1 in order of decreasing effect within each measure.
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<tr>
<td>Insulin</td>
<td>Adenovirus</td>
<td>Human (250)</td>
<td>SCID</td>
<td>Glucose reduced 200mg/dl</td>
<td>[360]</td>
</tr>
<tr>
<td>HGF</td>
<td>Adenovirus</td>
<td>NHP (500IE)</td>
<td>NOD-SCID</td>
<td>Glucose reduced 200mg/dl</td>
<td>[323]</td>
</tr>
<tr>
<td>Prohormone Convertase (PC) 1/3</td>
<td>Adenovirus</td>
<td>C57BL/6 (200)</td>
<td>C57BL/6</td>
<td>Glucose reduced 180mg/dl</td>
<td>[361]</td>
</tr>
<tr>
<td>AKT (const. active)</td>
<td>Adenovirus</td>
<td>Human (1500IE)</td>
<td>SCID</td>
<td>Glucose reduced 175mg/dl</td>
<td>[325]</td>
</tr>
<tr>
<td>HGF</td>
<td>Adenovirus</td>
<td>CD-1 (300)</td>
<td>SCID</td>
<td>Glucose reduced 100mg/dl</td>
<td>[320]</td>
</tr>
<tr>
<td>VEGF</td>
<td>Adenovirus</td>
<td>Balb/c (300)</td>
<td>Athymic NIH nude</td>
<td>Glucose reduced 100mg/dl</td>
<td>[362]</td>
</tr>
<tr>
<td>SOCS-3</td>
<td>Transgenic (RIP)</td>
<td>C57BL/6 (300)</td>
<td>Balb/c</td>
<td>7 fold increase time to reject</td>
<td>[363]</td>
</tr>
<tr>
<td>BCL-2</td>
<td>Adenovirus</td>
<td>NHP (2000IE)</td>
<td>C57BL/6</td>
<td>5.6 fold increase time to reject</td>
<td>[364]</td>
</tr>
<tr>
<td>Decoy TNFR-Ig</td>
<td>Adenovirus</td>
<td>Balb/c (400)</td>
<td>C57BL/6</td>
<td>1.8fold increase time to reject</td>
<td>[365]</td>
</tr>
<tr>
<td>MnSOD</td>
<td>Adenovirus</td>
<td>NOD (400)</td>
<td>NOD-SCID*</td>
<td>1.7 fold increase time to reject</td>
<td>[366, 367]</td>
</tr>
<tr>
<td>IL-1Ra</td>
<td>Adenovirus</td>
<td>NOD (400)</td>
<td>NOD-SCID*</td>
<td>1.6 fold increase time to reject</td>
<td>[366]</td>
</tr>
<tr>
<td>indoleamine 2,3-dioxygenase (IDO)</td>
<td>Adenovirus</td>
<td>NOD (400)</td>
<td>NOD-SCID*</td>
<td>1.5 fold increase time to reject</td>
<td>[366]</td>
</tr>
</tbody>
</table>

**Table 3.1. Therapeutic approaches to improve outcome from islet transplantation.**

This table summarizes a survey of published therapeutic treatments aimed at improving islet transplant efficacy and outcome. Unless indicated by “spontaneous”, diabetes in recipients was chemically induced. If species was not indicated under donor or recipient columns, then the animal was mouse and the strain is listed. Special characters denote additional information as follows. « 150 wild type donor islets compared to 120 rip13 → Irs2 donor islets. * denotes hepatic portal islet transplant. ** 6.2 fold increase in time to reject in remaining 40% of recipients. # denotes omental pouch islet transplant. ## denotes intraperitoneal islet transplant. $ denotes epidymal fat pad implantation. NHP = Non-human primate. PNA = Peptide Nucleic Acid. @ denotes syngeneic challenge with auto-reactive splenocytes. I.S. = Immune Suppression. ¥ No effect seen in 50% of recipients. ∞ Mice immune function was reconstituted with human lymphocytes. §AAV-IL10 infection of skeletal muscle 4weeks before syngeneic NOD transplant increased cure rate 40% [330]. € IL-12p40 expression in islets resulted in reduced IFNγ and increase TGFβ expression in graft.

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### B. Reduce Pro-apoptotic

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Donor Species</th>
<th>Recipient Species</th>
<th>Treatment Effect</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATF3 Knock Out</td>
<td>C57CL/6 (250)</td>
<td>C57BL/6</td>
<td>80% increase in cure rate</td>
<td>TBA</td>
</tr>
<tr>
<td>MIF Knock Out</td>
<td>C57BL/6 (4000IE)</td>
<td>C57BL/6</td>
<td>8% increase in cure rate</td>
<td>[328]</td>
</tr>
<tr>
<td><strong>C. Alter Immunogenicity</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR-4 Dom. Negative. Adenovirus</td>
<td>C57BL/6 (500IE)</td>
<td>Balb/c</td>
<td>75% increase in cure rate</td>
<td>[368]</td>
</tr>
<tr>
<td>soluble CD40-Ig Adenovirus</td>
<td>C57BL/6</td>
<td>Balb/c</td>
<td>56% increase in cure rate</td>
<td>[369]</td>
</tr>
<tr>
<td>soluble anti-CD4 Ab. Adenovirus</td>
<td>C57BL6 x Balb/c (400)</td>
<td>CBA</td>
<td>40% increase in cure rate</td>
<td>[370]</td>
</tr>
<tr>
<td>IL-12p40 Adenovirus</td>
<td>NOD (400)</td>
<td>Spontaneous NOD</td>
<td>9 fold increase time to reject</td>
<td>[371]</td>
</tr>
<tr>
<td>TGF-β1, express Adenovirus</td>
<td>NOD</td>
<td>Spontaneous NOD</td>
<td>3 fold increase time to reject</td>
<td>[372, 373]</td>
</tr>
<tr>
<td>β2 Microglobulin Knock Out</td>
<td>C57BL/6 (N/A)</td>
<td>Spontaneous NOD</td>
<td>2.5 fold increase time to reject</td>
<td>[374]</td>
</tr>
<tr>
<td>CCR5 inhibitor PNA</td>
<td>Balb/c (300-400)</td>
<td>C57BL/6</td>
<td>1.8 fold increase time to reject</td>
<td>[375]</td>
</tr>
<tr>
<td>CXCR3 inhibitor PNA</td>
<td>Mouse</td>
<td>Allogenic</td>
<td>1.4 fold increase time to reject</td>
<td>[376]</td>
</tr>
<tr>
<td>CTLA4-Ig Adenovirus</td>
<td>BN Rat (2000)</td>
<td>Lewis Rat</td>
<td>1.3 fold increase time to reject</td>
<td>[377]</td>
</tr>
<tr>
<td>soluble ICAM-1-Ig Adenovirus</td>
<td>C3H/HeJ (750)</td>
<td>Balb/c</td>
<td>1.3 fold increase time to reject</td>
<td>[378]</td>
</tr>
<tr>
<td>IL-4 or IL-10 Adenovirus</td>
<td>NOD (500)</td>
<td>Spontaneous NOD</td>
<td>No Effect§</td>
<td>[371, 379]</td>
</tr>
<tr>
<td>IL-4 AAV</td>
<td>C57BL/6 (300)</td>
<td>C57BL/6</td>
<td>52% reduction in cure rate</td>
<td>[380]</td>
</tr>
<tr>
<td>IL-10 Adenovirus</td>
<td>BB/OK Rat (2000)</td>
<td>BB/OK Rat</td>
<td>2.4 fold reduced time to reject</td>
<td>[381]</td>
</tr>
<tr>
<td>FasL Adenovirus</td>
<td>Wistar Rat (1000)</td>
<td>SD Rat</td>
<td>1.8 fold reduced time to reject</td>
<td>[382-384]</td>
</tr>
</tbody>
</table>

### 2. In Vitro Treatment

#### A. Chemical Agent

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Donor Species</th>
<th>Recipient Species</th>
<th>Treatment Effect</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>EP1013 Inhibit Casp.</td>
<td>Balb/b (500)</td>
<td>Ball/b*</td>
<td>100% increase in cure rate</td>
<td>[385]</td>
</tr>
<tr>
<td>EP1013 Inhibit Casp.</td>
<td>Human (150IE)</td>
<td>B6-Rag**</td>
<td>70% increase in cure rate</td>
<td>[385]</td>
</tr>
<tr>
<td>EP1013 or zVAD-FMK Inhibit Casp.</td>
<td>Balb/b (250)</td>
<td>Balb/b</td>
<td>70% increase in cure rate</td>
<td>[385]</td>
</tr>
<tr>
<td>zVAD-FMK Inhibit Casp.</td>
<td>Balb/b (500)</td>
<td>Balb/b*</td>
<td>65% increase in cure rate</td>
<td>[385]</td>
</tr>
<tr>
<td>Z-DEVAD-FMK Inhibit Casp-3</td>
<td>Human (1000IE)</td>
<td>Nude</td>
<td>60% increase in cure rate</td>
<td>[386]</td>
</tr>
<tr>
<td>AEOL10113 and AEOL10150 SOD mimetic</td>
<td>Human (200IE)</td>
<td>NOD-scid</td>
<td>50% increase in cure rate</td>
<td>[387]</td>
</tr>
<tr>
<td>JANEX-1 Inhibit Jak/Stat</td>
<td>C57BL6 (400)</td>
<td>Balb/c</td>
<td>10% increase in cure rate</td>
<td>[388]</td>
</tr>
<tr>
<td>MK0431 (sitagliptin) Dipeptidyl peptidase-IV inhibitor</td>
<td>C57BL/6 (300)</td>
<td>C57BL/6</td>
<td>Glucose reduced 450mg/dl</td>
<td>[389]</td>
</tr>
<tr>
<td>Val-Pro-Met-Leu-Lys, (V5) Inhibit Casp.</td>
<td>Balb/c (150)</td>
<td>Balb/c</td>
<td>Glucose reduced 100mg/dl</td>
<td>[390]</td>
</tr>
</tbody>
</table>

Continued
### Table 3.1 continued

#### B. Biological Agent

<table>
<thead>
<tr>
<th>Biological Agent</th>
<th>Encapsulation Effect</th>
<th>Donor Species</th>
<th>Recipient Species</th>
<th>Treatment Effect</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mesench. Stem Cells</td>
<td>anti-inflamm / immuno-reg</td>
<td>Lewis Rat (600)</td>
<td>Lewis Rat #</td>
<td>90% increase in cure rate</td>
<td>[392]</td>
</tr>
<tr>
<td>JNK-1 Peptide</td>
<td>anti-stress response</td>
<td>Mouse (syngeneic)</td>
<td>Spontaneous BB Rat</td>
<td>83% increase in cure rate</td>
<td>[393]</td>
</tr>
<tr>
<td>IL-6</td>
<td>anti-inflamm / immuno-reg</td>
<td>Lewis Rat (1200-1500)</td>
<td>C3H/HeJ (200)</td>
<td>60% increase in cure rate</td>
<td>[391]</td>
</tr>
<tr>
<td>Methylprednisolone</td>
<td>anti-inflamm</td>
<td>Human (600IE)</td>
<td>SCID</td>
<td>50% increase in cure rate</td>
<td>[394]</td>
</tr>
<tr>
<td>42 days in 37° Culture</td>
<td>anti-inflamm / immuno-reg</td>
<td>ACI Rat (200)</td>
<td>4 Rat allo-models</td>
<td>50% increase in cure rate</td>
<td>[395]</td>
</tr>
<tr>
<td>Sertoli Cells</td>
<td>immuno-reg</td>
<td>NOD (250)</td>
<td>Spontaneous NOD</td>
<td>45% increase in cure rate</td>
<td>[396]</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>anti-apoptosis</td>
<td>DBA/2 (500IE)</td>
<td>B6AF1</td>
<td>40% increase in cure rate</td>
<td>[397]</td>
</tr>
<tr>
<td>Mesench. Stem Cells</td>
<td>anti-inflamm / immuno-reg</td>
<td>Wistar Rat (600-800)</td>
<td>Lewis Rat #</td>
<td>40% increase in cure rate</td>
<td>[392]</td>
</tr>
<tr>
<td>Prolactin</td>
<td>Angiogenic</td>
<td>C57BL/6 (200)</td>
<td>C57BL/6</td>
<td>30% increase in cure rate</td>
<td>[398]</td>
</tr>
<tr>
<td>14 days in 37° Culture</td>
<td>anti-inflamm / immuno-reg</td>
<td>Lewis Rat (1200-1500)</td>
<td>BB Rat</td>
<td>20% increase in cure rate</td>
<td>[391]</td>
</tr>
<tr>
<td>anti-Tissue Factor</td>
<td>anti-IFNγ</td>
<td>Monkey (5000IE/Kg)</td>
<td>Monkey (allogenic)</td>
<td>3.5 fold increase time to reject</td>
<td>[321]</td>
</tr>
<tr>
<td>14 days in 37° Culture</td>
<td>anti-inflamm / immuno-reg</td>
<td>Lewis Rat (1200-1500)</td>
<td>Wistar Rat</td>
<td>2 fold increase time to reject</td>
<td>[391]</td>
</tr>
<tr>
<td>14 days in 37° Culture</td>
<td>anti-inflamm / immuno-reg</td>
<td>Lewis Rat (1200-1500)</td>
<td>Spontaneous BB Rat</td>
<td>1.6 fold increase time to reject</td>
<td>[391]</td>
</tr>
<tr>
<td>Sertoli Cells</td>
<td>immuno-reg</td>
<td>C57BL/6 (300-400)</td>
<td>CH3</td>
<td>1.5 fold increase time to reject</td>
<td>[399]</td>
</tr>
<tr>
<td>VEGF</td>
<td>Angiogenic</td>
<td>Lewis Rat (1000IE)</td>
<td>Lewis Rat</td>
<td>Normoglycemia achieved 1 day early</td>
<td>[400]</td>
</tr>
</tbody>
</table>

#### 3. Materials Engineering

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>alginate-polylysine-alginate</td>
<td>Microcapsule</td>
<td>Allograft (N/A)</td>
<td>Spontaneous BB Rat #</td>
<td>100% increase in cure rate</td>
<td>[401]</td>
</tr>
<tr>
<td>Calcium Alginate</td>
<td>Microcapsule</td>
<td>Porcine (N/A)</td>
<td>Lewis Rat ##</td>
<td>100% increase in cure rate</td>
<td>[402]</td>
</tr>
<tr>
<td>Sodium Alginate</td>
<td>Tubular Chamber</td>
<td>Canine (25000IE)</td>
<td>Spontaneous BB Rat ##</td>
<td>100% increase in cure rate</td>
<td>[403]</td>
</tr>
<tr>
<td>Barium Alginate</td>
<td>Microcapsule</td>
<td>Lewis Rat (3500)</td>
<td>Lewis Rat #</td>
<td>90% increase in cure rate</td>
<td>[404]</td>
</tr>
<tr>
<td>Agarose</td>
<td>Microcapsule</td>
<td>NOD (2000)</td>
<td>Spontaneous NOD #</td>
<td>85% increase in cure rate</td>
<td>[405]</td>
</tr>
<tr>
<td>High Gluronic Alginate</td>
<td>Microcapsule</td>
<td>Canine (2000-4000)</td>
<td>Nude ##</td>
<td>80% increase in cure rate</td>
<td>[406]</td>
</tr>
<tr>
<td>Sodium Alginate</td>
<td>Microcapsule</td>
<td>Human (1800)</td>
<td>Balb/c #</td>
<td>66% increase in cure rate</td>
<td>[407]</td>
</tr>
<tr>
<td>Sodium Alginate</td>
<td>Microcapsule</td>
<td>SD Rat (1800)</td>
<td>Balb/c #</td>
<td>63% increase in cure rate</td>
<td>[407]</td>
</tr>
<tr>
<td>Calcium Alginate-poly Lysine Alginate</td>
<td>Microcapsule</td>
<td>Wag Rat (1000)</td>
<td>Balb/c #</td>
<td>60% increase in cure rate</td>
<td>[408]</td>
</tr>
<tr>
<td>Barium Alginate</td>
<td>Microcapsule</td>
<td>Wistar Rat (3500)</td>
<td>Lewis Rat #</td>
<td>20% increase in cure rate</td>
<td>[404]</td>
</tr>
<tr>
<td>Biodriltin</td>
<td>Microcapsule</td>
<td>Human (3500IE)</td>
<td>Balb/c</td>
<td>Glucose reduced 350mg/dl</td>
<td>[409]</td>
</tr>
</tbody>
</table>

Continued
Table 3.1 continued

<table>
<thead>
<tr>
<th>ECM coated PGL</th>
<th>Hollow Fiber Agarose carrier</th>
<th>Lewis Rat (9000)</th>
<th>Lewis Rat ##</th>
<th>Glucose reduced 250mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethisorb Dura Patch</td>
<td>Scaffold</td>
<td>C57BL/6 (150)</td>
<td>C57BL/6</td>
<td>Glucose reduced 100mg/dl</td>
</tr>
</tbody>
</table>

4. Combination Therapy

<table>
<thead>
<tr>
<th>Secondary Agent</th>
<th>Donor Species</th>
<th>Recipient Species</th>
<th>Treatment Effect</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ad-Bcl-2</td>
<td>Porcine (5000IE)</td>
<td>NOD-SCID*∞</td>
<td>100% increase in cure rate</td>
<td>[413]</td>
</tr>
<tr>
<td>Barium alginate encapsulation 12day 22°C culture in vitro</td>
<td>Wistar Rat (3500)</td>
<td>Lewis Rat ##</td>
<td>70% increase in cure rate</td>
<td>[404]</td>
</tr>
<tr>
<td>Sodium Alginate microcapsule</td>
<td>hemoglobin x-linked to capsule</td>
<td>Dawley Rat (500)</td>
<td>Balb/c</td>
<td>Glucose reduced 300mg/dl</td>
</tr>
<tr>
<td>Ad-HGF</td>
<td>Ad-IL-1Ra</td>
<td>Human</td>
<td>NOD-SCID</td>
<td>Glucose reduced 100mg/dl</td>
</tr>
<tr>
<td>Superoxide dismutase glutathione peroxidase</td>
<td>CBA x C57BL6 (1500 IE)</td>
<td>CBA x C57BL6</td>
<td>Glucose reduced 90mg/dl</td>
<td>[416]</td>
</tr>
<tr>
<td>IGF-II Pre-treatment Encapsulation</td>
<td>Rat (150)</td>
<td>scid/SzJ</td>
<td>7.6fold increase time to reject</td>
<td>[417]</td>
</tr>
<tr>
<td>Sertoli Cells</td>
<td>ALS</td>
<td>Rat (500)</td>
<td>Balb/c</td>
<td>6 fold increase time to reject</td>
</tr>
<tr>
<td>Ad-CTLA4-Ig</td>
<td>FK506</td>
<td>BN Rat (2000)</td>
<td>Lewis Rat</td>
<td>4.8 fold increase time to reject</td>
</tr>
<tr>
<td>Agarose encapsulation 7 day culture in vitro</td>
<td>Porcine (6000IE)</td>
<td>Balb/c ##</td>
<td>2.7 fold increase time to reject</td>
<td>[419]</td>
</tr>
<tr>
<td>IGF-II Pre-treatment Encapsulation</td>
<td>Rat (300)</td>
<td>scid/SzJ</td>
<td>2 fold increase time to reject</td>
<td>[417]</td>
</tr>
<tr>
<td>Ad-MnSOD</td>
<td>Ad-IL-1Ra</td>
<td>NOD (400)</td>
<td>NOD-SCID*</td>
<td>1.8 fold increase time to reject</td>
</tr>
</tbody>
</table>
This survey illustrates several important aspects regarding the efforts aimed at improving islet transplantation. First, the broad scope of therapies that have been evaluated for their potential to improve islet transplant outcome are highlighted and placed into perspective with respect to their relative impact on outcome. Importantly, benefit is observed not only from the treatment of islets with biological agents, chemical agents or material encapsulation, but also from genetic modification of the islets. Furthermore, significant improvements in outcome were observed with combination treatments, such as the adenoviral expression of the pro-survival gene BCL-2 in conjunction with islet encapsulation. This suggests that efforts directed both at bolstering the resistance of islets to apoptosis as well as shielding them from the immune system represent effective therapeutic approaches.

Second, this summary emphasizes that ex vivo targeting of islets is a viable approach to improve transplant outcomes. In each case, the results achieved in these reports were obtained in the absence of immune suppression. It is generally accepted that graft death and rejection following allogenic and xenogenic transplantation is dominantly controlled by the recipients immune response. Therefore, treatments that have been evaluated in the clinical setting have primarily been focused on optimizing immunosuppressive regimes. These results suggest that targeting islets prior to transplantation may represent an effective alternative or supplement for systemic immunosuppression of recipients and merits clinical evaluation.

Finally, this summary reveals the dominant use of adenoviral based gene targeting vectors in islet transplantation. This is likely due to the absence of an alternative vector capable of infecting islets as efficiently as adenovirus. However, to be clinically viable
as a gene therapy vector, the vehicle must be safe, efficient, and long lasting. While adenoviruses are efficient and generally considered to be safe, as there has only been one death related to adenoviral administration in over 80 clinical trials, they do not result in long term gene expression in vivo [420]. This is in large part due to the generation of a humoral and cellular immune response directed against specific adenoviral capsid proteins[421, 422]. Furthermore, adenoviral infection of islets results in the expression of chemokines and chemokine receptors which leads to islet death secondary to an enhanced local inflammatory response [335]. Therefore, islets infected with adenovirus may not only lose the expression of the transgene upon transplantation, but may also be more susceptible to death induced by pro-inflammatory cytokines.

Despite these drawbacks, table 3.1 demonstrates that adenoviral based gene therapy efforts in animal models can still be successful. However, in the clinical setting, the rate limiting factor for the success of human islet transplantation is the retention of functional islet mass. Therefore, an ideal gene therapy vehicle for human islet transplantation is one that does not intrinsically compromise the efficacy of the therapeutic gene being delivered.

AAV represents an attractive gene therapy vehicle for islet transplantation because it is non-immunogenic and results in long lasting transgene expression. However, the islet infection efficiency of AAV varies between serotype and generally only infects 15-20% of the cells within the islet [423]. Strategies aimed at improving the utility of AAV have focused on increasing the efficiency of transgene expression as well as optimizing viral entry to the cell. The generation of double stranded genomic constructs or the utilization of strong islet specific promoters such as insulin have
dramatically increased the level of transgene expression within the cells. Furthermore, identifying the optimal species specific pseudotypes or modifying viral coat proteins to promote the use of alternative receptors for viral entry to the cell have overall enhanced reported infection efficiencies.

Work presented in this chapter demonstrates that fusion of RGD, the prototypical cell adhesion motif, to the AAV1 capsid markedly enhanced its infection efficiency for murine, non-human primate, and human islets. This infection resulted in persistent transgene expression that did not compromise islet function following transplantation to the kidney subcapsule. Consequently, the results from this study demonstrate the superiority of AAV1/RGD as a gene therapy vehicle for islet transplantation and leaves open its use for many therapeutic applications.

ACKNOWLEDGEMENTS

I would like to thank Drs. Lucia Rosas, Douglas McCarty, and Jeffery Bartlett for the development and production of the AAV viruses. Their expertise in AAV biology made this work possible. I would also like to thank Dr. Amer Rajab and Jill Buss for providing the precious human and non-human primate islets used in this study. Finally, I would like to thank several current lab members for their assistance. Specifically, I thank Dr. Stephen McConoughey for his assistance with the peptide competition experiments and Stephanie Warnock and Adrian Zoller for their assistance in maintaining the mouse colony.
FIG. 3.5. Dot blot examination of AAV pseudotype titers. Viral titers were re-evaluated by dot-blot to confirm that comparable viral doses were being used for islet infections. $2.5 \times 10^7$ AAV vector genomes were first incubated in 0.5 NaOH, then blotted onto a nylon membrane along with a serial dilution of a standard plasmid. The membrane was hybridized with a P32-labeled probe specific for GFP to quantitate the amount of viral particles packaged with DNA.
FIG. 3.6. GFP expression compromises transplanted islet function. Mouse islets were picked into groups immediately following isolation for mock infection or infection with GFP expressing AAV1/RGD virus. Six days after infection, islets were washed free of virus and transplanted under the kidney capsule of syngeneic STZ-induced diabetic mice. (A) Non-fasted blood glucose of mice transplanted with either a curative (300) or marginal (150) number of non-infected or GFP expressing AAV1/RGD infected islets. Data represent means ±S.E.M. * p< 0.05 300 Non-infected islets vs. 300 GFP expressing AAV1/RGD infected islets at the indicated POD.
FIG. 3.7. Addition of a RGD motif to AAV improves non-human primate islet infection efficiency. Non-human primate islets were infected with the indicated GFP expressing AAV pseudotypes immediately following isolation and imaged daily by confocal microscopy for GFP expression. Shown are representative images from a survey of the relative islet infection efficiency of AAV1/RGD and the non-RGD modified AAV pseudotypes.
CHAPTER 4
THE ROLES OF ATF3, AN ADAPTIVE-RESPONSE GENE, IN HIGH FAT DIET INDUCED DIABETES

SUMMARY

The insulin secreting β-cells of the pancreatic islet play an important role in the pathogenesis of diabetes. Previously, we demonstrated that activating transcription factor 3 (ATF3) is a stress inducible gene and pro-apoptotic in various cell types, including β-cells. Our results support a model that ATF3 plays a role in the pathogenesis of diabetes, partly by its apoptotic effect in β-cells. However, this model was established in vitro using diabetes relevant stresses (i.e. inflammatory cytokines and high concentrations of glucose & lipid). Therefore, the goal of the work performed in this chapter was to test in vivo whether ATF3 plays a role in the development of high fat diet (HFD) induced diabetes. To accomplish this goal, I placed wild type (WT) and ATF3 knockout (KO) mice on a HFD and monitored the development of diabetes. This work also involved in vitro functional comparison of islets from WT and KO mice. I found that ATF3 was induced in the islets of mice fed a HFD and, to our surprise, played a protective role, as evidenced by the more severe diabetic phenotype observed in KO mice. The investigation into this phenotype revealed that ATF3 promoted insulin gene expression. This finding was first suggested by in vivo data that showed KO mice on HFD had
significantly reduced serum insulin levels when compared to WT controls. Subsequent *in vitro* investigation using gain and loss of function approaches to examine steady-state mRNA levels, reporter activity, in vivo DNA binding, and in vivo transcription, strongly indicated that insulin is a target gene directly up-regulated by ATF3. Therefore, this work identifies a role for ATF3 in islet function by contributing to insulin gene expression. While ATF3 can also play a role in β-cell death by repressing pro-survival genes, under alternate stress contexts, it may improve islet function by regulating additional target genes. This has significant implications for our understanding of the role ATF3 plays in maintaining cellular homeostasis.

**INTRODUCTION**

Although classically referred to as non-insulin dependent diabetes mellitus (NIDD), type 2 diabetes (T2D) has been recognized as a dual disease of insulin resistance and insulin deficiency, some reviews [424], [425], [243], [255], [426], [271], [272]). It is well established that T2D develops only after the pancreatic β cells fail to produce enough insulin to meet the metabolic demand (some reviews, [271], [426], [255], [425]). This failure is the consequence of an acquired reduction in functional β-cell mass, resulting from a combination of reduced β-cell number and function. During the development of T2D, β cells are induced to undergo apoptosis by various stimuli and mechanisms (for some reviews, see [426], [271]). These stimuli include extracellular factors such as amyloid plaque deposits and high concentrations of lipid and glucose and intracellular mechanisms such as mitochondrial dysfunction, triglyceride/free fatty acid (TG/FFA) cycling dysfunction, reactive oxygen species (ROS), and endoplasmic reticulum (ER)
stress. When apoptosis outpaces the compensatory mechanisms (hypertrophy, moderate replication and neogenesis) to increase β cell mass, problems arise. The importance of β-cell mass is underscored by the observation that most people with T2D have reduced β-cell mass ([265]). Thus, β-cell apoptosis has emerged as a critical cellular process that becomes dysregulated during the development of T2D (some reviews, [271], [426]).

Deterioration in β-cell function has long been linked to T2D and is now well documented to play an important role in the pathogenesis of the disease (some reviews [425], [424], [243], [255], [426], [271]). Defects have been identified in numerous aspects of β-cell function, including firsts-phase insulin response (the burst of insulin secretion by β cells within 30 minutes after glucose challenge), pulsatile secretion of insulin, and insulin biosynthesis (see above reviews). The first-phase insulin response is a process linked to acute elevations in plasma glucose, which directly increases the concentration and metabolism of glucose within β-cells. This increased rate of glucose metabolism decreases the abundance of adenosine diphosphate (ADP) and induces the closure of the ADP-sensitive K⁺ channel. Closure of the K⁺ channel alters the membrane potential by about 15mV, which is sufficient to activate voltage gated Ca²⁺ channels and allow calcium influx. The increased cytosolic Ca²⁺ concentration then activates the physical process by which insulin is secreted. This process includes insulin granule recruitment, docking, priming, and then fusion with the plasma membrane. In patients with T2D, the first-phase insulin response to glucose is lost; however, non-glucose stimuli are still capable of inducing insulin secretion [427-430]. This suggests that defects in the steps leading to glucose induced calcium influx give rise to the loss of first-phase insulin response in T2D patients.
A second aspect of defective β-cell function in T2D is the loss of pulsatile insulin secretion. Under normal circumstances, serum insulin levels fluctuate at both low and high frequencies, with a period of approximately 120 minutes and 5-8 minutes respectively [431, 432]. Measuring oscillations in islet Ca$^{2+}$ concentration and β-cell membrane potential in vitro has revealed patterns of pulsatility consistent with insulin secretion, and found that individual islets oscillate as a single unit independent from other islets [433-435]. However, the in vivo observation that serum insulin concentration oscillates suggests the possibility that islets within the pancreas can be ‘entrained’ to produce synchronized secretory periods. While several mechanisms have been proposed to synchronize the pancreatic secretory pattern, such as an intrapancreatic ganglion pacemaker [436-438] or a circulating factor [439], evidence to support these hypotheses have yet to be produced. Furthermore, no definitive explanation exists as to why islets would function to secrete insulin in this pulsatile manner. However, it has been proposed that the sustained elevation in Ca$^{2+}$ concentration needed to meet the demand for insulin secretion would likely lead to cellular damage. Therefore, oscillation provides a less toxic mechanism for β-cells to maintain glucose homeostasis. Additionally, studies in humans have found the pulsatile administration of exogenous insulin to be more effective at reducing serum glucose concentrations than continuous infusion [440]. Thus, pulsatile insulin secretion may represent the most biologically efficient mechanism for β-cells to maintain glucose homeostasis. Therefore, the loss of this β-cell function may contribute to the development of overt T2D.

A third aspect of defective β-cell function in T2D is linked to abnormal insulin biosynthesis. The production of a functional insulin molecule is a complicated process
wherein glucose and other metabolic signals influence transcriptional, translational and post-translational events. Given the role of β-cells as metabolic regulators of systemic glucose concentration, it is fitting that insulin biosynthesis responds to glucose exposure. Glucose regulation of insulin transcription occurs through multiple pathways, ultimately resulting in the activation of sequence specific transcription factors. Specifically, three of these transcription factors are NFAT, MafA, and PDX-1 and their activity is regulated, in part, by glucose induced alterations in glycolysis, intracellular Ca^{2+} concentration, and PI3K activity [441-443]. In addition to promoting insulin transcription, glucose stimulation is also known to increase steady state levels of insulin mRNA by increasing pre-RNA processing and decreasing insulin mRNA degradation [444, 445]. Glucose regulation of insulin translation has also been demonstrated. This regulation primarily occurs at the level of initiation as it involves a redistribution of cytosolic pools of insulin mRNA to RER associated polysomes. As a result, the rate of insulin synthesis is increased to a level which exceeds that of general protein synthesis within the β-cell. Whether this apparent “exclusive” mechanism is entirely due to mRNA redistribution or also involves cis acting elements within the mRNA has yet to be determined. Lastly, glucose is also capable of regulating the post-translational modification of insulin. Two post-translational events convert the preproinsulin molecule into its fully functional form. The first is the cleavage of the 24 amino terminal residues, known as the signal peptide, which targets the newly translated insulin molecule to the RER membrane for vesicular packaging. This cleavage event is performed by the signal peptidase that resides within the membrane of the RER and results in a proinsulin molecule that consists of two anti-parallel β-helices (A and B chains) joined by disulfide bonds and a loop region called the C-peptide. The
second modification occurs within the acidic environment of the secretory vesicle and involves the removal of the C-peptide by the PC1 and PC2 endopeptidases. Glucose contributes to the post-translational processing of insulin by inducing the transcription and translation of PC1 and PC2, thus increasing β-cell capacity to meet the increased demand for insulin secretion. In T2D, defects in insulin biosynthesis are linked to two main aspects of its production; transcription and post-translation. β-cell exposure to chronic hyperglycemia leads to a reduction in insulin transcription secondary to the development of an oxidative environment within the cell that inhibits the function of the above mentioned transcription factors. This gluco-toxic response presents as an outright failure to produce insulin and is typically associated with late stage T2D. Prior to this precipitating development in the disease, β-cells compensate for increased metabolic demand by producing and secreting more insulin. However, in patients who ultimately develop diabetes, this increased rate of insulin secretion is associated with an elevated serum ratio of pro-insulin to insulin. Given that the insulin receptor displays a lower affinity for pro-insulin than insulin [446], it has been proposed that this altered ratio reflects a contributing factor to the relative insulin deficiency that gives rise to pathological glucose levels in T2D. Therefore, the failure to complete post-translational processing of the insulin molecule prior to its secretion has been proposed to be a contributing factor to the development of T2D. Ultimately, the aspects of β-cell function discussed here are all interdependent upon each other to achieve maximal control over glucose homeostasis. It is unlikely that a single defect is necessary or sufficient for the development of T2D; instead, the accumulation of multiple defects in β-cell function is requisite for disease onset.
Recently, genome-wide association analyses aimed to identify polymorphisms that increase the risk of T2D in humans provided the exciting revelation that most T2D-risk variants affect genes regulating β-cell function and/or mass, rather than insulin signaling (reviews, [447], [448]). Taken together, these results are consistent with a model that T2D results from the combination of gene-and-environment interactions: genetic predisposition that makes β cells vulnerable (to metabolic demand) and environment factors that induce insulin resistance ([447], [449], [243]). Overwhelming evidence implicates hyper-nutrition and obesity as dominant environmental factors contributing to the induction of insulin resistance.

In this chapter, I present data that implicates a role for ATF3, an adaptive-response gene, in the induction of T2D by high fat diet. ATF3 is a member of the ATF/CREB family of transcription factors related by their basic region-leucine zipper (bZip) DNA-binding domain and ability to bind to the same consensus sequence TGACGTCAC in vitro ([5]). Previously, we and others showed that ATF3 is induced in β cells by various stress signals relevant to T2D, including high glucose, high fatty acid, ROS, and ER stress ([60], [7], [316], [62], [105]). Functionally, ATF3 is pro-apoptotic in β cells ([7]), in part by repressing the pro-survival gene IRS2 ([316]). Due to the importance of β cells in T2D development, I set out to test whether ATF3 contributes to the development of T2D by comparing wild type (WT) and ATF3 knockout (KO) mice in HFD-induced diabetes. To our surprise, I found that ATF3 enhances the ability of β cells to produce insulin. Thus, ATF3 plays a role in both β-cell apoptosis (from previous work) and function (from this work). Results described below are consistent with a model that, before β-cell apoptosis
becomes an issue, expression of ATF3 is beneficial and helps β cells to cope with higher metabolic demand.

MATERIALS AND METHODS

Animals and dietary treatment. Wild type and ATF3 KO C57BL/6 mice were housed under conventional conditions and cared for according to Ohio State University Laboratory Animal Resources guidelines. ATF3 KO mice were described previously in chapter 2 Materials and Methods. At 6 weeks of life, mice were divided into groups of 10-15 and fed either normal chow (Harlan Labs, #8640) or a high fat diet (Research Diets) consisting of 10% (#D12450B), 45% (#D12451) or 60% (#D12492) fat for up to 16 weeks. On caloric basis, the normal chow consisted of 5% from fat, 22.58% from protein, and 72.42% from carbohydrate with a total energy composition of 3.82 kcal/gm. High fat diet caloric compositions were 10/45/60% from fat, 20% from protein, and 70/35/20% from carbohydrates with total energy compositions of 3.85/4.73/5.24 kcal per gm respectively.

Glucose tolerance tests, insulin tolerance test, and ELISA. Glucose tolerance tests were performed on 16 hour fasted mice by intraperitoneal injection with 2 grams of glucose/kg of body weight as previously reported [354]. Insulin tolerance tests were performed on 4 hour fasted mice by intraperitoneal injection with 0.75 units of insulin/kg of body weight. For both assays, blood samples were obtained from the snipped tail and analyzed using an Ascensia Contour portable glucometer at 15, 30, 60, 120 and 180 minutes after injection. Fasting serum insulin, IL-1β, IL-6 and TNFα levels were measured from tail blood samples using a radioimmunoassay (Linco).
**Cell culture, adenoviruses and luciferase assay.** INS-1 and INS-r3 cells were grown as previously described [318]. Both cell lines were used for mRNA analysis and chromatin immunoprecipitation and yielded consistent results. Unless otherwise indicated, all cells were cultivated with standard humidified cell culture conditions (37 °C, 5% CO₂, 20% O₂). Adenoviruses were constructed using the Invitrogen Gateway technology and purified by cesium chloride ultracentrifugation [350]. For luciferase experiments, INS cells were nucleofected (Amaxa) in triplicate with a Nucleofector I using solution V and program D-15. Luciferase activity in 100 μl of extract was measured 36 hours later in a Lumat (Berthold) luminometer and all values were normalized to wild type insulin1 promoter in the absence of ATF3.

**Islet isolation.** Islets were isolated from adult mice as previously described [319, 349]. Briefly, 0.25mg/ml of rat liberase (Roche) was perfused to the pancreas through the common bile duct. Pancreata were removed and incubated at 37 °C for 13-16 minutes prior to filtration through a 419μm wire mesh. Islets were then isolated using Histopaque (Sigma) gradient, capture on 100μm nylon filter, and hand pick. Once isolated, islets were allowed to recover for 72 hours at 27 °C in a humidified cell culture incubator prior to being used in experiments. For additional details see Appendix A.

**RNA analyses and chromatin immunoprecipitation assay.** Total RNA was isolated by Trizol (Invitrogen) and subjected to RT-PCR or quantitative RT-PCR in triplicate using the
Bio-Rad iQ SYBR Green Supermix. Chromatin immunoprecipitation (ChIP) assay was carried out as described in Appendix C. All PCR primers are listed in Table 4.1.

**Immunoblot and immunohistochemistry.** Fifty to 100μg of protein was resolved on 10-12% polyacrylamide gels and transferred to nitrocellulose membrane for analysis by immunoblot. The antibodies used were the following: anti-ATF3 (Santa Cruz), anti-actin (Sigma), anti-AKT (Cell Signaling), anti-phospho-AKT (Cell Signaling), anti-cleaved caspase 3 (Cell Signaling), anti-insulin (Dako), and anti-F4/80 (Caltag Laboratories). For immunohistochemistry, the following antigen retrieval steps were necessary. ATF3: No antigen retrieval necessary, use non-fasted mice, and develop with alkaline phosphatase blue. Caspase 3: 4 hours in 60°C incubator, citrate buffer and pressure cooker. F4/80: citrate buffer and 1 hour in rice steamer.

**Image analysis and statistics.** Immunohistochemistry and densitometry analysis was done using the NIH ImageJ Software (version 1.41a). All quantitative data are expressed as means ±S.E.M., and comparisons are made by the Student’s t test.

**Glucose stimulated calcium influx.** Intracellular Ca2+ was measured using the ratiometric Ca2+ indicator fura-2 AM using a modification of previously published methods [450]. Islets were maintained in 2.5 mM glucose at all steps prior to studies to prevent transient states in calcium influx due to shifts in glucose prior to or during the experiment. Islets were loaded with 2 μM fura-2 AM for 30-40 min, washed, and transferred to a small volume chamber (MatTek Corporation) and placed on the stage of an
Olympus BX51WI fluorescence microscope (Olympus). Islets were perfused with 2.5 mM glucose Krebs-Ringer HEPES-buffered (KRB) solution with a peristaltic pump (Gilson) at ~35°C by an in-line heater (Warner Instruments). Islets were incubated within the chamber in these conditions for 10-15 min before beginning the experiment. Excitation light from a xenon burner was supplied to the preparation via a light pipe and filter wheel (Sutter Instrument Company). Images were taken sequentially from 340 nm and then 380 nm excitation using a Hammamatsu ORCA-ER camera (Hammamatsu) to produce each Ca2+ ratio from emitted light at 510 nm. Paired images were recorded every 5-sec for 15 min. After recording Ca2+ in 2.5 mM glucose for ~3 min, islets were then recorded to determine response to 25 mM glucose stimulation. The glucose-stimulated Ca2+ influx (GSCI) was defined as the difference between ratio measurements (340/380 nm fluorescence) in 25 mM vs. 2.5 mM glucose. Data were analyzed with IP Lab® software version 4.0 (Scanalytics). All quantitative data are expressed as means ±S.E.M., and comparisons are made by the Student’s t test.

RESULTS

The role of ATF3 in high fat diet (HFD)-induced diabetes. To examine whether ATF3 plays a role in the development of T2D, I compared WT and ATF3 KO mice using an HFD-induced diabetes model. Six week old mice were fed with high fat (60%) or normal (5% fat) chow and monitored for weight gain and glucose intolerance over a 16-week period. Previously, we reported that the ATF3 KO mice developed normally and exhibit no obvious phenotypes under unstressed conditions ([7]). However, they are slightly and consistently lower in body weight at weaning than the WT mice (<3%
difference). Despite this subtle difference, they gained weight and consumed chow at similar rates as the WT mice as shown in Fig. 4.7A and 4.7B. Intraperitoneal glucose tolerance tests (IPGTT) indicated that both WT and KO mice became glucose intolerant, when assayed at 6 weeks and 10 weeks after HFD (Fig. 4.1A). However, WT mice performed better than the KO counterparts as indicated by the area under the curve analysis (Fig. 4.1B), suggesting that ATF3 has a protective role in maintaining glucose homeostasis under HFD. The difference was not due to a difference in insulin resistance, since the WT and KO mice performed similarly in insulin tolerance test (Fig. 4.1C) and showed similar insulin-induced Akt phosphorylation in the epidymal fat pad (Fig. 4.1D).

As expected from the well-known ability of HFD to induce insulin resistance (some reviews, [451], [452]), mice under HFD had reduced insulin-induced Akt phosphorylation in their fat pad as compared to their counterparts under normal chow. However, this reduction was comparable in both WT and KO mice (Fig. 4.1D). Since ATF3 was induced in the WT fat pad by HFD (Fig. 4.1D), these results indicate that, at the time of assay, the status of ATF3 expression did not impact the ability of adipocytes to respond to insulin. HFD and obesity have been shown to induce macrophage infiltration into the adipose tissues, leading to local and systemic inflammation (some reviews, [451], [452]). As shown in Fig. 4.1E and Fig. 4.7C, WT and KO mice had a similar macrophage infiltration in their epidymal fat pad. Taken together, these results suggest that ATF3 plays a protective role in obesity-induced diabetes; however, the protective effect is not due to the reduction in insulin resistance. This prompted us to hypothesize that ATF3 may enhance β-cell functional mass under HFD, thus contributing to better glucose homeostasis in WT than KO mice.
FIG. 4.1. ATF3 dampens the hyperglycemic effect of high fat diet without altering insulin sensitivity. WT and ATF3 KO C57BL/6 mice were placed on HFD and monitored for onset of diabetes. Data from WT mice are graphed as black line/black triangle or black bar while KO data points are grey line/grey square or grey bar. HFD groups are solid line while normal chow groups are dashed line. (A) Intraperitoneal glucose tolerance tests performed after the indicated weeks on HFD. (B) Area under the glucose tolerance test curve. (C) Intraperitoneal insulin tolerance test performed after the indicated weeks on HFD. (D) Insulin signaling in epidymal fat pad was evaluated 5 minutes following hepatic portal vein (HPV) insulin injection. The indicated proteins were analyzed by immunoblot. Shown is a representative blot where extracts from 4 fat pads per group were pooled for analysis. (E) After 12 weeks on HFD, epidymal fat pads were analyzed for inflammatory cell recruitment by F4/80 immunohistochemistry. Shown are representative fields from the HFD groups with control IgG staining on adjacent sections. Data represented as means ±S.E.M. * p< 0.05, ** p< 0.001 WT vs. KO on HFD or within each genotype normal chow vs. HFD.
The role of ATF3 in HFD-induced hyperinsulinemia. One compensatory response of β cells to the higher metabolic demand under HFD is to produce and secrete more insulin. Analysis of fasting (Fig. 4.2A) and non-fasting (Fig. 4.2B) serum insulin levels indicated that both WT and KO mice exhibited this compensatory response — higher serum insulin levels under HFD than under normal chow. However, it was much dampened in KO mice, as indicated by the lower serum insulin levels in KO mice than in WT mice when assayed under fasting (Fig. 4.2A) or non-fasting (Fig. 4.2B) conditions. This result was reproduced in four independent HFD experiments (Fig. 4.8 and data not shown), indicating a beneficial role of ATF3 in the compensatory increase of serum insulin under HFD and providing an explanation for the more pronounced glucose intolerance of the KO mice. I also examined the serum insulin levels within 30 minutes after glucose challenge. As shown in Fig. 4.2C, WT mice had more robust response than the KO mice, again supporting a beneficial role of ATF3 in β-cell function.
FIG. 4.2. ATF3 contributes to high fat diet induced hyperinsulinemia. Fasting (A) and non-fasting (B) serum insulin levels were measured from WT (black bars) and ATF3 KO (grey bars) C57BL/6 mice fed HFD for the indicated weeks. (C) Serum insulin levels were measured following intraperitoneal glucose injection. Data from WT mice are graphed as black line/black triangle or black bar while KO data points are grey line/grey square or grey bar. HFD groups are solid line while normal chow groups are dashed line. Data represented as means ±S.E.M. * p< 0.01, ** p< 0.005 WT vs. KO on HFD.
To investigate the causes for the reduced serum insulin levels in KO mice, I first examined pancreatic insulin-positive β cells. As shown in Fig. 4.3A-E, WT and KO mice had similar β-cell area (Fig. 4.3B) and islet number (Fig. 4.3C) under HFD. Importantly, in both groups of mice these parameters are higher under HFD than under normal chow, a well-known compensatory response under obesity. Immunohistochemistry showed that ATF3 is induced in the WT islets under HFD, consistent with previous studies that ATF3 is induced by glucolipotoxicity in β cells and primary islets in vitro (see Chapter 4 Introduction). The lack of ATF3 signals in the KO islets (Fig. 4.3A) confirmed its deficiency. Since ATF3 is pro-apoptotic in β cells as shown previously ([7], [316]), it is surprising that WT and KO islets had similar β-cell area. Thus, I examined activated caspase 3, an apoptosis marker, by immunohistochemistry. Fig. 4.3A showed that no signals were detected in the WT or KO pancreata under normal chow or HFD (Fig. 4.3AC). A side-by-side analysis of a diabetic NOD pancreas was used as a positive control for the assay (inset). Therefore, at the time of analyses (12 weeks after HFD), β-cell apoptosis had not become a major problem yet. Under these conditions, ATF3 expression appears beneficial, contributing to increased serum insulin level (Figs. 2A, 2B). Since WT and KO mice had comparable islet area and number, we hypothesized that ATF3 increases insulin biosynthesis, secretion, or both.
FIG. 4.3. ATF3 does not contribute to high fat diet induced pancreatic compensation in islet number or size. (A) Pancreata from mice on HFD for 12 weeks were analyzed by immunohistochemistry with the indicated antibodies. Bar = 100μm. Inset picture for cleaved caspase 3 is positive control from a diabetic NOD mouse. Shown are representative images. Pancreatic islet size (B) and number (C) were quantified from insulin staining done in A. Data represented as means ±S.E.M.
The role of ATF3 in insulin gene expression in β cells. Two clues supported the notion that ATF3 may up-regulate the transcription of insulin gene. First, ATF3 is a transcription factor; second, the insulin promoter contains a highly conserved ATF/CRE site, a potential binding site for ATF3, at the proximal region around -200 bp from the transcriptional start site (TSS) ([453], [454]). To test the potential of ATF3 to regulate insulin gene, I examined the endogenous insulin 1 and insulin 2 mRNA levels after infecting the INS-1 β cells with adenovirus expressing ATF3 or βGal as a control. Fig. 4.4A (left panel) shows that ectopic expression of ATF3 increased insulin 1 and 2 steady-state mRNA levels. Reporter assay indicated the ability of ATF3 to up-regulate the rat insulin 2 promoter (Fig. 4.4A, right panel). I also examined the ability of ATF3 to bind to the insulin promoter using the chromatin immunoprecipitation (ChIP) assay. Panel 4.4B shows that ATF3 indeed binds to insulin 1 promoter around the ATF/CRE site. Control antibody (IgG) showed no signals on the insulin promoter and control primers showed no binding of ATF3 to the α- or β-actin promoters (Fig. 4.4B), which lack recognizable ATF/CRE sites. In addition, antibody against RNA Polymerase II (Pol II) showed PolII binding to the promoter of β-actin (a β-cell gene) but not α-actin (a non-β-cell gene) promoter, further validating the ChIP procedure in this experiment.

The binding of ATF3 to the insulin promoter, combined with the increased insulin mRNA levels upon ATF3 expression, suggests (but does not prove) that ATF3 up-regulates the transcription of insulin gene in vivo. Two commonly used assays for vivo transcription are the PolII occupancy assay (which examines PolII binding on the coding region downstream of the TSS) and the pre-mRNA assay (which examines the production of mRNA precursors). I carried out ChIP analysis to determine PolII occupancy on the
FIG. 4.4. ATF3 contributes to insulin gene expression in INS-1 cell line. INS-1 cells were infected with Ad-βGal (black bar) or Ad-ATF3 (grey bar) 36 hours prior to analysis for insulin mRNA by RT-PCR (A) or for ATF3 and Pol II recruitment to the insulin gene by ChIP (B-C). RIP2 promoter activity was measured by luciferase on the right side of (A) using INS-1 cells nucleofected with wild type or CRE mutant luciferase reporter construct along with a control or ATF3 expressing construct. In B, PCR reactions using primers for proximal promoters of insulin, β-actin and α-actin were terminated in the linear phase of amplification and run out on an agarose gel. A representative result from two experiments is shown. In C, real time PCR reactions were done on ChIP samples using primers for the following regions of the insulin gene; D = distal (-1200bp from TSS), P = proximal (-200bp from TSS), 3’ = 3’ UTR (+1200bp from TSS). Data represent means ±S.E.M. * p<0.02, ** p<0.022, βGal vs. ATF3 for the indicated gene or primer pair.
insulin promoter and used quantitative PCR (qPCR) to quantify the ChIP signals. As shown in Fig. 4.4C, PolII antibody precipitated DNA fragment at the coding region ~1.5kb downstream from the TSS (bar 12, detected by the primer set designated as 3’), but the control IgG did not (bar 6). The signal strength was 3 fold in the presence of ectopic expression of ATF3 than that of βGal (compare bars 12 to 11), indicating that ATF3 enhances PolII occupancy in the coding region, a surrogate measurement of in vivo transcription. This enhancement was not observed in the distal promoter region ~1.5kb upstream from TSS (bars 7 and 8, using primer set designated as D) nor in the proximal region around the TSS (bars 9 and 10, using primer set designated as P), supporting the specificity of this in vivo transcription assay. Taken together, these results—increased steady-state mRNA levels, reporter activity, in vivo DNA binding, and in vivo transcription—strongly indicate that insulin is a direct target gene of ATF3 and is up-regulated by ATF3.

**The role of ATF3 in primary islets.** To test whether ATF3 plays a role in insulin gene expression in primary islets, I compared WT and KO islets in the absence or presence of high concentration of glucose (33 mM for one hour), which is known to induce the transcription of insulin gene [455-457]. As shown in Fig. 4.5, glucose treatment increased the endogenous ATF3 protein level (Fig. 4.5A) and the steady-state insulin 1 mRNA levels (Fig. 4.5B) in the WT but not KO islets, indicating that ATF3 contributes to glucose induced insulin expression. To test whether this is due to an increase in transcription, I analyzed glucose stimulated insulin 2 pre-RNA levels.
**FIG. 4.5. ATF3 contributes to insulin gene expression in primary islets.** Primary islets were isolated from WT (black bars) and ATF3 KO (grey bars) mice and allowed to recover for 72 hours prior to initiation of a 1 hour treatment with 5.5mM or 33mM glucose. (A) ATF3 protein was analyzed by immunoblot following treatment with 33mM glucose for the indicated times. (B) Mature insulin and ATF3 mRNA were measured by real time PCR following glucose treatment. (C) Pre-insulin2 RNA, ATF3 mRNA and actin mRNA levels following glucose treatment were measured from random hexamer synthesized cDNA pools. Glucose induction of pre-insulin2 was evaluated using primers that amplify through the Exon2/Intron2 junction of mouse insulin2. Shown are representative gels from 2 experiments. (D) Quantitation of pre-insulin2 data as performed in panel C from 5 experiments. (E) Intra-islet insulin content was measured by ELISA from intact islets. Data represented as means ±S.E.M. * p< 0.05, ** p< 0.006 WT vs. KO for the indicated glucose treatment.
This approach exploits the existence of an unspliced form of insulin that is unique to mouse insulin 2. This unspliced pre-RNA has a half-life on the order of minutes (rather than up to 80 hours for mature insulin 1/2). It has been proposed that measurement of the short half-life insulin 2 transcript more accurately reflects changes in transcription than the longer half-life species [456]. Thus, the level of this precursor, which can be assayed by RT-PCR using a primer specific to intron 2 and a primer targeted to the preceding exon, is a good indicator for transcription as demonstrated previously ([458]). As shown in Fig. 4.5C, glucose treatment increased insulin 2 pre-mRNA levels in the WT but not KO islets, supporting the notion that ATF3 enhances insulin gene transcription. Parallel experiments omitting reverse transcriptase in the RT-PCR assay (the minus RT control) showed no signals for all lanes (data not shown). Analysis of ATF3 mRNA level confirmed the induction of ATF3 in WT islets by glucose treatment and confirmed the lack of ATF3 in KO islets. Fig. 4.5D shows the quantitation of insulin 2 pre-mRNA levels from five independent experiments. Consistent with the mRNA data, the intracellular insulin protein level was lower in KO islets than that in WT islets (Fig. 4.5E). Thus, KO islets had a reduced ability to produce insulin and this may be, at least in part, due to a decrease in transcription.

I next examined glucose-induced insulin secretion. As shown in Fig. 4.6A, the amount of insulin in the KO supernatant was much lower than that in the WT supernatant. Because KO islets have reduced insulin content, reduced secretion could simply be a secondary effect. Alternatively, the deficiency in insulin output could be a combination of reduced insulin content and reduced function of the glucose stimulated secretory response. A key regulatory step for glucose-induced insulin secretion is
glucose-stimulated calcium influx (GSCI) [459]. At low (non-stimulatory) glucose concentrations, β-cell intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)) is low (50-100nM); however, upon stimulation, a large influx of Ca\(^{2+}\) occurs. This increase in [Ca\(^{2+}\)]\(_i\) is preceded by a small drop (from the basal Ca\(^{2+}\) level) due to Ca\(^{2+}\) sequestration into the ER and followed by a nadir and oscillation that persists as long as the stimulation is present ([460]). Ultimately, increased [Ca\(^{2+}\)]\(_i\) leads to insulin secretion through the activation of Ca\(^{2+}\)-calmodulin protein kinase II (Cam-kinase II) [461]. This kinase co-localizes with insulin secretory granules and is believed to play a significant role in mobilizing granule exocytosis.

To address the possibility that decreased insulin output from KO islets was, in part, a consequence of a role for ATF3 in GSCI, I examined the [Ca\(^{2+}\)]\(_i\) of WT and KO islets in real time as they were stimulated with glucose. Fig 4.6B shows a representative result from a GSCI assay using Fura-2 to detect changes in intra-cellular calcium concentration. No differences in GSCI were observed between WT and KO islets, suggesting that the reduction in insulin output observed in the KO islets was not secondary to reduced calcium influx. Quantitation of the average Fura-2 minimum (low calcium) and maximum (high calcium) from three separate stimulations confirmed that there was no defect in GSCI, however KO islets consistently maintained a slightly elevated [Ca\(^{2+}\)]\(_i\) (Fig. 4.6C). Representative traces in Fig. 4.6D show that no differences existed between WT and KO islets in calcium oscillation with prolonged glucose exposure. Quantitation of the average period (Fig. 4.6E) and amplitude (Fig. 4.6F) from three separate stimulations confirmed this observation. Collectively, these results indicate that KO islets do not have a defect in their glucose induced calcium response.
FIG. 4.6. ATF3 contributes to insulin output in primary islets. Primary islets were isolated from WT (black bars) and ATF3 KO (grey bars) mice and allowed to recover for 72 hours prior to glucose stimulation. (A) Glucose induced insulin secretion was measured by ELISA from the supernatant of intact islets following a 1 hour treatment with 5.5mM or 33mM glucose. (B) Fura2 ratiometry was used to assess glucose stimulated calcium influx in isolated islets. Shown are representative traces from multiple islets. (C) Average Fura2 pre-glucose minimum and post-glucose maximum ratios from three experiments performed as in panel B. (D) Glucose stimulated calcium oscillation. Shown are three representative traces from WT and KO islets. Quantitation of period (E) and amplitude (F) of glucose stimulated calcium oscillation as performed in panel D. Data represented as means ±S.E.M. * p< 0.05, ** p< 0.00001 WT vs. KO for the indicated glucose treatment.
Therefore, the deficiency in glucose stimulated insulin output from KO islets may in large part be due to reduced insulin transcription.

**DISCUSSION**

In summary, I investigated the roles of ATF3, an adaptive-response gene, in HDF-induced diabetes. Because of its pro-apoptotic role in β cells, we originally hypothesized that WT mice would perform worse than KO mice in the glucose tolerance test. However, the results were opposite: WT mice performed better. Further analyses indicated that, compared to WT mice, KO mice had significantly reduced serum insulin levels and attenuated insulin release upon glucose challenge. However, WT and KO mice performed similarly in insulin tolerance test and had comparable insulin-induced Akt phosphorylation in their fat pad, suggesting no obvious difference in their insulin resistance. I note that in adipocytes ATF3 was shown to repress the expression of adiponectin ([24], [90]), an insulin-sensitizing adipokine ([462]). However, we did not observe higher insulin sensitivity in the KO mice than in the WT. One potential explanation is that ATF3 is multi-functional and its functions in other tissues may have counteracted this undesired adiponectin-repressing action. As an example, in macrophages ATF3 represses the expression of several pro-inflammatory genes (including IL6, IL1β, and TNFα) ([96], [30], [51]), a function that will dampen the inflammatory response both locally and systemically and thus provide beneficial effects. Therefore, the balance maintained by ATF3 between the insulin-resistance inducing effect of repressing adiponectin and the insulin-sensitizing effect of reduced inflammation in macrophages may be preserved in ATF3’s absence, just inverted.
Clearly this is speculation, however the important point is that no differences in insulin resistance were observed between WT and KO mice on HFD.

The reduced glucose tolerance in the KO mice combined with the lack of difference between WT and KO mice in insulin resistance pointed to a reduced β-cell functional mass in the KO mice. This is counter-intuitive, since ATF3 has been shown to be pro-apoptotic in β cells (see introduction). This discrepancy can be reconciled by the finding that, at the time of analyses, β-cell apoptosis was not a problem yet as evidenced by the lack of caspase 3 activation (Fig. 4.3A). This supposition is also supported by two other observations. First, the mice still had normal compensatory response—increased β-cell mass (Fig. 4.3B and 4.3C). Second, neither WT nor KO had developed insulin resistance in their liver. As shown in Fig. 4.7C, intra-portal injection of insulin induced Akt phosphorylation in both WT and KO mice and the level of induction was comparable to that in their counterparts fed under normal chow. Thus, the mice were at an early-stage of disease development. In the absence of apparent β-cell apoptosis, the effects of ATF3 to improve β-cell function predominate, leading to the better performance of WT mice than KO.

The lack of apoptosis indicates that expression of ATF3 is not sufficient to induce β-cell apoptosis, despite its potential to do so. Considering the context-dependent nature of ATF3 and its multi-functional capacity, this is not surprising. One example of the context-dependency of ATF3 is its dichotomous role in breast cancer cells: It enhances stress-induced apoptosis in untransformed cells but protects malignant cancer cells from stress-induced detrimental effect ([463]). The fact that β cells did not undergo apoptosis at the stage of my analyses allowed me to investigate ATF3 for its potential functions
other than pro-apoptosis. This led to the identification of ATF3 in β-cell functions. My studies primarily focused on transcription of the insulin gene. Previously, glucose was demonstrated to increase insulin biosynthesis at the transcriptional, post-transcriptional, and translational levels (for some reviews, [464], [271]). At the transcriptional level, the increase has been shown to occur within minutes after glucose stimulation ([455]). However, due to the long half-life of the mature insulin mRNAs ([444]), transcriptional regulation is thought to be a mechanism to increase insulin production in response to long-term, rather than acute, glucose stimulation ([464], [271]). In light of this view, we interpret that the decreased serum insulin levels in the KO mice under HFD—a long-term stress model—was, at least partly, due to the decrease in insulin gene transcription in the KO β cells.

In addition to insulin gene transcription, ATF3 deficiency affected glucose-stimulated insulin release. Since the KO islets had reduced insulin content, the reduction in insulin release could be simply a secondary effect of this deficit. Alternatively, the KO islets may also have an impaired secretory process. I found that glucose-induced calcium influx—the triggering step during the secretory process—is not dramatically different between WT and KO islets; however, I did not address the other steps regulating insulin secretion, which include the amplification step in Ca\(^{2+}\) action and the exocytosis step that is composed of granule recruitment, docking, priming, and fusion ([460]). In addition, I did not address the issue of calcium-independent secretion ([460]). Further investigation is required to address whether ATF3 affects any of these processes.

The novelty of my findings is that they linked ATF3, a pro-apoptotic gene, to β-cell function. Since ATF3 is an adaptive-response gene induced by many stress signals
encountered by β cells during the development of T2D, these results have significant implications. Of particular interest in this regard is the β-cell exhaustion concept proposed by Leahy ([465], [466]) and Butler ([467]). According to this concept, hyper-stimulation of β cells to produce and release insulin is a non-sustainable event; β cells become exhausted and eventually dysfunction/failure ensues (reviews, [424], [425]). A key point of this concept is that hyperglycemia is the stimulus for β-cell dysfunction. In lieu of this concept, we propose the following speculative model to explain the roles of ATF3 in T2D. At the early stage of disease development, stress-induced ATF3 helps the β cells to cope with high metabolic demands, at least in part by increasing insulin gene transcription. However, this adaptation or coping mechanism comes with a cost, since ATF3 is a double-edged sword. Its detrimental, pro-apoptotic function comes into play when the proper cellular context emerges after prolonged β-cell hyperactivity. Clearly, this is a speculation and more investigation is required to test this model.

In conclusion, ATF3 regulates β-cell apoptosis (previous work) and function (this report)—two important aspects of β cells in T2D development. Since ATF3 is an adaptive-response gene whose expression can be modulated by many stress signals, our work identified a molecular target for the environmental factors and potential therapeutic agents to impact β cells and T2D development.
ACKNOWLEDGMENTS

I would like to thank Dr. Raghavendra Mirmira and Dr. Michael Zhu for their intellectual and physical contribution to the calcium studies performed in this chapter. Their expertise and equipment made these experiments possible. I would also like to thank Dr. Martha Belury, Dr. Ling Qi and Dr. Marc Montminy for sharing their expertise with HFD induced diabetes. A special thanks to Drs. Qi and Montminy for performing the cytokine serum analysis presented in this chapter. Finally, I would like to thank Shawn Behan for his diligence and meticulous attention to detail while assisting with the animal work presented in this chapter.
FIG. 4.7. Supplemental phenotype of WT and KO mice on HFD. WT and ATF3 KO C57BL/6 mice were placed on HFD and monitored for onset of diabetes. Data from WT mice are graphed as black line/black triangle or black bar while KO data points are grey line/grey square or grey bar. HFD groups are solid line while normal chow groups are dashed line. Body weight (A) and diet consumption (B) were monitored throughout administration of HFD. (C) Insulin signaling in liver was evaluated 5 minutes following hepatic portal vein (HPV) insulin injection. The indicated proteins were analyzed by immunoblot. Shown is a representative blot where extracts from 4 fat pads per group were pooled for analysis. (D) Epidymal fat from WT and ATF3 KO C57BL/6 mice fed a HFD were examined for signs of inflammation. Shown are representative images from H&E stain after 12 weeks on diet. (E) Serum from fasted WT and ATF3 KO C57BL/6 mice fed a HFD was collected and analyzed for TNFα by ELISA. Data represented as means ±S.E.M. from 5 mice. (F) After 12 weeks on diet, tissues from WT mice were surveyed for ATF3 mRNA abundance. Black bar represents data from normal chow mice while white bar is from HFD mice. Data represented as means ±S.E.M. * p< 0.001 WT mice normal chow vs. HFD.
FIG. 4.8. ATF3 contributes to insulin production in mice fed a 10% or 45% fat diet. WT and ATF3 KO C57BL/6 mice were placed on a mild (10%) or medium high (45%) fat diet and monitored for onset of diabetes. Data from WT mice are graphed as black line/black triangle or black bar while KO data points are grey line/grey square or grey bar. HFD groups are solid line (45% fat) and dashed line (10% fat). (A) Intraperitoneal glucose tolerance tests performed after 8 weeks on diet. Body weight (B) and diet consumption (C) were monitored throughout administration of diet. (D) Intraperitoneal insulin tolerance test performed after 8 weeks on diet. As controls, diabetic db/db mouse (solid line/open circle) and non-diabetic C57BL/6 mouse (dashed line/open circle) were included. (E) Fasting serum insulin measured after 8 weeks on diet. Data represented as means ±S.E.M. * p< 0.02, ** p< 0.005 WT vs. KO on HFD.
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<th>Species</th>
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<th>Reverse primer (5’ to 3’)</th>
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**Table 4.1. Primers used in Chapter 4.** Rat and mouse primer sequences were designed for each gene using PerlPrimer (version 1.1.14). Genomic and mRNA sequence information was extracted from the Human Genome Browser Gateway.
CHAPTER 5

FUTURE PERSPECTIVES

Work presented in this dissertation identifies and characterizes two opposing functions of ATF3 in β-cell stress response. This work also identifies a new tool that may have applications towards the pursuit of basic science research as well as clinical islet studies. Data presented in chapter 2 describes the role ATF3 plays in β-cell death during islet transplantation and provides insight into the mechanism by which ATF3 functions in this stress context. Chapter 3 reports the identification of a modified non-immunogenic gene therapy vehicle that possesses enhanced infection efficiency for murine, non-human primate, and human islets. The properties of this modified AAV make it an ideal gene therapy vehicle for islet transplantation. Data shown in chapter 4 describes an unexpected role for ATF3 in a high fat diet model of diabetes. ATF3 was observed to enhance insulin gene expression and therefore promote the function of islets during periods of metabolic demand. Given the previously known pro-apoptotic function of ATF3 in islets, it was expected that ATF3 induction in this stress context would induce β-cell death rather than the observed stimulation of an adaptive response. In conclusion, this body of work offers insight into the significance of ATF3 expression during two
distinct and separate stress contexts, islet transplantation and high fat diet induced diabetes, while identifying potential mechanisms by which ATF3 may function in each case. Furthermore, this dissertation identifies the RGD modified AAV1 viral vector as a highly infectious and non-immunogenic gene therapy tool that may contribute towards efforts aimed at improving outcomes from clinical islet transplantation.

While data presented in this dissertation begins to address the biological significance of ATF3 expression and the mechanism by which it functions in β-cell stress response, much work remains to be done. Islet transplantation represents an interesting and clinically relevant context to pursue these studies because it involves two major biological processes regulated by ATF3; inflammation and apoptosis. During transplantation, the islet derived inflammatory response plays a large role in initiating and modulating the larger recipient immune response against the islets. Specifically, islet expression of MHC molecules, tissue factor, and CCL2 (MCP1) have all been demonstrated to influence graft rejection, presumably through their pro-inflammatory activities [321, 394, 468, 469]. Given that inflammatory stress potently induces apoptosis in islets, islets have the potential to play a role in determining their own fate. Therefore, the dual functions of ATF3 in promoting both the intracellular process of β-cell apoptosis as well as the islet derived inflammatory response, which ultimately leads to even more apoptosis, makes ATF3 a potent mediator of islet graft rejection. The islet transplants that were performed in this dissertation involved syngeneic (self) transplants into chemically induced (non-autoimmune) diabetic recipients. Since this approach does not evoke an adaptive immune response and does not involve auto-immunity, it offers the opportunity to study the response of transplanted islets to primary non-function (PNF) in
the absence of the complications that arise due to cell mediated immunity. PNF is an important aspect of islet transplant biology in of itself as this phenomenon is responsible for the loss of approximately 50% of functional islet mass. However, aside from the induction of PNF, syngeneic islet transplantation has little clinical relevance with respect to treating type 1 diabetes. Rather, clinical islet transplantation in the strictest sense involves the use of allogenic (non-self) islets transplanted into auto-immune recipients. In this allo/auto-immune environment, the combination of PNF and cell mediated immunity challenge the islets with a broader spectrum of stresses that combine to increase the risk of graft failure and rejection. Given that work in this dissertation has identified CCL2, NOXA, and bNIP3 as downstream targets for ATF3 in the syngeneic transplantation context, it is plausible that ATF3 may also contribute to islet graft response to allo/auto transplantation. The role for ATF3 in this more clinically relevant transplantation context remains to be examined experimentally.

The potential role of ATF3 in the host immune response to islet transplantation is an area that has also not be tested experimentally yet. In recent years, several papers have been published on the role of ATF3 in dampening the inflammatory response of immune cells exposed to acute stresses [29, 30, 96, 470]. In these studies, bacterial or allergic stimuli were used to elicit inflammatory responses in mice. In each case, ATF3 deficient mice demonstrated a hyperacute, sometimes lethal, immune response. This work suggests that while ATF3 expression in transplanted islets is deleterious to the survival of the graft, ATF3 expression in the recipient immune cells may be important to promote the survival of the graft, presumably by dampening the inflammatory response. However, the immune response elicited by acute exposure to bacterial or allergic stimuli
may involve distinctly different processes from those activated during allogeneic islet transplantation. Experiments using wild type islets transplanted into wild type and ATF3 knock out recipients are planned in order to address this issue.

The identification of AAV1/RGD as a non-immunogenic gene therapy vehicle with improved islet infection efficiency is an exciting finding that has many implications. Of the many applications for gene therapy, islet transplantation in particular will benefit a great deal from this advancement. As mentioned previously, the long term success of clinical islet transplantation is limited by the loss of islets to primary non-function (PNF) and adaptive immunity. Therefore, any measures that function to intervene in these processes will greatly improve clinical outcomes. Currently, the standard clinical approach to block the adaptive immune response towards the transplanted islets involves the systemic immunosuppression of recipients. However, the systemic doses needed to achieve efficient immunosuppression mediate several off target effects, one of which involves islet toxicity and death. Therefore, targeting the islets directly ex vivo to reduce their immunogenicity and promote their intrinsic ability to survive represents an attractive alternative to systemic immunosuppression. Until now, adenovirus based delivery vehicles have been the vectors used for gene therapy in mouse and rat islet transplant studies. This is likely due to their relatively high islet infection efficiency when used at high doses [315]. Despite the known drawbacks to adenoviral delivery vehicles, including the interferon response and high immunogenicity, many of the reports using adenovirus still achieved therapeutic benefit. However, the potency of their gene therapy efforts may have been limited by adverse consequences from the adenovirus infection. Therefore, in moving forward with the AAV1/RGD project, our lab intends to
use what we have learned from the literature regarding the relative potency of adenoviral delivered gene therapy constructs as well as our experience with islets isolated from ATF3 knock out mice to guide our efforts in selecting the most potent gene therapy approach for islet transplantation. As an example, we are currently validating an AAV1/RGD-ATF3-miRNA construct for its ability to block the expression of ATF3 in islets cultured in vitro. If effective in blocking the expression of ATF3, this construct will be used on islets prior to transplantation to determine whether knocking down ATF3 is as efficient at promoting graft survival as knocking ATF3 out was. If so, we may be able to apply what we have learned from research presented in chapter 2 of this dissertation towards other animal models where ATF3 knock out is not possible.

Given our interest in improving outcomes from clinical islet transplantation we have developed, in addition to the AAV1/RGD project, a materials science project in collaboration with Dr. Jianjun Guan in the College of Engineering at The Ohio State University. This project involves the encapsulation of islets with a thermosensitive biocompatible “hydrogel” polymer. Islet encapsulation is a technique previously reported in the literature to protect islets from the allo-immune response following transplantation to the intraperitoneal cavity. Encapsulation creates a physical barrier that protects islets from cell mediated attack while still allowing for the exchange of nutrients and oxygen. The novelty of our encapsulation approach is three fold. First, the hydrophobicity of hydrogel is engineered to be thermosensitive, in that it is hydrophilic and a liquid at 4°C but becomes hydrophobic at 37°C and turns into a gel. The formation of a gel around the islets as they are warmed to 37°C has the advantage of inducing less stress than other encapsulation approaches that involve super-physiological temperatures or
harsh cross-linking treatments to form a solid capsule. In addition, islets can be mixed with hydrogel and poured into a casting apparatus for gelation. This affords great flexibility in creating capsules shaped to enhance function. Second, polymers making up the hydrogel contain reactive side chains that can be covalently modified with functional side groups. The addition of molecules such as polyethylene glycol, collagen, or RGD peptides have the potential to greatly enhance the stability of the capsule as well as promote islet function by proving biologically relevant points for islet cell adhesion. Third, our approach allows for the addition of soluble growth factors within the capsule. Growth factors such as the GLP-1 or VEGF have the potential to enhance islet function and promote graft vascularization. Collectively, these novel features of hydrogel make it an ideal material for islet encapsulation. Data from this project is not presented in this dissertation, but we have generated some encouraging preliminary results regarding the performance of hydrogel in vitro and in vivo. Ultimately, our goal is to combine a gene therapy approach using the AAV1/RGD vector to block islet death induced by primary non-function with islet encapsulation to further protect the islets from the adaptive immune response. If used together, these technologies may significantly increase the survival of transplanted islets and enhance the success of transplantation.

With respect to the role of ATF3 in the development of high fat diet induced diabetes, several aspects of ATF3’s contribution towards this disease remain to be clarified. Most importantly, the use of a β-cell specific ATF3 knock out mouse would strengthen our conclusion that ATF3 enhances pancreatic insulin gene expression in obesity. Insulin gene expression can be influence by several systemic factors, especially in the context of high fat diet induced obesity. Given that we and others have observed
high fat diet to induce ATF3 in islets, adipocytes, hepatocytes, and macrophages of atherosclerotic plaques, with many other tissues likely, it is possible that ATF3 deficiency in these tissues may contribute the defect in insulin transcription in the ATF3 knock out mouse used in this study. While work presented in this dissertation demonstrates that factors known to influence insulin demand, such as insulin resistance and inflammation, are not altered in the ATF3 knock out mice, other unknown variables may be influencing insulin transcription. Therefore, using β-cell specific ATF3 knock out mice to examine the contribution of ATF3 towards insulin transcription during periods of metabolic demand would be a more appropriate experimental design. Unfortunately, the β-cell specific ATF3 knock out mouse did not exist at the time these studies were done. However we are currently developing a conditional ATF3 knock out mouse in the lab and the β-cell specific knock out will be available in the near future.

Work presented in this dissertation demonstrated a role for ATF3 in the induction of insulin by glucose. However the upstream and down stream mechanisms by which this occurs have yet to be addressed. In regards to the events upstream from ATF3 induction, the signaling pathways activated by glucose in β-cells have been identified and include calcium, MAPK, and mTOR. Calcium signaling and MAPK activation are classical pathways known to induce the expression of ATF3 in other cell types. Therefore, activation of these pathways by glucose in β-cells may play a role in the induction of ATF3. Experiments using pathway selective inhibitors combined with glucose stimulation would allow the direct examination of which pathway, if any, plays a role in glucose induction of ATF3 in β-cells. In regards to the events downstream from ATF3 induction, the mechanism by which ATF3 regulates the insulin promoter also was
not addressed. Data presented in chapter 4 demonstrated that ectopic expression of ATF3 was sufficient to induce insulin gene expression under static physiological glucose concentrations. However, data from additional experiments that were performed but not shown revealed that ATF3 is not sufficient to induce insulin expression under conditions of glucotoxicity. Since glucotoxicity is reported to induce the degradation and nuclear exclusion of two known master regulators of insulin gene expression (PDX1 and MafA), ATF3 may require these factors in order to enhance insulin expression. Therefore, additional mechanistic insight may be obtained by evaluating the profile of DNA occupancy, recruitment and interaction of ATF3 with that of PDX-1 and MafA.
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Appendix A: A Method For Isolating Islets From The Mouse Pancreas

This is the protocol for mouse islet isolation. See another protocol for rats (islet isolation, Rat). This revised protocol is a combination of protocols from Gordon Weir’s lab, Shane Grey’s lab and the Roche website with modifications based on our results. Matt Hartman went to Gordon’s lab in 2001 to learn the method and we subsequently received tips from Shane Grey and Adolfo Ocana-Garcia about technique. The Roche website (http://www.roche-applied-science.com) has a lot of information about islet isolation. Mi-Lyang Kim and Erik Zmuda have optimized 4 batches of Liberase stock as of 2009 and have not noticed much variation in stock to stock digestion time.

1. Preparation of reagents and equipment
   a. Liberase. Liberase RI (Roche, cat# 11 815 032 001, 100mg/vial, 800-845-7355, ~$249/vial, ~160 mice/vial, ~$16/prep) for mouse and rat islet isolations. Other species require different liberase products from Roche.
      i. Buy 4-6 vials at a time and make a large batch. Five vials will make 80 aliquots that are very stable when stored at -80°C.
      ii. Re-suspend lyophilized powder in sterile HPLC grade water (4ml/vial). Place vials on ice for 30 minutes with gentle swirling every few minutes. Check to ensure powder is completely dissolved at the end of 30 minutes.
      iii. Pool all dissolved liberase together and mix with gentle swirling (do NOT vortex or shake). Aliquot 0.25ml to eppendorph tubes, quick freeze on liquid nitrogen (treat it as other enzymes) and store at -80°C. Each aliquot is for single use and should not be stored or re-frozen once thawed.
      iv. For each new batch, the optimal incubation time must be calibrated. Calibrate the frozen stock, not the freshly dissolved stock as you want to mimic the real experimental conditions as much as possible
   1. Calibrate the water bath that you intend to use to exactly 37°C using a mercury thermometer and not an alcohol thermometer (less accurate). Use the same incubator in future experiments.
   2. Use one or two mice for each incubation time and test 13, 14, 15, 16, 17 minute incubation times. If possible, include 30 second intervals between times as the timing of digestion is very important. Ultimately the yield shouldn’t change too much between peak times, but the quality of islets at those intervals may vary. Only use the conditions that give you the largest number of healthy islets 48 hours after isolation. Shane’s condition changed from 22 to 15.5 minutes. Perhaps recent liberase is stronger (9-2002). The age of mice appears to affect the optimal incubation time. So, use mice within a similar age range. Ideally, use 8-12 week old mice, however, Erik has found
that even 8-10 month old mice can yield good islets. For calibration purposes, use younger mice.

3. Prior to use, thaw a stock tube of liberase and dilute by adding the 0.25ml aliquot to 22.5ml serum free RPMI. The desired final working concentration is 0.275mg/ml. Other labs conditions are as follows.
   a. Gordon’s lab = 0.373mg/ml, 30min (0.5ml stock added to 33ml buffer)
   b. Shane’s lab = 0.25mg/ml, 15-22min (optimized first)
   c. Roche web = 0.233mg/ml, 30min (0.14ml stock added to 14.86ml buffer)

   i. 0.419mm diameter wire mesh from Thomas Scientific (cat# 8321-Q49, 800-345-2100)
   ii. 0.1mm diameter nylon cell strainer from BD Falcon (fisher cat# 08-771-19, BD falcon cat3 352360, 800-766-7000)

c. Histopaque 1077. From Sigma, for making gradient (cat#H1077, 800-262-9141)

d. Dissecting microscope. Zeiss Stemi SV8 set to 8x magnification.

e. Centrifuges and surgical equipment. Centrifuge must have a swing bucket rotor, be capable of 900xG, be refrigerated, and have a break that can be disengaged. Erik Zmuda has used Dr. Xin-an Pu’s centrifuge, a Sorvall RT6000D with the Sorval H1000B rotor. 900xG speed is 2400RPM. Slow spins are done at 800RPM. Surgical equipment needed includes; 1 pair of sharp scissors for abdominal cut, 2 pairs of forceps, 1 hemostat to clamp off the bile duct, 3 27-gauge needles bent with a 90 degree angle (towards the beveled face) using the hemostat, and a 5ml syringe.

f. Media.
   i. RPMI1640 from Invitrogen (cat#23400021, 800-955-6288)
   ii. Fetal bovine serum (Current low price vendor). FBS is needed for cultivation of islets and for quenching liberase activity.
   iii. 70% Ethanol in a spray bottle for wetting the mouse hair. This keeps hair from spreading inside the abdominal cavity and contaminating the prep.

2. Surgical procedures
   a. Set up equipment and media. All reagents and instruments (touching the samples) should be sterile. Use the laminar flow and tissue culture hoods for all steps (if the samples are exposed to air). Except during liberase digestion at 37°C, the samples should be kept at 4°C or on ice. Erik Zmuda has found that isolation and hand picking of islets can be performed outside of the hood without increased incidence of contamination. Sterilization of tools and wire mesh are critical for avoiding contamination.
      i. Autoclave or flame sterilize instruments before use, bend needles, and fill spray bottle with 70% ethanol.
      ii. Set up dissecting microscope on lab bench or in the hood.
      iii. Pre-chill centrifuge to 4°C.
iv. Place histopaque, RPMI (1 liter with 10% serum, 200ml without serum), 50ml conical tubes (1 for each pancreas) and 5ml syringe on ice.

v. Thaw 0.25ml aliquot of liberase and dilute in 22.5ml serum free RPMI. Serum, BAS and EDTA inhibit liberase. Zinc and calcium are cofactors. Store on ice and use within 1.5 hours. This is enough for ~11 mice (2ml/mouse).

vi. Warm 37°C water bath.

vii. Bring as many mice as you can cannulate in 1 hour (~10 mice) to the lab.

b. Preparing for cannulation.

i. Euthanize the mouse by cervical dislocation or CO2 asphyxiation.

ii. Fill syringe with 2ml diluted liberase and spray down abdomen of mouse with 70% ethanol.

iii. Open abdomen of mouse with a V-incision from the pubic region to both front legs. Fold skin over chest to reveal abdominal cavity.

iv. Move mouse to dissecting microscope and position it with the head pointing towards you. Locate the duodenal entry of the common bile duct as highlighted in FIG.1. This can be done without looking through dissecting scope objective.

v. Clamp duodenal opening with hemostat as demonstrated in FIG.2. Clamp
position is critical for blocking flow of liberase into the intestines. If clamp is too high or too low, liberase will not perfuse the pancreas. Position clamp so that when compressed, the clamp runs exactly along the pancreatic/intestine boarder.

c. **Cannulation of common bile duct.**
i. Reposition the liver if necessary to expose entire length of common bile duct. (FIG.3)

![FIG. 3.](image)

**Common Bile Duct**

**Liver**

ii. Cannulate the common bile duct with the bent 27-gauge needle attached to the liberase filled syringe. There are two techniques for cannulating the bile duct.

1. **Free hand method.** Using the hemostat clamped on the duodenum, pull away from the head of the mouse towards the tail so that the common bile

![FIG. 4.](image)

**Confluence**
duct becomes taught. There is a confluence in the bile duct close to the liver where bile draining from the gall bladder and enzymes from the liver come together before entering the intestines. The inside of the V formed by this confluence is exposed by pulling the bile duct tight and is an ideal place to initiate cannulation of the duct (FIG.4). If positioned correctly, the needle will slide right through the V, and directly cannulate the lower portion of the duct. Feed the needle several millimeters past the end of the bevel before dispensing the liberase to prevent backflow into the liver and gall bladder. There is a mostly invisible branch to the common bile duct at its midpoint that drains the splenic tail of the pancreas, an islet rich area of the pancreas. If your cannula slides past this branch you will have less complete perfusion of the splenic tail and potentially reduced digestion of this area. Optimal islet yield occurs when your cannula depth is far enough that no liberase escapes to the liver/gall bladder but not so far that you’ve passed this difficult to see branch of the common bile duct. You can test for successful cannulation by dispensing a small amount of liberase. If you see the liberase filling the duct then dispense the rest of the 2mls. If the area around the duct begins to fill, stop dispensing, reposition the needle, and try again.

2. **Forceps assist method.** Using the hemostat clamped on the duodenum, pull away from the head of the mouse towards the tail so that the common bile duct becomes taught. Using the bent 27-gauge needle attached to the 5ml syringe puncture the fascia below the common bile duct close to the liver. Use the needle to clear the fascia from the duct (FIG.5). With the needle still in place, set the hemostat down and pick up a pair of forceps.

![FIG. 5. Clear duct](image1)

![FIG. 5. Insert forceps](image2)

Use one arm of the open forceps to lift up the bile duct where the needle has cleared the fascia. The ridges in the forceps create a tract that you can use to guide the needle into the duct. While pulling the duct and forceps towards you, slide the needle into the duct using the forceps as a backstop. Test for cannulation by dispensing a small amount of liberase. If the duct begins to fill (FIG.5 Arrow), dispense the rest of the 2mls. If the area around the duct begins to fill, stop dispensing, reposition the needle and try again.
d. **Perfusion and removal of pancreas.** As the 2mls of liberase fills the pancreas, the area near the duodenum begins to expand first, followed by the region on top of the stomach, and finally the splenic tail (FIG.6). Look for even expansion of the pancreas (FIG.6). If one area begins to expand and you don’t see the white tissue evenly expanding and spreading out you probably aren’t in the common bile duct. Rather, you are inside the pancreatic capsule.

Filling the capsule with liberase will not result in a high islet yield as the surface area exposed to the enzyme will be low. If this occurs, stop perfusion and reposition the needle. To remove the perfused pancreas from the mouse it must be pulled free from its points of contact with the intestines, stomach, and spleen. Start by removing the hemostat from the duodenum. Then, using forceps, lift the duodenum and separate the pancreas from the intestines with a second pair of forceps (FIG.7a-b). This is done by holding the second pair of
forceps steady while pulling the intestines out of the abdomen. Next, pull the pancreas free from the top of the stomach and the spleen (FIG.7c-d). Finally, lift the pancreas out of the abdomen and cut it free from the remaining fascia connections (FIG.7e-f). Place in a 50ml conical tube on ice while the remaining pancreata are perfused. For maximal islet yield, place only 1 pancreas in each tube. Start a 1 hour timer after the first pancreas has been perfused. It is not recommended to allow any of the perfused pancreata to sit on ice for more than 1 hour as the liberase will begin to degrade the tissue. Promptly move on to the next step at the end of the hour.

**e. 37 °C digestion.** Group 50ml tubes together either with tape or in a rack that fits into the 37°C water bath. Ensure that all caps are well secured and submerge tubes in 37°C water bath for the precise amount of time indicated for the current batch of liberase (See step 1.a.iv.). At the end of the incubation, move the tubes to ice and add RPMI1640 with serum to 20mls per tube. Serum containing media will stop the liberase digestion. Re-cap tubes and shake vigorously (~40 shakes in 10 seconds).

**f. Purifying islets from digested pancreas.** To separate the islets from the digested pancreas, complete the following steps.

i. Pool digested pancreata so that there are 2.5 pancreata per tube. Ten tubes should be condensed to 4 tubes each with 50mls of media.

ii. Centrifuge tubes for 2 minutes at 800RPM and 4°C.

iii. Pour off supernatant and resuspend pellet in 25mls media with FBS by vortexing for several seconds on setting #4.

iv. Pour slurry through the 0.419mm wire mesh and funnel into a fresh 50ml conical tube. This separates out the non-digested tissue, fat and lymph. Rinse tubes with an additional 10mls of media with FBS and pour through filter. Centrifuge tubes for 2 minutes at 800RPM and 4°C.

v. Pour off supernatant and invert tubes on a paper towel. Watch to make sure the islets don’t slide off. Wipe the inside of the tubes with a paper towel to remove residual media, being careful not to disturb the cell pellet.

vi. Resuspend the pellet in 5mls cold histopaque 1077 by gently vortexing on setting #4 until the suspension is homogeneous. Add an additional 5mls of histopaque to wash the islets off the wall of the tube.

vii. Overlay histopaque with 10mls of serum free RPMI, being careful to maintain a sharp interface between the histopaque and media. Add the media by pipetting slowly down the side of the tube at a rate of 1ml per 10 seconds. The media should be on the top, and histopaque on the bottom. Serum affects the density of media and should not be used during this step.

viii. Centrifuge at 2400RPM (900xG) for 20 minutes with very slow acceleration and no braking at 4°C.

ix. Collect the islets from the histopaque/media interface with a disposable 10ml serological pipet. Islet tissue sticks to glass, therefore glass pipets should not be used unless they have been siliconized. Place islets into a fresh 50ml conical tube (2 tubes can be pooled) and fill with serum containing RPMI.
x. Centrifuge tubes for 2 minutes at 1200RPM and 4°C.

xi. Pour off supernatant and resuspend islets in 50mls of serum containing RPMI. Centrifuge tubes for 90 seconds at 800RPM and 4°C. Repeat wash 1 more time.

xii. Pour off supernatant and take tubes to the tissue culture hood. Add pen/strep to the serum containing RPMI and resuspend the islets in 6mls. Place a 0.1mm nylon cell strainer upside down on a 15ml conical tube. Collect islets on the top of the cell strainer by pipetting the media thru it. Rinse the 50ml conical tube with an additional 6mls of media and pipet thru the strainer.

xiii. Place 3mls of culture media as a large drop in a 10-cm petri dish. Invert the cell strainer used to capture the islets and dip it into the media drop. This will transfer most of the islets into the petri dish. Pipet an additional 5-7mls of culture media through the strainer into the petri dish to wash any residual islets off of the strainer into the dish. Check islet yield and quality under the 4x microscope objective. Swirling the dish in a tight oval will collect the islets in the center of the dish. If islets look good, they can either be picked immediately for experimental use or separated evenly between 6-8 petri plates (per 10 mice). Culturing too many islets in the same dish causes the islets to become necrotic and degrade. For experiments, 250-300 islets per 6cm dish with 2-3mls of media is the upper limit of culture density that doesn’t appear to stress the islets. Do not use tissue culture treated dishes for incubating islets as the islets will stick to the plates and cannot easily be separated into experimental groups later. For the most part, islets will not adhere to petri plates, so they must be used for culturing the islets.

xiv. There will be some usable islets in the 15ml conical tube that have passed through the 0.1mm nylon cell strainer. These can be recovered following 3-4 rounds of gravity sedimentation. After ~4 minutes of sedimentation, aspirate all but ~1ml of media from the tube and refill it to the top. Cap the tube and mix. Repeating this process 3-4 removes most of the single cell exocrine cells that are slow to sediment, and enriches for the heavier aggregates of endocrine cells (islets) that quickly sink. After the final aspiration, resuspend in 7mls of culture media and plate in a fresh petri dish.

3. Culturing isolated islets
   a. Post isolation incubation. The process of isolating islets is fairly stressful on the islets; histopaque is toxic, shaking and centrifugation steps induce a lot of sheer stress, and the removal from host blood supply as well as in vitro culture can induce hypoxia. We and others have shown that isolation induces β-cell death, ATF3 expression, and JNK signaling. The effect from these stresses is seen by the sloughing of cells from the periphery of the islet and the darkening and expulsion of the central core of the islet (central necrosis). While the sloughing of cells from the periphery can be tolerated, the central necrosis of
the islet disqualifies it for use in experiments. Typically, the larger the islet, the greater the chance that central necrosis will be a problem. Efforts aimed at reducing the islet’s post isolation stress response will increase the yield of usable islets. Therefore, we use a previously reported technique of incubating the islets in a 25-27°C tissue culture incubator for the first 48-72 hours following isolation. This reduced temperature dampens the islets stress response and smoothes the transition to in vitro culture. To achieve the desired temperature range, turn off the heat control to the incubator that will be used and fill the bottom with freezer bricks from the -80°C freezer. Five to eight bricks are adequate for 16-24 hours of reduced temperature. Replace the bricks with fresh -80°C bricks every 24 hours. No additional benefit has been observed after 72 hours of incubation at this lower temperature. If anything, moving the islets to 37°C after longer incubations at the lower temperature seems to induce central necrosis in the islets. It is also a good idea to change the media on the islets 24-48 hours after isolation. Secreted factors from stressed and dead islets promote additional islet stress. Gravity sedimentation is used to change the media on islets. Harvest all islets in one 50ml conical tube (if possible), allow islets to sediment to the bottom, aspirate all but 1 or 2mls of media, then resuspend the islets in 3-4mls and aliquot evenly the necessary number of petri plates. Bring the final volume of media up to 6-8mls/plate. Do this every 3-5 days in culture thereafter. Do not mix islets from different isolations as experimental groups are set up. The molecular baseline of the islets is influenced by many things, including the amount of time they have been in culture and the isolation stress that varies from prep to prep. To reduce variability, islet experiments must be performed on islets that were isolated at the same time.

b. Picking islets for experiments. Islets can be “picked” for experiments under the 4x or 10x microscope objective open to room air. It is a good idea to pool all of the islets into a single dish before setting up an experiment. This can reduce variability induced by subtle differences in culture conditions between plates during the post-isolation recovery period. Try to use islets in experiments before they have reached 7 days in culture. When picking islets into experimental groups, first swirl the plate to collect the islets into the center. Then, using a P-200 pipet set to 180μl, begin picking healthy islets. Healthy islets have smooth boarders and no dark center regions. Try to maintain an even distribution of islet sizes throughout experimental dishes as islet size can alters its response to treatment. One islet has approximately 1000-2000 cells and will yield 0.3-1μg of protein and ~25ng of total RNA. Therefore, experimental groups should be ~100-250 islets in number plated in 35mm or 6cm dishes with 1-3ml of media. To avoid variation induced by “picking fatigue” pick 25 to 50 islets at a time to each experimental dish. Repeat until all groups have the desired number of islets. It is difficult to maintain the same standard for islet quality and size as you pick thousands of islets into experimental groups. Using this approach will minimize the effect
of picking fatigue on experimental groups by spreading it relatively evenly across all groups.

c. Islet images.

![Images showing islet structures and their changes over time.]

- **Necrotic center being expelled**
- **Cells sloughing from periphery**
- **Exocrine contamination**
- **Exocrine cell aggregate**
- **Debris**
- **Central necrosis**

<table>
<thead>
<tr>
<th>Immediately following isolation</th>
<th>16 hours after isolation</th>
<th>36 hours after isolation</th>
<th>60 hours after isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>![Image of islets immediately following isolation]</td>
<td>![Image of islets 16 hours after isolation]</td>
<td>![Image of islets 36 hours after isolation]</td>
<td>![Image of islets 60 hours after isolation]</td>
</tr>
</tbody>
</table>
Tsonwin Hai Lab Mouse Islet Isolation Protocol Summary

Pre-isolation Setup

1. Sterilize instruments, bend needles, and fill spray bottle with 70% ethanol.
2. Set up dissecting microscope and pre-chill centrifuge to 4°C.
3. Place histopaque, media, 50ml conical tubes and 5ml syringe on ice.
4. Thaw 0.25ml aliquot of liberase and dilute in 22.5ml serum free RPMI.
5. Warm 37°C water bath.
6. Bring as many mice as you can cannulate in 1 hour (~10 mice) to the lab.

Surgical Procedure

1. Euthanize mouse and spray with 70% ethanol.
2. Fill syringe with 2ml diluted liberase.
3. Open abdomen of mouse with a V-incision and move to dissecting scope.
5. Cannulate and perfuse common bile duct.
6. Remove perfused pancreas and place on ice in 50ml conical tube.
7. Repeat steps for remaining mice within 1 hour.

Purifying Islets

1. Digest pancreata at 37°C for indicated time.
2. Stop digestion by adding media +FBS and vigorously shaking.
3. Pool 2.5 digested pancreata per tube.
4. Centrifuge tubes for 2 minutes at 800RPM and 4°C.
5. Pour off supernatant and resuspend pellet in 25mls media + FBS.
6. Filter through the 0.419mm wire mesh and funnel into a 50ml conical tube.
7. Centrifuge tubes for 2 minutes at 800RPM and 4°C.
8. Pour off supernatant and remove residual media.
9. Resuspend the pellet in 5mls cold histopaque 1077.
10. Add an additional 5mls of histopaque to wash the islets off the wall of the tube.
11. Overlay histopaque with 10mls of serum free RPMI.
12. Centrifuge at 2400RPM (900xG) for 20 minutes with no braking at 4°C.
13. Collect the islets from the histopaque/media interface.
14. Fill add media to 50mls and centrifuge tubes for 2 minutes at 1200RPM.
15. Pour off supernatant and resuspend islets in 50mls of serum containing RPMI.
16. Centrifuge tubes for 90 seconds at 800RPM and 4°C.
17. Repeat wash 1 more time.
18. Pour off supernatant and collect islets on the top of a cell strainer.
19. Rinse the 50ml conical tube and pipet media thru the strainer.
20. Transfer the islets to a petri dish and separate evenly between 6-8 plates.
21. Complete 3-4 rounds of gravity sedimentation on the cell strainer flow thru.
22. After the final aspiration, resuspend in 7mls of media and plate in a petri dish.
Appendix B: An ImageJ Macro Program To Delineate The Islets Automatically.

// "Islets" V1.0
// © M. Viapiano and T. Hai laboratories, The Ohio State University, 2008
//
// This macro automates the identification of pancreatic islets in culture
// (large spheres cells stained blue with Hoechst 33258) and apoptotic cells
// within the islets (stained red with propidium iodide).
// The macro separates the channels of an original RGB color image, allowing
// the use of color digital cameras to take a single picture of the cultures
// (instead of separate pictures for each channel), thus speeding up the imaging process.
//
// Briefly, the macro separates the two channels (Red & Blue) of the image
// and calculates the area of Red pixels above threshold within each islet
// identified in the Blue channel
//
// Certain parameters, such as upper/lower limits for thresholds or the constrains
// for particle size were selected ad-hoc for the images to be analyzed. Those
// parameters should be re-calculated or adapted for other applications.
//
// For documentation purposes, the macro creates a final stack with two slices showing
// the original Red and Blue channels, two extra slices showing the corresponding thresholded
// images and one final slice with the identified particles.
//
// --BEGIN
// The macro takes the name of the original file to name the final stack. If the
// original image was dragged into older versions of ImageJ, "File.name" may
// be empty, so a token name "image.tif" is given. Then the last four characters
// of the file name (usually the file extension) are dropped for simplicity.

V=getImageID();
selectImage(V);
imagewidth = getWidth;
imageheight = getHeight;
Dir=File.directory;
Name=File.name;
if (lengthOf(Name)==0) { Name="image.tif"; }
if (lengthOf(Name)<4)  { Name=File.name+"xxxx"; }
Name2=substring(Name, 0, lengthOf(Name)-4);

// Optional: The scale 1 pixel=1.85 micrometer was calculated from the size of a
// CCD element in the digital camera (7.4x7.4 micrometers) and the magnification
// used in the microscope objective (4X).
run("Set Scale...", "distance=1.85 known=1 pixel=1 unit=µm global");

// The RGB image is separated in channels and the Green channel is dropped.
setPasteMode("Copy");
run("RGB Stack");
run("Stack to Images");
selectWindow("Green");
close();

// The original image from the Blue channel is copied into slice 1 of the final stack
selectWindow("Blue");
run("Select All");
run("Copy");
nNewImage(Name2+"_stack", "RGB White", imagewidth, imageheight, 5);
setSlice(1);
run("Paste");

// Now the original Blue channel is thresholded
selectWindow("Blue");
run("Enhance Contrast", "saturated=0.5");
setAutoThreshold();
run("Threshold...");
waitforUser("Please adjust the threshold until the islets are covered and click 'OK' to proceed");
getThreshold(lower, upper);
bluelow=lower;
blueup=upper;
run("Convert to Mask");

// A watershed filter is applied to fracture the large particles and
// approximate their real number. A white border is drawn in the image to
// prevent particles in the borders from getting recognized as a single large particle
run("Close-");
run("Watershed");
setColor(255,255,255);
setLineWidth(2);
drawRect(0,0, imagewidth, imageheight);
run("Select All");
run("Copy");
selectWindow(Name2+"_stack");
setSlice(2);
run("Paste");

// Back to the binary image, the particles are identified and recorded
// In this macro we chose a lower limit of 1000 square micrometers to
// filter out small particles (debris, etc.)
selectWindow("Blue");
run("Set Measurements...", "area limit redirect=None decimal=3");
run("Analyze Particles...", "size=1000-Infinity circularity=0.00-1.00 show=Nothing include record");
Np=nResults();
TA= newArray(Np);
TC= newArray(Np);

// The perimeter of each particle is recorded in the ROI manager and saved as a separate file
for (i=0; i<Np; i++) {
    TA[i]=getResult('Area', i);
    X= getResult('XStart', i);
    Y= getResult('YStart', i);
    doWand (X,Y);
    roiManager("Add");
}
roiManager("Save", Dir+Name2+_"particles.zip");

// The Blue channel is finally closed
selectWindow("Blue");
close();

// Now the Red channel is copied to the stack and the original image is thresholded
selectWindow("Red");
run("Enhance Contrast", "saturated=0.5");
run("Select All");
run("Copy");
selectWindow(Name2+_"stack");
setSlice(3);
run("Paste");
selectWindow ("Red");
setThreshold(128, 255);
run("Threshold...");
waitForUser("Please adjust the threshold until the particles are covered and click 'OK' to proceed");
getThreshold(lower, upper);
redlow=lower;
redup=upper;
run("Convert to Mask", " ");
run("Select All");
run("Copy");
selectWindow(Name2+_"stack");
setSlice(4);
run("Paste");

// The ROIs saved for the blue channel are now used to
calculate the total area of red pixels within each islet.
selectWindow("Red");
V=getImageID();
selectImage(V);
run("ROI Manager...");
run("Set Measurements...", "area limit redirect=None decimal=3");
RA= newArray(Np);
for (i=0; i<Np; i++) {
    roiManager("Select", i);
    run("Measure");
    RA[i]=getResult("Area", i);
}

// The particles are finally visualized
run("RGB Color");
setForegroundColor(255, 128, 0);
setFont ("SansSerif", 20, "bold");
setColor (255,0,0);
for (i=0; i<Np; i++) {
    roiManager("Select", i);
    getSelectionBounds(topX, topY, Width, Height);
    roiManager("Draw");
    drawString(i+1, topX+(Width/2), topY+(Height/2));
}
// Finally the image is copied to the stack
run("Select All");
run("Copy");
selectWindow(Name2+"_stack");
setSlice(5);
run("Paste");

// run("Invert", "slice");
// The stack is saved as a TIFF file and the particle areas are tabulated
saveAs("Tiff", Dir+Name2+"_stack");
selectWindow("Red");
close();
selectWindow("ROI Manager");
runk("Close");
selectWindow("Threshold");
runk("Close");
selectWindow("Results");
runk("Close");
print("Parameters:");
print("Blue image threshold: "+bluelow+" -"+blueup);
print("Red image threshold: "+redlow+" -"+redup);
for (i=0; i<Np; i++) {
    print("particle "+(i+1)+" total area(um2)= "+TA[i]+" red area(um2)= "+RA[i]);
}
//
// -- END
Appendix C: A Method For Chromatin Immunoprecipitation

This protocol is based upon protocols from Dr. Farnham’s and Upstate’s protocols plus a fair amount of trial and error. It has successfully been used with MIN6 and INS832/13 cells. However, it is necessary to optimize conditions for your specific cell type.

**Day1**

1. Add formaldehyde directly to tissue culture media to a final concentration of 1%. I generally use 1-2 x 10^7 cells per antibody per time point. Fewer cells can be used but usually results in a lower signal to noise ratio. Incubate adherent cells on a shaking platform for 10 minutes at room temperature. Crosslinking for longer periods of time (>30 minutes) tends to cause cells to form into a giant crosslinked aggregate that can not be efficiently sonicated.

2. Stop the crosslinking reaction by adding glycine to a final concentration of 0.125 M. Continue to rock at room temp for 5 minutes. (I make 1.4M glycine as 10X stock)

3. Pour off media and rinse plates twice with cold 1X PBS.

4. Add an appropriate volume (I use 5ml per 10cm dish) of 1X PBS or a 20% 1X trypsin/EDTA (tissue culture grade) solution in 1X PBS. Incubate at 37 °C for 10 minutes if using trypsin (I have found this step useful for recovery of cells which stick to the plates after crosslinking).

5. Following addition of trypsin or PBS, scrape adherent cells from dishes. If you have used trypsin, inactivate the trypsin by adding a small amount of serum. Centrifuge scraped adherent and wash pellet once with 1X PBS plus PMSF(10ul per ml).

6. Resuspend cell pellet in cell lysis buffer plus the protease inhibitors PMSF (10ul per ml), aprotinin (1ul per ml) and leupeptin (1ul per ml). The final volume of cell lysis...
buffer should be sufficient so that there are no clumps of cells (1ml per 1.5ml tube). Incubate on ice for 10 minutes. Cells can also be dounced on ice with a B pestle several times to aid in nuclei release.

7. Microfuge at 5,000 rpm for 5 minutes at 4°C to pellet the nuclei.

8. Resuspend nuclei in ChIP buffer plus the same protease inhibitors as the cell lysis buffer. Incubate on ice for 10 minutes.

9. Sonicate chromatin to an average length of about 600 -3000bp while keeping samples on ice (the time and number of pulses will vary depending on sonicator, cell type and extent of crosslinking). For beta cells, I normally use microtip with the following conditions: ~1X10^7 cells in 550ul ChIP buffer per 1.5ml tube, output 5.0, 6”on/30”off X 15 pulses. I normally got an average size around 2kb-3kb. Microfuge at 14,000 rpm for 10 minutes at 4 °C. At this point, chromatin can be snap frozen in liquid nitrogen and stored at -70 °C for up to several months.

10. Carefully remove the supernatant and transfer to a new tube. Preclear chromatin by adding blocked protein A beads. Use 20-40 uls of 50% slurry of preblocked protein A beads for every sample.

11. Incubate on a rotating platform at 4 °C for 1h minutes. Microfuge at 14,000 rpm for 5 minutes.

12. Transfer supernatant to a clean tube and divide equally among your samples. Be sure to include a "no antibody" sample or IgG sample as negative control. You can also include a "mock" sample which contains 1X ChIP buffer instead of chromatin (no antibody and mock are critical to control for nonspecific interactions and DNA contamination of IP and wash solutions....the final output of this experiment is analyzed by PCR). Adjust the final volume of each sample with IP dilution buffer plus protease inhibitors if necessary. Samples volumes should be between 500ul and 1ml. Save 10% of each sample as total input and store at -20°C. Add 1-2 ug of antibody to each sample.

13. Incubate on the rotating platform at 4 °C for at least 3 hours. Overnight is fine.
**Day2**

14. Add 20ul of 50% slurry of preblocked protein A beads to each sample. Incubate on the rotating platform at 4 °C for 1h, no longer.

15. Microfuge samples.

16. Wash pellets once with 1ml of 1X Low Salt wash buffer, 1ml High Salt wash buffer, 1ml LiCl wash buffer, 1ml TE buffer (twice). For each wash, rock at 4 °C for 5-10min. **Efficient washing is critical to reduce background.**

17. After the last wash, microfuge and remove the last traces of buffer. Elute antibody/protein/DNA complexes by adding 250ul of IP elution buffer. Rock on a rotator for at least 15 minutes. Microfuge at 10,000 rpm for 1 minute. Transfer supernatants to clean tubes. Repeat once with 200ul elution buffer and combine both eluents in the same tube.

18. After the second elution, microfuge samples at 14,000 rpm for 5 minutes to remove any traces of Protein A beads. Transfer supernatants to clean tubes. Process the total input sample together with IPs (I normally add ChIP buffer to total input samples to bring up the volume to ~450ul). Add 3ul of high concentration RNase A (10mg/ml) and 30ul 5M NaCl to a final concentration of 0.3 M. Incubate samples in the 65 °C water bath for 4-5 hours to reverse formaldehyde crosslinks.

19. Add 20ul 1M Tris.Cl (pH6.5), 10ul 500mM EDTA and 3 uls of proteinase K to each sample. Incubate in 55°C water bath for 1-2 hours. (Tris buffer of pH 6.5 helps to adjust pH of the sample and prevent SDS precipitation)

21. Extract once with 500ul of phenol/chloroform/ isoamyl alcohol. Total input samples may need to be extracted twice.

22. Add 20ug of glycogen to each sample. Mix well then add 1ml of pure ethanol. Precipitate in -20 °C freezer overnight.
**Day 3**

23. Microfuge samples at 14,000 rpm for 15min at room temperature. Allow pellets to dry in a vacuum. Resuspend DNA in water or TE and analyze by PCR. I generally resuspend each ChIP sample in 20ul and total input sample in 100ul and use 2-3ul for each PCR reaction.

**Solutions**

**Cell Lysis buffer**
- 5mM PIPES pH 8.0
- 85mM KCL
- 0.5%NP40
- Protease inhibitors

**Nuclei Lysis buffer**
- 50mM Tris-Cl pH 8.1
- 10mM EDTA
- 1%SDS
- Protease inhibitors

**IP Dilution buffer**
- 0.01%SDS
- 1.1%Triton X 100
- 1.2mM EDTA
- 16.7mM Tris-Cl pH 8.1
- 167mM NaCl

**Elution buffer**
- 100mM NaHCO3
- 1%SDS
5X PK buffer
50mM Tris-Cl pH 7.5
25mM EDTA
1.25%SDS

ChIP buffer
1X nuclei lysis buffer diluted 1:10 in 1X IP dilution buffer

Protease Inhibitors
100mM PMSF in ethanol, use at 1:100
10mg per ml aprotinin in 0.01 M HEPES pH 8.0, use at 1:1,000
10mg per ml leupeptin in water, use at 1:1,000

Low Salt Immune Complex Wash Buffer (Catalog # 20-154)
Formulation: 24ml of 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl. Liquid.

High Salt Immune Complex Wash Buffer (Catalog # 20-155)
Formulation: 24ml of 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl. Liquid.

LiCl Immune Complex Wash Buffer (Catalog # 20-156)
Formulation: 24ml of 0.25M LiCl, 1% IGEPALCA630, 1% deoxycholic acid (sodium salt), 1mM EDTA, 10mM Tris, pH 8.1. Liquid.

TE Buffer (Catalog # 20-157)
Formulation: 24ml of 10mM Tris-HCl, pH 8.0, 1mM EDTA. Liquid.

Blocked Protein A beads
Protein a beads suspended in 1X ChIP buffer and rock for >1h at room temperature to swell. Block beads with 0.1% BSA and 200ug/ml tRNA at 4°C for 1h. Keep at 4°C with 0.02% sodium azide if not use immediately.
Appendix D: A Method For Sub-capsular Kidney Islet Transplantation

Summary: This protocol includes a method that was developed to induce a diabetic state in mice that is similar to type I diabetes, wherein all endogenous islets have been destroyed. This protocol differs from type I diabetes in that it doesn’t induce islet death through an autoimmune response, but rather utilizes a cytotoxic glucose analogue, Streptozotocin (STZ), that is taken up through the GLUT2 transporter. This selectively targets STZ to islets and neurons. Therefore, at higher doses of STZ the mice will die due to neurotoxicity. So it is important to find the right dose that induces the least death (within the first week) with the highest incidence of hyperglycemia. The second protocol in this method pertains to transplanting islets to the kidney subcapsule of diabetic mice.

IMPORTANT: Mice should fast for four (4) hours prior to STZ induction. This results in a greater yield of diabetic mice following STZ administration.

IMPORTANT: The STZ-Na Citrate buffer solution should only be prepared immediately before injection as the drug degrades after 15-20 minutes in the Na-Citrate buffer.

Protocol for inducing diabetes with single high dose STZ injection
1. Mice should be fasted prior to injection; four to six hours of fast is the critical window in which injections must be performed. Place mice in a fresh cage at the start of the fast as food frequently falls into the bedding of their cages and this will compromise the fast.

2. Prepare Na-Citrate Buffer three and a half hours after starting the fast. Dissolve 1.47g of Na-Citrate (Fisher Scientific Cat.# BP 327-1) in 50ml ddH₂O. Adjust the pH of the buffer to 4.5 pH with HCl. This buffer should be made fresh with each day’s worth of injections.

3. Weigh out 0.05g STZ and place in a sterile 1.5ml eppendorph tube. Keep tube protected from light and on ice until the STZ is rehydrated in Na-Citrate Buffer. Prepare 1 tube for every 8 mice that will be injected. This amount of STZ/tube when resuspended in 1ml of Na-Citrate Buffer will produce a 50mg/ml solution. We have found that the optimal dose of STZ for making mice diabetic is 190mg/kg body weight. Our collaborators Dr. Shane Grey and Adolfo Garcia use 160mg/kg and 250mg/kg respectively in their labs. However, these doses in our hands either did not induce a high incidence of diabetes or killed the mice within 3 days in the absence of hyperglycemia. Given this narrow range of activity, it is important to pay careful attention to how much STZ the mice are being given.
4. Collect the following and proceed to the animal facility.
   - Several 0.5ml insulin syringes.
   - P1000 pipette and tips.
   - Calculator, Timer, and Small animal scale (frequently already in mouse room)
   - Tubes of 0.05g STZ
   - 50ml conical tube of Na-Citrate buffer pH 4.5

5. Immediately before injecting the mice, resuspend one tube of STZ with 1ml of Na-Citrate Buffer. Mix well with pipetting so that all clumps of STZ go into solution. **Start the timer counting down from 15 minutes. Discard the tube if it hasn’t been used up before the 15 minutes are over.**

6. Weigh the mouse and quickly determine the volume of 50mg/ml STZ to inject. Goal is to inject 190mg/kg of mouse. Therefore, multiply body weight by 190 and then divide by 50. This gives the volume in μl needed to deliver 190mg/kg per mouse. For example, if a mouse weighs 25 grams, the volume of STZ to inject is 95μl.

7. Using a 0.5ml insulin syringe draw up the volume indicated in step 6, tap out the air bubbles and ensure the volume is accurate, and inject into the lower right quadrant of the mouse’s peritoneal cavity.

8. Repeat steps 6 and 7 for each mouse. Each mouse should be given only one injection. Resuspend STZ as needed. After all mice have been injected, add food to the cages and place them back on the rack.

9. Mice should be tested for sufficient levels of hyperglycemia three days after injection and 4 weeks post-injection. Mice should be severely diabetic. Mice with non-fasting blood glucose levels above 350mg/dl are considered to be diabetic.

**Protocol for transplanting islets to the kidney sub-capsular**

1. Mice that have demonstrated 3 consecutive days of non-fasting blood glucose above 350mg/dl can be used as recipients for islet transplants.

2. Isolate donor islets to be transplanted. The number of islets needed for each recipient varies depending on several parameters, including recipient body weight and whether the therapy is intended to have marginal or curative effects. Typically, 300 islets will cure a recipient that weights ~20 grams at the time of transplant. 150 islets to a similar recipient will produce a marginal cure where glucose levels are reduced some, but not back to normal levels (normal is between 100mg/dl and 170mg/dl).

3. Hand pick the islets to be transplanted and place them in a 1.5ml eppendorph tube. Take care to ensure that good quality islets are selected for transplant and that their sizes are evenly distributed between the groups. Think about “islet mass” being transplanted and not just islet number. Groups that have a greater number of larger islets have the
potential to receive a greater mass of β-cells and will therefore respond differently than mice receiving smaller islets. Alternatively, larger islets are more susceptible to transplantation stresses, and as a result groups with larger islets may experience more cell death than groups of smaller islets. This may result in a net greater β-cell mass in the small islet group resulting in a different response. It's important to avoid bias introduced through the picking process, so think about how to develop a picking technique that works best for you to achieve an even distribution of healthy islets.

4. Hand picked islets can be stored on ice for several hours while the transplant procedure is being performed.

5. Collect the following and proceed to the animal facility
   - 200μl Hamilton Syringe
   - transplant tubing
   - P200 pippette, P200 tips and gel loading tips
   - extra 1.5ml eppendorph tubes and an eppendorph tube rack
   - sterile surgical tools
     (2 forceps, 1 hemostat, 1 sharp scissors, 6-0 polyurethane suture, wound clips)
   - sterile PBS in a 50ml conical tube on ice
   - sterile q-tips
   - hand picked islets on ice.
   - flame pulled and polished glass capillary probe for making pouch under capsule
   - 27gauge needle

6. Anesthetize recipient mouse and shave fur on the flank over the kidney. Lay mouse on its side and palpate the kidney to identify its location.

7. Make a small incision using scissors perpendicular to the length of the mouse directly over the kidney. The incision should be barely large enough for the kidney to pass through and become exposed outside of the body. A small incision will allow the kidney to remain “propped up” outside of the body. This facilitates the transplantation process. With larger incisions, the kidney continually slips back inside the abdomen making it difficult to deliver the islets.

8. Once the kidney is exposed, wet the surface with a PBS soaked q-tip. Repeat this step as necessary to ensure that the kidney does not dry out.

9. Using the 27 gauge needle, make a small incision through the kidney capsule towards the surface closest to the mouse’s head. Insert the flame pulled and polished glass capillary probe through this incision and feed it towards the “tail” end of the kidney to create a pouch in which the islets will be placed. Ideal pouches are longer and narrower, rather than shorter and wider. Narrow pouches prevent backflow of islets around the transplant tube as they are being placed into the pouch.
10. Load islets into the transplant tubing. This is done by first allowing the islets to sediment into a gel loading tip that has been bent (but not kinked) and placed in a 1.5ml eppendorph tube. Islets can be collected from the 1.5ml tube they were picked into using the P200 pipetter and a regular P200 tip. Once all of the islets have been aspirated into the P200 tip, dispense them into the bent gel loading tip and allow them to sediment into the narrow end of the tip. Kinks in the gel loading tip will cause the islets to shear and become destroyed during step #12 below.

11. After islets have sedimented, aspirate as much of the supernatant as possible without disturbing the pelleted islets. This will reduce the volume the islets have to spread out into during the transplant process and will increase the efficiency of transplantation.

12. Remove the gel loading tip from the 1.5ml eppendorph tube and affix the transplantation tubing to the narrow end of the gel loading tip. Place the other end of the tip onto a P200 pipette and carefully pass the islets into the transplant tubing. Stop when the islets are approximately 2 inches from the end of the transplantation tubing.

13. Remove the tubing from the gel loading tip and affix to the needle of the 200ml Hamilton syringe with the plunger already fully pulled out. Depress the plunger until the islets are just a few centimeters away from the open end of the transplant tube. Place the syringe aside allowing the tubing to hang free. Allow the islets to sediment towards the open end of the tube.

14. With the transplant tube still attached to the Hamilton syringe, insert the open end of the tube into the sub-capsular pouch that was created in step 9. Place the tubing to the very back of the pouch before depressing the syringe plunger to force the islets out. As the plunger is depressed, roll the transplant tube between your fingers to ensure the tube doesn’t stick to the capsule. This allows you to smoothly pull the transplant tube out as the islets are filling the pouch. This technique minimizes the backflow of islets around the transplant tube.

15. After all of the islets have been delivered to the sub-capsular pouch, use the flame pulled glass probe to push the islets towards the back of the kidney and away from the opening near the head of the mouse. This can be done by gently sliding the glass probe along the surface of the kidney moving away from the opening in the capsule near the head of the mouse. Use care to ensure that you don’t force the islets too far, as the capsule can break and spill the islets out into the peritoneal cavity.

16. Pass the kidney back inside the peritoneal cavity and place 2 or 3 sutures into the wall of the peritoneal cavity to close the opening. Use an additional wound clip or two to close the opening in the epidermis.

17. Remove mouse from anesthesia and monitor for recovery. Check blood glucose 24hrs later to determine if transplant was effective at restoring normo-glycemia. Normal blood glucose readings have been observed as early as 4 hours later.
Appendix E: A Method For Immunohistochemistry

The following protocol is from the IHC protocol of Cell Signaling (www.cellsignal.com) with minor modifications.

A. Solutions and Reagents

- Xylene or Clear-Rite 3 (Richard-Allan Scientific, for reaction product that is soluble in xylene, such as Vector Blue)
- Ethanol, anhydrous denatured, histological grade 100%
- Deionized water (dH₂O)
- Hematoxylin (optional, Richard-Allan Scientific)
- Wash Buffer:
  - 10X Phosphate Buffered Saline (PBS): To prepare 1 L add 80 g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4 g sodium phosphate, dibasic (Na₂HPO₄) and 2.4 g potassium phosphate, monobasic (KH₂PO₄) to 1 L dH₂O. Adjust pH to 7.4.
  - 10X Tris Buffered Saline (TBS): To prepare 1 L add 24.2 g Trizma® base (C₄H₁₁NO₃) and 80 g sodium chloride (NaCl) to 1 L dH₂O. Adjust pH to 7.6 with concentrated HCl.
- Antigen Unmasking Solution:
  - 10 mM Sodium Citrate Buffer: To prepare 1 L, add 2.94 g sodium citrate trisodium salt dihydrate (C₈H₇Na₃O₇•2H₂O) to 1 L dH₂O. Adjust pH to 6.0.
- 3% Hydrogen Peroxide: To prepare, add 10 ml 30% H₂O₂ to 90 ml dH₂O.
- Blocking Solution: 10% normal horse serum or goat serum (depending on the species where the 2nd Ab is produced) diluted in recommended wash buffer (+0.02% sodium azide).
- Biotinylated secondary antibody.
- **ABC Reagent:** (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA) Prepare according to manufacturer’s instructions 30 minutes before use.
- **DAB Reagent or suitable substrate:** Prepare according to manufacturer’s recommendations.

B. Deparaffinization/Rehydration

**NOTE:** After baking, do not allow slides to dry at any time during this procedure.

1. Bake paraffin sections of 4 um thickness overnight at 37 °C, followed by 30 min at 60 °C.

**Deparaffinize/hydrate sections:**
2. Incubate sections in two washes of xylene/Clear-Rite 3 for 5-10 minutes each.
3. Incubate sections in two washes of 100% ethanol for 5 minutes each.
4. Incubate sections in two washes of 95% ethanol for 5 minutes each.
5. Incubate sections in 2 washes of 70% ethanol for 5 minutes.
6. Wash sections twice in dH2O for 5 minutes each.

C. Antigen Unmasking

1. **For heating plate:** Bring slides to a boil in 10 mM sodium citrate buffer pH 6.0 then maintain at a sub-boiling temperature for 10 minutes (turn the heating plate to 0). Cool slides on bench top for 30-60 minutes.
2. **For pressure cooker:** it takes ~40 min for 2 L buffer to reach high-pressure stage at maximum power. Keep at high pressure for 5-10 min. Then release the pressure, turn off the heat and open the lid partially to let it cool down. (This method works the best and consistent for most Abs that requires heat-induced antigen retrieval for IHC).
3. **For steamer:** Bring slides to boil in antigen unmasking solution in microwave and transfer the slide container to a boiling steamer and keep the steamer ON for 60 min, turn of the steamer and let the slides cool for 30 min.
D. Staining

1. Wash sections in dH$_2$O three times for 5 minutes each.
2. Incubate sections in 3% hydrogen peroxide for 10 minutes to block the endogenous peroxidase activity.
3. Wash sections in dH$_2$O twice for 5 minutes each.
4. Wash section in wash buffer for 5 minutes. Use Pan Pen to outline tissue (at a distance so that tissue won’t be affected). Block each section with 100-400 μl blocking solution for 1 hour at room temperature.

**NOTE:** For tissues with high endogenous biotin, biotin-binding proteins, lectins or nonspecific binding substances, such as breast tissue, block the section with Avidin/Biotin Blocking kit (Vector Laboratories, SP-2001) is preferred. After blocking with normal serum, incubate section with Avidin D solution for 15 min. Rinse briefly with wash buffer, then incubate for 15 min with Biotin solution. These steps should be performed prior to addition of primary Ab.

5. Remove blocking solution and add 100-400 μl diluted primary antibody to each section. (Dilute antibody in blocking solution.) Incubate overnight at 4°C.
6. Remove antibody solution and wash sections in wash buffer three times for 5 minutes each. You may block the section again at RT for 20-30 min.
7. Add 100-400 μl secondary antibody, diluted in blocking solution per manufacturer’s recommendation (normally start with 1:200 dilution), to each section. Incubate 30 minutes at room temperature.
8. If using ABC avidin/biotin method, make ABC reagent once start the secondary Ab incubation according to the manufacturer’s instructions and incubate solution for at least 30 minutes at room temperature. For ABC reagent from Vector Laboratories (PK-6100), add 2 drops of Reagent A to 5 ml wash buffer, mix and then add 2 drops of Reagent B. Mix again and let it stand @ RT for at least 30 min.
9. Remove secondary antibody solution and wash sections three times with wash buffer for 5 minutes each.
10. Add 100-400 μl ABC reagent to each section and incubate for 30 minutes at room temperature.

11. Remove ABC reagent and wash sections three times in wash buffer for 5 minutes each.

12. Add 100-400 μl DAB or suitable substrate to each section and monitor staining closely. For DAB substrate from Vector Laboratories (SK-4100), immediately before use, to 5 ml of distilled water, add 2 drops of Buffer Stock Solution and mix well. Add 4 drops of DAB Stock Solution and mix well. Add 2 drops of the Hydrogen Peroxide Solution and mix well. If a gray-black stain is desired, add 2 drops of the Nickel Solution and mix well. Incubate tissue sections with the substrate at room temperature until suitable staining develops. Development time should be determined by the investigator but generally 2-10 minutes provides good staining intensity.

13. As soon as the sections develop, immerse slides in dH2O.

14. If desired, counterstain sections in hematoxylin per manufacturer’s instructions.
   To use hematoxylin 7211 from Richard-Allan Scientific for counterstain, dip the slides in Hematoxylin 3-5 times, then rinse with tap water for ~ 1 min till no more color wash off.

15. Dehydrate sections:
   Incubate sections in 95% ethanol two times for 10 seconds each.
   Repeat in 100% ethanol, incubating sections two times for 10 seconds each.
   Repeat in xylene, incubating sections two times for 10 seconds each.

## Antibody: Source and Usage

<table>
<thead>
<tr>
<th>Name</th>
<th>Company/Source</th>
<th>Dilution</th>
<th>Wash buffer &amp; AR method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaved Caspase 3 (Asp 175)</td>
<td>Cell Signaling (#9661), Rabbit, polyclonal</td>
<td>1:100 ~ 1:400</td>
<td>Citrate/PBST, heating plate or pressure cooker. May need 4hr 65 °C bake first.</td>
</tr>
<tr>
<td>Anti-phospho-Histone H3</td>
<td>Cell Signaling (#9701), Rabbit, polyclonal</td>
<td>1:50</td>
<td>Citrate/TBST, heating plate or pressure cooker</td>
</tr>
<tr>
<td>Phospho-Akt</td>
<td>Cell Signaling (#9277), Rabbit, polyclonal</td>
<td>1:50</td>
<td>Citrate/TBST, heating plate or pressure cooker</td>
</tr>
<tr>
<td>IRS2</td>
<td>Upstate (06-506), Rabbit, polyclonal</td>
<td>1:50</td>
<td>Citrate/TBST, Microwave: High power 9-10min/1L to boil followed by 15 min Mid-Low power to maintain slow boiling</td>
</tr>
<tr>
<td>ATF3</td>
<td>Santa Cruz (sc-188), Rabbit, polyclonal</td>
<td>1:1000 for RIP-ATF3; 1:50 for endogenous HFD induced. Atlas works better for TXP induced.</td>
<td>No antigen retrieval required for exogenous ATF3. For endogenous ATF3, use pressure cooker on TXP samples and Atlas AB. No retrieval required for HFD Pancreas detection, but must use Alkaline Phosphatase Blue and nuclear fast red development.</td>
</tr>
<tr>
<td>Pan-keratin</td>
<td>Cell Signaling (#4545) Mouse, monoclonal</td>
<td>1:500</td>
<td>Citrate/TBST, heating plate or pressure cooker</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>Cell Signaling (#2926), Mouse, monoclonal</td>
<td>1:50</td>
<td>Citrate/TBST, heating plate or pressure cooker</td>
</tr>
<tr>
<td>ATF3</td>
<td>Atlas (HPA001562), Rabbit, polyclonal</td>
<td>1:100</td>
<td>For endogenous ATF3 in transplanted islets, use pressure cooker and citrate buffer. Develop with DAB and hematoxylin counterstain.</td>
</tr>
<tr>
<td>p53</td>
<td>Cell Signaling (#2527), Rabbit monoclonal</td>
<td>1:40</td>
<td>Citrate/TBST, heating plate or pressure cooker</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>Cell Signaling (#4065), Rabbit polyclonal</td>
<td>1:50</td>
<td>Citrate/TBST, heating plate or pressure cooker</td>
</tr>
<tr>
<td>CD31</td>
<td>Abcam (ab28364), Rabbit polyclonal</td>
<td>1:50</td>
<td>Citrate/TBST, pressure cooker</td>
</tr>
<tr>
<td>F4/80</td>
<td>Invitrogen (MF48000), Rat monoclonal</td>
<td>1:40</td>
<td>Citrate/TBST, steamer</td>
</tr>
<tr>
<td>CD68</td>
<td>Dako (M0876), Mouse monoclonal</td>
<td>1:50</td>
<td>Citrate/TBST, pressure cooker</td>
</tr>
<tr>
<td>Ki67</td>
<td>Dako (M7249)</td>
<td>1:25-1:50</td>
<td>Citrate/TBST, pressure cooker</td>
</tr>
</tbody>
</table>
Appendix F:

A Method For Southern Blot Screening of Embryonic Stem Cell Clones Grown On A 96-well Plate
### Reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ES Lysis Buffer</td>
<td>10ml</td>
</tr>
<tr>
<td>0.5% Sarcosyl</td>
<td>500ul of 10%</td>
</tr>
<tr>
<td>300mM NaCl</td>
<td>600ul of 5M</td>
</tr>
<tr>
<td>10mM EDTA (ph 8.0)</td>
<td>200ul of 0.5M</td>
</tr>
<tr>
<td>10mM Tris.HCl (pH 8.0)</td>
<td>100ul of 1M</td>
</tr>
<tr>
<td>1mg/ml Proteinase K</td>
<td>1000ul of 10mg/ml</td>
</tr>
</tbody>
</table>

**Nucleic Acid Transfer Buffer (20x SSC)**

- 88.3g Tri-sodium citrate
- 175.32g NaCl
- 800ml diH2O
- ph to 7-8
- QS to 1000ml and Autoclave

**0.25M HCl**

- 10.4ml Concentrated HCl
- 490ml diH2O

**0.4N NaOH**

- 24ml 10N NaOH
- 576ml diH2O

**50x Denhardt’s Reagent**

- 5g Ficoll (Type 400)
- 5g Polyvinylpyrrolidone
- 5g BSA
- 500ml di H2O

**Pre-hybridization/Hybridization Buffer (Church Buffer)**

- 1 Liter
- 7% SDS
- 342mM Na$_2$HPO$_4$
- 158mM NaH$_2$PO$_4$
- 1mM EDTA
- di H2O
- 2ml of 0.5M
- QS to 1 L

**Wash Buffers**

- A. 2x SSC, 0.5% SDS
- B. 0.5x SSC, 0.5% SDS
Protocol

DNA Extraction from ES-Cells in 96-well Plate
1. Use duplicate 96well plates for DNA isolation. (2 wells worth of DNA for each clone)
2. Remove media with multi-chanel pipette or by aspiration.
3. Add 100ul PBS, remove.
4. Add 50ul lysis buffer.
5. Wrap the plate with wet paper towel then seal it in a plastic bag.
6. Incubate at 50-55 C overnight.
7. Add 100ul ice cold 100% EtOH. (100mM salt final concentration)
8. Leave at room temperature for 1hr.
9. Do Not Spin plate as this appears to loosen the DNA from the walls of the plate. Carefully pour off EtOH.
10. Wash 3x with 150ul 70% EtOH.
11. Gingerly pat on paper towel until very little liquid comes out, then leave uncovered at room temperature for 10-15minutes.
12. Dissolve in 25ul TE overnight at 50-55 C.
13. Digest the DNA in 40ul total volume overnight at 37 C. Use 5 units of enzyme per 1ug of DNA. Add an additional dose of enzyme the following morning and allow digest to continue 4 more hours.
14. Place the contents of one of the plates in to the corresponding wells of the duplicate plate to combine the DNA samples.
15. Place at 55 C for 1-2 hours. This allows evaporation to reduce the sample volume so that it fits into a single well on the gel.

DNA Electrophoresis and Transfer to Nylon Membrane
1. Load entire volume of digested DNA to 0.8% agarose gel.
2. Run gel at 60-80V for 12-19 hrs and take a picture with a ruler.
3. (Optional) Depurinate the DNA in the gel by rocking it in 0.25M HCl for exactly 10min.
4. Neutralize and Alkaline denature the gel in 0.4N NaOH 3x 15minutes each.
5. Equilibrate the gel in 20x SSC for 5minutes.
6. Set up the transfer-
   a. Fill a large dish with 20x SSC with a glass plate on top (or invert the gel pouring mold and place in the dish so the top is not submerged).
   b. Lay 2 pieces of wick filterpaper cut to the width of the gel across the platform so that both ends are submerged in 20x SSC. Wet the wick with 20x SSC and roll out any air bubbles.
   c. Place agarose gel face down on the wick and roll out air bubbles. Cover any exposed wick surface with saran wrap or transparency paper. The gel is upside down because it is the flatter side and will make a better contact.
surface with the membrane. Covering the exposed wick prevents a short
circuit in the transfer that will cause the experiment to fail.
d. Prepare membrane according to manufacturers instructions and place on
top of the gel. Smooth out air bubbles. Use Hybond N+ membrane
e. Cut 4 pieces of filter paper to the size of the gel. Wet the first with 20x
SSC and place it over the membrane. Leave the remaining 3 filter pieces
dry and place on top.
f. Place a stack of paper towels on top of the filter papers and apply a
weight.
g. Allow transfer to go overnight.
7. Take apart the transfer assembly being careful not to move the membrane.
8. Take off the gel and membrane together and flip. Use a pencil to mark the wells.
9. Cross Link membrane by baking at 80 °C for 2 hours.
10. Proceede to pre-hybridization or store membrane for later use.

Prehyb and Probe Hybridization
1. Set water bath to 65 °C and freshly prepare 50ml of pre-hybridization (Church)
buffer.
2. Incubate membrane with prehybridization solution for at least 2 hours.
3. Incubate membrane with hybridization solution over night.
   a. hybridization solution is pre-hybridization solution plus enough probe for
      10e6 to 10e7 specific activity per ml of solution. Manniantis suggests 10-
      20ng probe/ml solution with probe specific activity of 1e9.cpm/ug for
      Southern on 10ug genomic DNA.
4. Wash Membrane
   a. One room temperature rinse with wash buffer A.
   b. Two 30min washes at 65 C with was buffer B.
5. Wrap membrane in saran wrap and expose 2-3 days at -80 C.

Calculating the Specific Activity of the Probe
1. Calculate the stock concentration of radiolabled nucleotide.
   a. What is the specific activity of radionucleotide stock (Ci/mmol)?
   b. What is the concentration of radionucleotide stock (mCi/ml)?
      
      Equation: (concentration / specific activity) * 1Ci/1000mCi * 1000ml/L * 
      1000umol/mmol = uM radionucleotide

2. Calculate the moles of labeled nucleotide in your labeling reaction.
   a. What is the volume of radionucleotide in the labeling reaction (ul)?
      Equation: volume of radionucleotide (ul) * concentration of 
      radionucleotide (uM) * 1L/10e6ul * 1mol/10e6umol * 1e12 pmol/mol = 
      pmol nucleotide
3. Determine the efficiency of radiolabled nucleotide incorporation.
   a. Spin column method.
b. What was the final reaction volume before spin column (ul)?
c. What was the volume of reaction aliquoted before before spin column (ul)?
d. How many counts per minute came from pre spin aliquot?
e. What was final volume after spin column (ul)?
f. What was the volume of aliquot post spin (ul)?
g. How many counts per minute came from post spin aliquot?

Equation: \( \frac{\text{CPM per ul in (g) / volume in (f) * volume in (e)}}{\text{CPM per ul in (d) / volume in (c) * volume in (b)}} \times 100\% = \text{percent of radionucleotide incorporation} \)

4. Calculate the moles of labeled nucleotide incorporated into product DNA

\[ \text{Equation: } \frac{\text{percent incorporation/100\% * ul radionucleotide added * uM radiolabeled nucleotide * } 1L/10^6 \text{ ul * } 1\text{ mol/10e6umol * } 10e12 \text{ pmol/mol}}{\text{pmol nucleotide incorporated}} \]

5. Calculate the amount of DNA synthesized.

\[ \text{Equation = pmol nucleotide incorporated * 330g/mol * 4 equimolar amount of all 4 nucleotides * 1mol/10e12 pmol * 10e9ng/g = ng DNA synthesized} \]

6. Calculate the total amount of DNA.

\[ \text{Equation = ng template DNA + ng DNA synthesized} \]

7. Determine the counts per minute incorporated into product.

\[ \text{Equation = percent incorporation/100\% * vol radiolabeled nucleotide(ul) * concentrarion of radiolabeled nucleotide (mCi/ml) * 2.222 e6 dpm/uCi * } 1ml/1000ul * 1000uCi/mCi = \text{cpm incorporated}. \]

8. Calculate the specific activity of the probe.

\[ \text{Equation = cpm incorporated/ng total DNA * 1000ng/ug = specific activity (cpm/ug)} \]