Examining the Role of Jagn1 in Pancreatic β-Cells

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Physiology
University of Toronto

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ABSTRACT

Endoplasmic Reticulum (ER) stress and activation of the Unfolded Protein Response (UPR) has been implicated in causing pancreatic β-cell dysfunction leading to the development of Type II diabetes. Jagn1 was identified in a proteomic screen as an upregulated protein in response to ER stress caused by mutant proinsulin production. Jagn1 is an ER membrane protein with an unknown function in mammalian cells. This thesis examined the role of Jagn1 in pancreatic β-cells. Jagn1 mRNA was detected in insulinoma cell lines and rodent islets and its levels were increased in response to ER stress. Knock-down of Jagn1 in INS-1 832/13 insulinoma cells resulted in an increase in insulin secretion, proinsulin biosynthesis and ER content compared to control cells. Conversely, overexpression of myc-Jagn1 decreased insulin protein levels, whereas overexpression of mutant Jagn1 had no effect. Overall, these results suggest that Jagn1 may regulate insulin biosynthesis in pancreatic β-cells undergoing ER stress.
Acknowledgements

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<th>Full Form</th>
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<tr>
<td>4μ8c</td>
<td>4-methyl umbelliferone 8-carbaldehyde</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis initiation factor</td>
</tr>
<tr>
<td>ATF4</td>
<td>Activating transcription factor 4</td>
</tr>
<tr>
<td>ATF6</td>
<td>Activating transcription factor 6</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>Bax</td>
<td>Bcl-2 Associated X-Protein</td>
</tr>
<tr>
<td>Bak</td>
<td>Bcl-2 Homologous Antagonist/ Killer</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BiP</td>
<td>immunoglobulin heavy chain binding protein</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHOP</td>
<td>CAAT/enhancer binding protein (C/EBP) homologous protein</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>CReP</td>
<td>constitutive reverter of eIF2α phosphorylation</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>Dox</td>
<td>doxycycline</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDEM1</td>
<td>ER degradation enhancing a-mannosidase-like protein</td>
</tr>
<tr>
<td>eIF2α</td>
<td>eukaryotic initiation factor 2α</td>
</tr>
<tr>
<td>EIF2AK3</td>
<td>eukaryotic initiation factor 2α kinase 3</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ERAD</td>
<td>ER-associated degradation</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ERSE</td>
<td>ER stress response element</td>
</tr>
<tr>
<td>FFA</td>
<td>Free Fatty acids</td>
</tr>
</tbody>
</table>
GADD34  growth arrest and DNA damage-inducible gene 34
GFP    Green fluorescent protein
GRP78  glucose-regulated protein of 78-kDa
GRP94  glucose regulatory protein of 94-kDa
GSIS   Glucose Stimulated Insulin Secretion
HC-RT-PCR  High capacity RT-PCR
HEPES  4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
Herp   homocysteine inducible ER protein
HFD    high fat diet
HRD1   hypoxia responsive domain 1
HRP    horseradish peroxidise
IF     immunofluorescence
IRE1   Inositol-requiring enzyme 1
JNK    c-Jun N-terminal Kinase
K+     Potassium
KRBH   Krebs-Ringer Bicarbonate Buffer
kDa    kilodaltons
NF-Y   nuclear factor Y
MafA   V-Maf Avian Musculoaponeurotic
mRNA   messenger ribonucleic acid
MYTH   Membrane yeast two hybrid
NCD    Normal chow diet
NeuroD1 Neuronal differentiation 1
NuPage Novex® high performance polyacrylamide gel electrophoresis
PBS    Phosphate Buffered Saline
PERK   double-stranded RNA-activated protein kinase (PKR)-like ER kinase
PFA    paraformaldehyde
PDI    Protein disulfide isomerase
PDX-1  Pancreatic/Duodenal Homeobox-1
PMSF   phenylmethanesulfonylfluoride
PTB    polypyrimidine tract-binding protein
qPCR   qualitative polymerase chain reaction
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>RBP</td>
<td>RNA-binding protein</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RIDD</td>
<td>Regulated IRE1 dependent decay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RRP</td>
<td>Readily releasable pool</td>
</tr>
<tr>
<td>rRNA</td>
<td>ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SILAC</td>
<td>Stable isotope labeling by amino acids in cell culture</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
</tr>
<tr>
<td>SRP</td>
<td>Signal Recognition Particle</td>
</tr>
<tr>
<td>sXBP1</td>
<td>spliced X-box binding protein 1</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TFG</td>
<td>TRK-fused gene</td>
</tr>
<tr>
<td>Tg</td>
<td>Thapsigargin</td>
</tr>
<tr>
<td>Tm</td>
<td>Tunicamycin</td>
</tr>
<tr>
<td>TRB3</td>
<td>Tribbles homolog 3</td>
</tr>
<tr>
<td>UPRE</td>
<td>Unfolded protein response promoter element</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded Protein Response</td>
</tr>
<tr>
<td>UTR</td>
<td>Untranslated Region</td>
</tr>
<tr>
<td>VKOR</td>
<td>Vitamin K epoxide reductase</td>
</tr>
<tr>
<td>WFS1</td>
<td>Wolfram syndrome 1</td>
</tr>
<tr>
<td>WRS</td>
<td>Wolcott-Rallison Syndrome</td>
</tr>
<tr>
<td>XBP1</td>
<td>X-box binding protein 1</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION
1.1 Pancreatic β-Cells and Insulin Action

The endocrine portion of the pancreas consists of β-cells, α-cells, γ-cells, pancreatic polypeptide (PP) cells and Epsilon cells. Together, these cell types make up the islets of Langerhans and secrete hormones responsible for regulating nutrient metabolism\(^1\). Islets constitute approximately 1-2% of the total pancreas and the insulin-producing β-cells make up 60-80% of the total islet cells\(^2\). Pancreatic β-cells are specialized in insulin biosynthesis and secretion. Insulin is a protein hormone that is continuously secreted at basal levels. However, under stimulatory conditions such as following a nutrient load (“fed state”), insulin secretion is enhanced\(^2\). The central role of insulin is to tightly regulate circulating glucose levels within a very narrow range. It is able to do so by managing fuel storage within an individual\(^3\). Insulin acts on peripheral tissues, such as muscle cells and adipocytes to promote cellular glucose uptake, which signal to the cells to store glucose in the form of lipids and carbohydrates\(^4\). Insulin also suppresses hepatic glucose production, lipolysis and proteolysis. Both these inhibitory and excitatory effects of insulin occur simultaneously within these peripheral tissues\(^5\). Alternatively, in a “fasted state” in which circulating glucose levels are low, there would be lower levels of circulating insulin. As a result, peripheral glucose uptake would be reduced, while hepatic glucose production is enhanced in order to raise glucose concentrations within the normal range. Therefore, insulin plays an essential role in regulating glucose homeostasis and does so through the regulation of various metabolic actions\(^3\). However, the function of pancreatic β-cells and the ability of insulin to maintain glycemic control is perturbed in both type I and type II diabetes\(^6\,7\). Type I diabetes results from an autoimmune attack on the body’s pancreatic β-cells, whereas type II diabetes occurs as a result of genetic predisposition and environmental factors, such as over-nutrition and lack of exercise which over time, results in β-cell failure\(^6\).

1.2 The Secretory Pathway

Protein trafficking and secretion is an essential process within eukaryotic cells. Membrane proteins and proteins destined for secretion are trafficked along the secretory pathway that begins at the rough ER and continues with the Golgi complex. Cells involved in a high
degree of protein trafficking, such as pancreatic β-cells, have an abundance of these organelles\(^8,^9\).

Once a secretory protein is translated, an N-terminal signal sequence is first recognized by a signal recognition particle (SRP) within the cytoplasm. The SRP-ribosome-polypeptide complex migrates to the ER where it interacts with SRP receptors at the ER membrane. As a result, the SRP is released and translation continues simultaneous with translocation of the polypeptide being synthesized into the lumen of the ER\(^8,^9\). Once inside the ER, the signal peptide is cleaved and the protein begins to fold into its native conformation with the help of various enzymes and chaperone proteins\(^8\).

From the ER, properly folded proteins are then packaged into membrane-bound vesicles, known as COPII-coated vesicles, and are either transported to the cis-Golgi or fuse with each other to form an “intermediate compartment (IC).” It is within the IC where protein sorting occurs as ER-resident proteins are recognized by COPI coat proteins (discussed below). The vesicles uncoat following fusion with either the IC or the Golgi complex, allowing the COPII proteins to be recycled back to the ER to be reused\(^8-10\). As proteins migrate from the cis-Golgi to the trans-Golgi complex, via a process known as cisternal migration, necessary post-translational modifications are made (i.e. acetylation). It is within the trans-Golgi network where proteins are then sorted into either transport vesicles or secretory granules. Transport vesicles are targeted to the endosomal system or the plasma membrane, while secretory granules store their contents within the cell until they are stimulated for release\(^9\) (see figure 1.1).

1.2.1 Retrograde Transport

As proteins exit the ER via COPII vesicles, ER-resident proteins often escape into these vesicles and therefore, must be retrieved and returned. This reverse transport, from Golgi to the ER, is known as retrograde transport and is carried out by COPI-coated vesicles. ER-resident proteins are often recognized and retrieved at either the intermediate compartment or at the cis-Golgi cisternae. The ability to identify ER-localized proteins is by their C-terminal ER retention signals. ER luminal proteins, such as the molecular chaperone Grp78, have a KDEL signal sequence that interacts with the KDEL receptor at the IC or cis-Golgi in order to target it to
COPI vesicles. The KDEL receptor contains a COPI binding sequence, the dilysine motif (KKXX), that allows it to efficiently enter these vesicles. ER-localized membrane proteins also have a C-terminal dilysine motif (KKXX or KXKXX) that is recognized directly by the COPI coat\textsuperscript{10, 11} (Figure 1.1). Thus, ER-resident membrane proteins achieve steady-state localization within the ER due to efficient retrieval via COPI vesicles.

![Figure 1.1 The Secretory pathway and retrograde transport.](image)

This is a simplified schematic of vesicular transport from ER to Golgi and from Golgi to the plasma membrane (PM). Secretory or membrane proteins exit the ER and are transported to the intermediate compartment (IC) and Golgi complex via COPII-coated vesicles. Secretory granules containing secretory proteins are formed from the Trans Golgi Network and stored inside the cell prior to stimulation that results in exocytosis. ER-localized proteins containing either a KDEL or dilysine motif are recognized either at the IC or the cis Golgi and are transported back to the ER via COPI-coated vesicles. Adapted from Asp and Nilsson, 2008\textsuperscript{12}.

### 1.3 Insulin Biosynthesis and Secretion

Insulin is initially translated as a precursor protein, known as preproinsulin, which has an N-terminal signal peptide that interacts with a cytosolic signal recognition particle (SRP) to facilitate its translocation into the lumen of the endoplasmic reticulum (ER)\textsuperscript{13}. The signal sequence is then cleaved by signal peptidase, generating a proinsulin molecule. With the help of
various enzymes and chaperone proteins, proinsulin folds into its native conformation consisting of three disulfide bonds: two inter-chain bonds between the A and B chains and one intra-chain bond within the A chain\textsuperscript{13, 14}. The folded proinsulin molecule is then transported to the Golgi apparatus where it is packaged into secretory granules. It is within these granules that the proinsulin molecule is cleaved by prohormone convertases into mature insulin and C-peptide. Insulin-containing granules then await stimulation in order to release the insulin into the extracellular environment\textsuperscript{13}.

Glucose, as well as other nutrients such as amino acids and fatty acids, is capable of stimulating insulin secretion by \(\beta\)-cells, although glucose is the main nutrient that regulates metabolic homeostasis. Pancreatic \(\beta\)-cells sense high blood glucose levels by taking up glucose via GLUT-2 glucose transporters followed by phosphorylation by glucokinase\textsuperscript{15}. Glucose is then metabolized, thereby increasing the ATP/ADP ratio within the cell. This causes ATP-sensitive potassium (K\textsuperscript{+}) channels to close and allows potassium ions to accumulate within the cell, resulting in plasma membrane depolarization. Voltage-gated Ca\textsuperscript{2+} channels open in response to the plasma membrane depolarization, allowing calcium to rush into the cell. As a result, secretory granules (which store mature insulin) are able to fuse with the plasma membrane allowing for exocytosis of insulin\textsuperscript{16, 17} (Figure 1.2).
1.3.1 Biphasic Insulin Secretion and Glucose-Stimulated Insulin Biosynthesis

Glucose-stimulated insulin secretion occurs as a biphasic response. The initial phase, or the “first phase” of secretion, occurs within approximately 5-10 minutes after stimulation with glucose. The latter “second phase” of secretion originates from a secondary pool of secretory granules known as the reserve pool. Insulin secreted in the first phase occurs from a pool of secretory granules known as the readily releasable pool (RRP) which are docked granules present at the plasma membrane. Secretory granules in the reserve pool however, are transported to the plasma membrane in order to be available for secretion.

To replenish the insulin that is lost as a result of secretion, glucose also stimulates an increase in insulin transcription and to a larger degree, translation. Glucose is able to regulate the activity of several cis-acting transcription factors: Pdx-1, NeuroD1 and MafA, known to bind the promoter region of the insulin gene and enhance insulin gene transcription. Glucose also regulates insulin biosynthesis via translational mechanisms. In response to increased glucose concentrations, insulin mRNA stability is enhanced while its degradation is retarded. The mechanisms by which this occurs may include RNA-binding proteins, such as the polypyrimidine tract-binding protein (PTB), that interact with the 5’ or 3’ untranslated regions (UTR) of the insulin mRNA. This refers to long-term control of insulin translation. However, there are also short-term translational control mechanisms, in response to glucose stimulation. At least three of these short-term mechanisms have been identified: (1) increased transfer of insulin mRNA to cellular compartments containing ribosomes, (2) initiation of insulin mRNA transport from free ribosomes to membrane bound ribosomes and lastly (3) increased elongation of preproinsulin. Therefore, glucose is not only important for insulin secretion, but it also enhances and regulates insulin biosynthesis at multiple levels.
1.4 β-Cell Failure and Development of Type II Diabetes

Obesity has been implicated as a major risk factor for the development of type II diabetes (T2D) and the prevalence is continually increasing\textsuperscript{26}. Numerous studies have identified various factors which link obesity to the pathogenesis of diabetes. For example, some of these factors include increased circulating free fatty acids (FFA), pro-inflammatory cytokines, mitochondrial dysfunction, hyperglycemia, and insulin resistance\textsuperscript{26, 27}. Insulin resistance in peripheral tissues is a hallmark of T2D and each of these factors has been shown to contribute to its development. For example, FFA and its metabolites, as well as inflammatory cytokines can activate protein kinases that will phosphorylate insulin receptor substrates in an inhibitory manner, thus hindering insulin signaling\textsuperscript{27, 28}.

It is important to note that development of T2D only occurs when insulin resistance is concomitant with the loss of β-cell function\textsuperscript{29, 30}. During preceding stages of disease development, the pancreatic β-cells of an insulin-resistant individual respond to a nutrient overload by increasing insulin secretion. Pancreatic β-cells are able to do so by expanding their overall cell mass, enhance insulin biosynthesis and improve their sensitivity to nutrient-stimulated insulin secretion\textsuperscript{29}. Therefore, β-cells are able to compensate for the peripheral insulin resistance in order to allow for the individual to maintain normal blood glucose levels.

Glucose as well as free fatty acids (FFA) in the blood are key stimulants for increased β-cell mass. Possible sources of cells contributing to this increased mass include proliferation of existing β-cells or neogenesis from ductal cells\textsuperscript{29, 31}. There are also many factors that may contribute to enhanced β-cell function, such as upregulation of glucose metabolism\textsuperscript{32}. Therefore, this would result in an increase ATP production in the cell and subsequent increased insulin secretion. There is also an upregulation in insulin gene expression and biosynthesis\textsuperscript{29}. As a result, both of these factors are contributing to hyperinsulinemia seen during the compensation stage.

When the pancreatic β-cells can no longer maintain a compensatory response to peripheral insulin resistance, the individual begins to develop T2D. This is observed following the hyperinsulinemic/normoglycemic stage, in which blood glucose levels begin to rise above average as a result of β-cell dysfunction and loss of β-cell mass. The reason for this eventual loss of cellular function can be due to a variety of factors such as mitochondrial dysfunction,
oxidative stress, glucomlipotoxicity and ER stress\(^{29}\). All of these can be mediated by obesity/diet-induced factors mentioned above\(^{27}\).

1.4.1 Endoplasmic Reticulum Stress in Pancreatic $\beta$-Cells

Pancreatic $\beta$-cells undergo a high degree of protein trafficking as a result of insulin biosynthesis and secretion and therefore have a highly developed ER. The ER plays a key role in various cellular activities such as protein folding, post-translational modifications of secretory proteins and calcium storage. Disturbances in any of these activities can cause an accumulation of unfolded or misfolded proteins within the ER, causing ER stress and activation of the unfolded protein response (UPR)\(^{33}\). The UPR is activated in an attempt to alleviate the stress within the cell. However, under conditions of prolonged or chronic ER stress, apoptotic pathways are initiated\(^{33,\ 34}\). ER stress has been observed in T2D human islets\(^{35}\), in mouse models\(^{36}\) and in vitro studies\(^{37}\) and is believed to be a key factor that contributes to the development of T2D. Both genetic and environmental factors can generate stress within the ER and ultimately $\beta$-cell dysfunction\(^{26}\).

1.4.1.1 Mutations associated with ER stress and diabetes

Mutations in the insulin gene (INS) are the second most common causes of permanent neonatal diabetes, the first being mutations that affect the ATP-sensitive potassium channel. A study done by Edghill et al.\(^{38}\) identified 16 different heterozygous missense mutations in the INS gene out of 35 patients diagnosed with diabetes before six months of age. Among these 16 mutations, nine were identified to affect disulphide bond formation within the insulin protein, while others affected the orientation of the molecule to allow for proper alignment between the A and B chains. In either case, native protein folding is hindered, thereby causing misfolded proinsulin to accumulate in the ER, inducing ER stress and ultimately leading to pancreatic $\beta$-cell apoptosis. This manifested as hyperglycemia and ketodacidosis in these patients and is what led to their diagnosis. Some of the particular mutations that have been identified include H29D, R89C and C96Y missense mutations\(^{38,\ 39}\). The C96Y mutation is the same mutation that occurs in the Akita mouse\(^{40}\). The Akita mouse spontaneously develops diabetes, as a result of a loss of
pancreatic β-cell abundance. This mutation replaces a cysteine residue with a tyrosine residue in the A chain of the insulin protein and disrupts the formation of one of the interchain disulphide bonds. Considering its physiological relevance, the C96Y mutant insulin is used as a model of β-cell ER stress by the Volchuk lab (discussed in section 1.5).

Additional mutations have been identified to be associated with early onset diabetes, such as the EIF2AK3/PERK mutation observed in Wolcott-Rallison syndrome (WRS). WRS is an autosomal recessive disorder characterized by a reduction in pancreatic β-cells, neonatal diabetes, epiphyseal dysplasia, osteoporosis and growth retardation. The PERK mutation observed in WRS is a frameshift mutation which results in the formation of a truncated and non-functional protein. Therefore, when PERK is activated it is unable to phosphorylate EIF2α and block translation. Consequently, under glucose stimulation pancreatic β-cells will continue to make large amounts of insulin. However, the ER does not have the capacity to deal with such an imposition. As a result, misfolded proteins accumulate, oxidative stress ensues and cellular apoptosis occurs. A similar effect is seen in PERK−/− mice that develop severe hyperglycemia. These mice have reduced pancreatic β-cell mass due to enhanced apoptosis and initially have elevated levels of insulin. Inevitably, these mice develop diabetes, along with many other complications often seen in WRS.

Moreover, another gene that has been identified to play a role in T2D susceptibility is the gene encoding wolframin protein, wfs1. Two single nucleotide polymorphisms (SNPs) in the wfs1 gene have been identified and observed to be associated with T2D development. Wolframin is an ER membrane protein responsible for maintaining calcium homeostasis and loss of function mutations in this gene result in the development of Wolfram syndrome. Wolfram syndrome is characterized by diabetes insipidus, non-autoimmune diabetes mellitus, optic atrophy and deafness. Studies have shown that mice with a disrupted wfs1 gene have impaired insulin secretion and consequently develop glucose intolerance or diabetes. These mice also experience a loss in pancreatic β-cell mass, likely due to increased ER stress given the enhancement in phosphorylated PERK and XBP1 protein levels observed.
1.4.1.2 Glucolipotoxicity and ER stress in pancreatic β-cells

As previously discussed, pancreatic β-cell function deteriorates during development and even after diagnosis of type II diabetes. The cause of this dysfunction is likely the result of a combination of factors. However, a well studied area of research is the effect of glucolipotoxicity on β-cell function, which is believed to be a major contributor to this pathology and has been shown to induce ER stress\(^{49}\). Glucolipotoxicity refers to the deleterious effects of elevated levels of both glucose and fatty acids on pancreatic β-cell function and survival\(^{49}\). Some of these deleterious effects include inhibition of glucose-stimulated insulin secretion (GSIS), impaired insulin gene expression and apoptosis\(^{49,50}\). This has been observed in islets isolated from T2D individuals\(^{51}\) as well as in rat insulinoma β-cells (INS-1 832/13) when treated with high glucose and palmitate, which were also shown to have increased ROS production\(^{52}\). There are many ways in which high levels of glucose and fatty acids induce apoptosis, such as oxidative stress and inflammation. Both of which can contribute to ER stress-mediated cell death by way of depletion of ER calcium stores and JNK activation. Furthermore, NIT-1 cells, a mouse pancreatic β-cell line, treated with high glucose and palmitate for 72h underwent ER stress, as represented by increased levels of CHOP, Bip, phosphorylated eIF2α and caspase-3\(^{50}\). In addition, 72h exposure of palmitate to human islets and rat primary β-cells causes apoptosis as a result of depletion of calcium stores and consequent activation of the UPR\(^{53}\). Similarly, signs of ER stress can be detected in pancreatic islets from C57BL/6 mice on a HFD\(^{54}\). Therefore, prolonged exposure to high fat diet and nutrient loading are major contributors to the metabolic disorders observed in diet-induced obese individuals. Pancreatic β-cell function is significantly perturbed under these conditions as it contributes to the loss of the islet’s ability to maintain circulating glucose levels.

1.4.1.3 The Unfolded Protein Response (UPR)

As mentioned previously, when protein folding demand exceeds chaperone capacity or when the ER environment is perturbed such that protein folding is compromised, eukaryotic cells, including β-cells, activate the UPR. The UPR is mediated by three distinct signaling proteins: PERK, IRE1 and ATF6, each of which involves a signaling cascade in order to upregulate particular target genes (Fig. 1.3). Some of these genes encode chaperone proteins to improve the folding capacity within the ER, proteins involved in ER associated degradation
(ERAD) to remove and degrade terminally misfolded proteins from the ER and in conditions of irremediable stress, apoptotic genes\textsuperscript{55}. In addition to upregulating various target genes, the UPR also causes transient translational attenuation to temporarily reduce the protein folding burden on the ER\textsuperscript{56}. Therefore, the UPR allows the cell to both adapt to its increased protein folding demands, as well as to reduce further stress in the ER. Each of the pathways will be briefly discussed below.
1.4.1.3 PERK Signaling

When pancreatic β-cells are undergoing ER stress, one of the first ways in which the cell reacts to this stress is to transiently reduce overall protein translation. This response is carried out by the PERK pathway of the UPR. PKR-like ER kinase (PERK) is an ER transmembrane protein, which under normal physiological conditions binds the chaperone protein, BiP, by its luminal domain, which prevents its dimerization and activation. However, in the presence of unfolded proteins, BiP interacts with the hydrophobic regions of these proteins to prevent aggregation. As a result, this allows PERK to dimerize, autophosphorylate and activate its cytoplasmic kinase activity. The kinase domain then phosphorylates the eukaryotic translation initiation factor 2 alpha (eIF2α) in order to inhibit eIF2α activity and consequently reduce global protein translation and lower the protein folding load in the ER. EIF2 is involved in recruiting the first amino acid, methionine, to ribosomes in order to initiate translation. However, when the α subunit is phosphorylated on residue Ser51, EIF2 can no longer perform its role as a translation initiation factor. When bulk translation initiation is inhibited, the transcription factor ATF4 is selectively translated. When ATF4 is produced, it translocates to the nucleus, to induce transcription of multiple genes. A well known target of ATF4 is the gene encoding CHOP (C/EBP homologous protein), a transcription factor shown to be associated with increased cell death.

The enzymes involved in the dephosphorylation of PERK are not known. However, two enzymes have been identified to be involved in the dephosphorylation of eIF2α: GADD34 and CReP. Both GADD34 and CReP encode subunits of two independent phosphatases that are responsible for dephosphorylating eIF2α. GADD34 is induced by CHOP and is involved in a negative feedback loop in order to regulate eIF2α activity during ER stress, whereas CReP is...
constitutively expressed to help regulate eIF2α dephosphorylation at baseline levels. The dephosphorylation of eIF2α then allows for translation to resume\(^\text{58}\).

1.4.1.3.2 ATF6 Signalling

ATF6 is an ER-localized transmembrane protein that interacts with BiP through its luminal domain under normal cellular conditions. However, in the presence of unfolded proteins within the ER, BiP dissociates from ATF6 which allows ATF6 to translocate to the Golgi apparatus, where it is cleaved by site-1 and site-2 proteases\(^\text{56, 58}\). As a consequence of this cleavage, a 50 kDa cytosolic transcription factor is generated which migrates into the nucleus and binds the ER stress response element (ERSE) in the promoter of various target genes and upregulates their expression\(^\text{56, 58, 59}\). Some of these genes include ER chaperones such as Grp78 and Grp94, and components of the ERAD pathway such as Herp, HRD1 and EDEM1\(^\text{60}\). ATF6 has also been shown to induce the expression of XBP1 mRNA\(^\text{61}\). Two isoforms of ATF6 exist: ATF6α and ATF6β, and although there is a lot of redundancy among their respective target genes, it has been shown that ATF6α is the main inducer of these genes\(^\text{62, 63}\).

1.4.1.3.3 IRE1 Signalling

The third main arm of the UPR is the IRE1 (Inositol requiring enzyme 1) pathway. Mammalian cells express two isoforms of IRE1: IRE1α and IRE1β. The alpha isoform is ubiquitously expressed, whereas the beta isoform is only found in gut epithelial cells\(^\text{64}\). Like the PERK enzyme, IRE1 is a type I transmembrane protein and becomes activated when BiP (Grp78) dissociates from its luminal domain to interact with unfolded or misfolded proteins. However, it has also been shown that the luminal domain of IRE1 directly binds to unfolded proteins within the ER and leads to its activation\(^\text{65}\). In either case, IRE1 is activated following oligomerization and subsequent trans-autophosphorylation\(^\text{66}\). Activated IRE1 consists of functional kinase and endoribonuclease domains at its cytoplasmic C-terminal end. The endoribonuclease domain (RNase activity) cleaves an XBP1 mRNA transcript when activated. This splicing removes a specific 26 nucleotide intron, resulting in a frameshift that allows for efficient translation of a functional XBP1 transcription factor. XBP1 can then translocate to the
nucleus where it forms a heterodimer with the NF-Y protein. This complex interacts with ERSEs as well as unfolded protein response elements (UPREs) within the promoters of target genes to up-regulate their expression\textsuperscript{67}. Some of the target genes of XBPI include genes involved in ERAD (EDEM1, EDEM2, EDEM3 and Herp), genes encoding proteins that assist with protein folding (PDI), and proteins involved in ER-Golgi transport\textsuperscript{68-70}.

Chronic or severe ER stress conditions can lead to the over-activation of the RNase domain of IRE1. The endoribonuclease begins to non-specifically cleave various mRNA transcripts within its vicinity, which can include insulin mRNA and therefore, hinder insulin production and secretion. This promiscuous cleavage is known as regulated IRE1-dependent decay (RIDD) and is believed to act as the “switch” that renders the UPR from being protective to destructive, as it may be degrading pro-survival transcripts as well\textsuperscript{62}. Conversely, RIDD activity may also be cytoprotective in pancreatic β-cells as it has shown to be active at basal levels and can modulate various transcript levels to reduce the load on the ER\textsuperscript{71}. Therefore, IRE1 signaling is important in cells involved in high protein production and secretion as it attempts to alleviate the stress and re-establish homeostasis under resolvable stress conditions. However, it may stimulate cellular apoptosis under chronic stress conditions.

Studies have also shown a role for IRE1 in regulating the rate of proinsulin biosynthesis in response to elevated glucose levels. Phosphorylated IRE1 was shown to increase in the presence of high glucose concentrations, which was correlated with increased proinsulin biosynthesis and insulin secretion. Interestingly, this type of activation did not require BiP dissociation from its luminal domain, nor was XBPI splicing induced\textsuperscript{39}. However, in response to prolonged exposure to high glucose, cellular insulin content began to decrease as phosphorylated IRE1 continued to increase. Therefore, this is likely to be indicative of hyper-activation of IRE1 and as a result, there is degradation of insulin mRNA by RIDD activity \textsuperscript{39, 57}. It has also been shown that IRE1 signaling is important for expansion of the ER lumen, a function that is essential during differentiation of secretory cells\textsuperscript{63}. Mice deficient in IRE1 (\textit{Ire1}\textsubscript{α}−/−) are not viable, \textsuperscript{67, 69} highlighting the vital role IRE1 plays, particularly during development.
1.4.1.4 ER-Stress Induced Apoptosis

Under conditions in which the UPR is unable to alleviate or resolve ER stress conditions, the cell will then activate apoptotic pathways in order to remove the damaged cell. There are several apoptotic pathways activated in response to ER stress, including the activation of CHOP, c-JUN NH₂-terminal Kinase pathway and activation of caspase-12, among many others. Several recent reviews have discussed this complex process.\(^{37,67,72}\)

Numerous studies have shown that under ER stress conditions such as tunicamycin (an N-linked glycosylation inhibitor) and thapsigargin (a SERCA inhibitor) treatment, as well as under high glucose conditions, the expression of CHOP is markedly induced.\(^{72}\) The primary inducer of CHOP is the PERK/ATF4 pathway of the UPR, however, in order to reach maximal induction of CHOP activation of all three pathways is required. CHOP is known to be involved in the initiation of apoptosis, as studies have shown that CHOP\(^{−/−}\) mice, Akita mice with a disrupted CHOP gene as well as cells with impaired CHOP activity, are more resistant to apoptosis in response to ER stress.\(^{72}\) CHOP is a transcription factor normally present in the cytoplasm; however, when it is activated (via phosphorylation of its serine residues) it is translocated to the nucleus in order to regulate its target genes.\(^{72,73}\) There are a variety of ways in which CHOP is believed to initiate apoptosis. For example, CHOP has been shown to induce the expression of genes involved in ROS production or may initiate mitochondrial-mediated apoptosis by regulating Bax/Bcl-2 expression.\(^{72,75}\)

An additional apoptotic pathway that has shown to be activated under ER stress conditions is the c-Jun-N-terminal kinase pathway (JNK). Numerous studies, including one done by Cunha et al. have demonstrated an increase in JNK activity in pancreatic β-cells in response to ER stressors, such as lipotoxicity, thapsigargin and tunicamycin treatment.\(^{53,76}\) Downstream of JNK activation are the Bcl-2 family of proteins. JNK phosphorylates Bcl-2 and inhibits anti-apoptotic activity. Therefore, pro-apoptotic BH3-only proteins (Bax and Bak) are no longer sequestered. Active Bax and Bak then alter the permeability of the mitochondrial membrane and allow for the release of mitochondrial apoptotic factors, such as cytochrome c and apoptosis inducing factor (AIF). Cytoplasmic cytochrome c and AIF can then directly activate caspases and therefore ultimately resulting in apoptosis.\(^{75}\)
Another mechanism of apoptosis initiation is the activation of caspase-12 cascade. Caspase-12 has been shown to play a key role in mediating ER stress-induced apoptosis. Studies have shown that cells deficient in caspase-12 have reduced apoptosis initiated by ER stressors such as tunicamycin or thapsigargin. In the presence of ER stress, caspase-12 homodimerizes and is activated as a result of auto-cleavage. This initiates a cleavage cascade ending in active caspase-3. Caspase-3 is the major effector caspase that is responsible for various intracellular modifications such as, DNA fragmentation, as well cleaving key cellular proteins and as a result, leading to eventual cell death\textsuperscript{78, 79}.

1.5 An ER stress pancreatic β-cell model system

In order to study ER stress and the UPR in pancreatic β-cells, a physiologically relevant model of ER stress was developed in the Volchuk lab\textsuperscript{80}. In this model, a doxycycline-inducible mutant proinsulin (C96Y) EGFP-tagged construct was stably incorporated into a Tet-ON rat INS-1 pancreatic β-cell line. When these cells are treated with doxycycline (Dox) it induces the expression of the C96Y proinsulin gene. This mutant proinsulin has a missense mutation replacing a cysteine residue with a tyrosine residue in the A chain of the proinsulin peptide, which prevents the formation of one of the disulphide bonds within the insulin protein (same mutation as in the Akita mouse). This prevents the proper folding of proinsulin causing it to accumulate within the ER, induce ER stress and activate the UPR. Following mutant proinsulin expression in response to Dox treatment for 24h, activation of the UPR was confirmed via increased phosphorylation of eIF2α, as well as increased expression of the active form of ATF6 (p50) and spliced XBP1. Numerous ER chaperone, co-chaperone (Erdj4/Dnajb9, P58\textsuperscript{IPK}, ERDj3/Dnajb11) and ERAD genes (Herp and Sel1) were also induced following Dox treatment for 24h, as well as some pro-apoptotic genes (Chop and Trb3). However, the profile of genes upregulated after 48h of Dox treatment differed compared to that of the 24h response. The number of genes induced increased and apoptotic gene expression was significantly enhanced\textsuperscript{80}. This indicates that depending on the duration and magnitude of the ER stress, the profiles of genes that are induced differ. Therefore, this cell line is a good model system to further study the various pathways of the UPR and to analyze the various target genes induced under a physiologically relevant model of ER stress.
Using the inducible mutant proinsulin-expressing stable cell line, we identified various proteins whose levels increased in response to ER stress caused by mutant proinsulin production. A stable isotope labeling by amino acids in cell culture (SILAC) screen was performed following Dox treatment in this cell line. A number of proteins were increased, many of which were expected and whose functions are well known. This included components of the ERAD pathway (HRD1), ER chaperone proteins (i.e. PDI, DNAjc3, cyclophilin B) and proteins involved in vesicular traffic (i.e Rab1A and Rab8). However, some proteins were identified in the screen whose function was not well known, one of these was Jagunal (Jagn1).

1.6 Jagunal

The Jagunal gene and its encoded protein, Jagn1, were first identified in Drosophila and this remains the only study on this protein. Lee and Cooley analyzed the role of Jagunal in the oocyte of D. melanogaster, during a process known as vitellogenesis, or egg (oocyte) development. During this process the oocyte doubles in size and develops into its mature, maximum size. In this study various mutations were identified in the Jagunal gene that resulted in a small oocyte phenotype. Some of these mutations identified were lethal and one was shown to be a semilethal mutation (D16N). Homozygous mutants were not viable however, heterozygous mutants appeared normal during development until reaching the stages of vitellogenesis. In this study they looked at the effect of the Jagn mutant expression on ER distribution, protein transport and secretion. Their results demonstrated that Jagunal is required for ER reorganization or clustering at the oocyte subcortex during vitellogenesis and mutant oocytes did not experience any ER luminal swelling that was seen in wildtype oocytes. ER luminal swelling is normally indicative of a highly active ER. Additionally, oocytes expressing mutant Jagunal had reduced vesicular transport and protein secretion, thereby resulting in a small oocyte phenotype.

The rat Jagunal gene encodes an ER membrane protein consisting of 183 amino acids. The gene is present in the genomes of various eukaryotic organisms, including humans, mice, rats, zebrafish, mosquitos and C. elegans, but is not present in lower eukaryotes such as yeast. The primary sequence of Jagn1 contains a highly conserved N-terminal region, four predicted transmembrane domains and a dilysine motif (KKXXK) at its C-terminus (Fig. 1.4).
mentioned in the introduction section 1.2.1, a C-terminal dilysine motif is involved in maintaining ER localization of membrane proteins by mediating interaction of escaped proteins with COPI retrograde transport vesicles in the IC and cis-Golgi. The expression or role of Jagn1 in mammalian cells has not been studied.

![Diagram of Jagn1 Protein](image)

**Figure 1.4 Structure of the Jagn1 Protein.**

The Jagunal gene encodes for a protein that contains four transmembrane domains and a C-terminal dilysine motif known to be an ER-retention signal. Arrow indicates the N-terminal semi-lethal mutation site. This mutation is a missense mutation replacing an aspartic acid residue with an asparagine residue. Adapted from Lee and Cooley, 2007.

### 1.7 Rationale and Hypothesis

Pancreatic β-cells are particularly prone to ER stress. Glucose stimulation induces the biosynthesis and secretion of insulin, thereby imposing a large protein folding load on the ER that is physiological. Furthermore, in the context of obesity and a sedentary lifestyle, an even greater stress can be imposed on the β-cell as a result of development of insulin resistance and various factors associated with obesity. Under these patho-physiological conditions, the UPR is unable to restore ER homeostasis due to chronic stress conditions, which can lead to β-cell dysfunction and apoptosis and development of T2D. Thus, ER stress is a fundamental process in β-cell biology with clear implications in disease. Understanding how β-cells respond to ER stress is vital to potential therapeutic options to maintain β-cell survival and function. The ER stress response can be studied using pharmacological agents such as tunicamycin, thapsigargin or dithiothreitol (DTT) to induce ER stress, but each of these has various drawbacks. Our lab was interested in the various genes or proteins whose levels increased under conditions of a more physiologically relevant ER stress inducer, expression of a mutant proinsulin.
Jagn1 was a protein identified in a SILAC screen following expression and accumulation of the mutant proinsulin within the ER. This was particularly interesting considering very little is known about the function of Jagn1. One study had examined its role during oocyte development in *D. melanogaster*. Jagn1 is an ER membrane protein and was shown to play an important role in ER organization and protein transport and secretion in oocyte development. Therefore, I hypothesized that Jagn1 is expressed in pancreatic β-cells and plays an important role in ER function and that loss of Jagn1 protein may alter the β-cell’s ability to synthesize and secrete insulin. In order to test this hypothesis three main aims were addressed (1) determine the localization of Jag1 in pancreatic β-cells (and other mammalian cells) (2) examine the effect of Jagn1 knockdown on ER morphology, insulin biosynthesis and secretion and (3) to examine the effects of overexpression of wildtype and a non-functional Jagn1 in pancreatic β-cells.
CHAPTER 2: MATERIALS AND METHODS
2.1 Cell Culture

Rat INS-1 (Insulin 2 C96Y-EGFP) cells (clone #4S2, generated as described\textsuperscript{[80]} and INS-1 832/13 (obtained from Dr. Chris Newgard, Duke University, Durham, NC\textsuperscript{[85]}) insulinoma cell lines were maintained in RPMI 1640 media (11.1 mM glucose, 1 mM sodium pyruvate, and 10 mM HEPES) supplemented with 10% FBS, 2 mM L-glutamine, and 55 µM β-mercaptoethanol, containing antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) at 37°C and 5% CO\textsubscript{2}. HeLa and AD293 human cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (25 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate) supplemented with 10% FBS at 37°C and 5% CO\textsubscript{2}. Media was changed every 3-5 days and the cells were trypsinized once 70% confluency was reached. Mouse and rat tissue samples were isolated by Liling Zhang (lab technician). All animal procedures were approved and were performed in accordance to the Animal UseProtocols at the Toronto Centre for Phenogenomics.

2.2 Cell Treatment and Lysis

In preparation for cell lysis, cells were grown in either six- or twelve-well plates and treated as indicated in the figure legends. Following treatments, plates were placed on ice and washed once with cold phosphate buffered saline (PBS). While remaining on ice, cells were then incubated in lysis buffer consisting of 1% Triton X-100, 100 mM KCl, 20 mM HEPES and 2 mM EDTA (pH 7.3), with the addition of 0.5 mM PMSF and protease inhibitors (Roche). After 20 minutes of incubation, lysates were collected and centrifuged at 13, 200 rpm for 10 min at 4°C. The supernatant was collected and protein concentrations were measured using the bicinchoninic acid (BCA) protein assay (Pierce Inc.).

2.3 Western Blot Analysis

Cell lysates were prepared as described above and equal amounts of protein per condition was mixed with 2x SDS sample buffer supplemented with 10% β-Mercaptoethanol. Samples were heated at 95°C for 5 min, vortexed and centrifuged at 13, 200 rpm for 15 seconds. Proteins were resolved by either a 10% SDS-PAGE gel or 4-12% NuPage gel (Invitrogen), and then transferred onto a nitrocellulose membrane. Once transferred, membranes were then incubated in blocking buffer consisting of 3% Skim milk in wash buffer (0.05% Nonidet P-40, 0.05% Tween-20 in PBS) for one hour at room temperature, with shaking. Membranes were then incubated
overnight at 4°C, with shaking, in primary antibody solution. The next day, membranes were washed by incubating in wash buffer for 15 minutes, at room temperature with shaking (repeated 3 times). Subsequently, the membranes were then incubated in HRP-conjugated secondary antibody for one hour at room temperature, with shaking and washed again in wash buffer (3x, 15 minutes each). As part of the enhanced chemiluminescence detection kit (Amersham Bioscience), ECL reagent was then used for signal detection. Membranes were then exposed to photographic films for varying times depending on the antibody used. Films were then developed and band density was measured and quantified using ImageJ software.

The following primary antibodies were used:
Jagn1 (1:500, Abcam, no. 107830), Jagn1 (1:500, Santa Cruz, no. 102630), α-Myc /9E10 (1:1000, Sigma, no. M4439), GFP (1:1000, Clonetech, no. 632381), GM130 (1:500, BD Transduction Laboratories, no. G65120), γ-Tubulin (1:1000, Sigma, no. T6557), Insulin (1:100, Santa Cruz, no. 9168), PDI (1:4000, StressGen, SPA-891), Calnexin (1:1000, StressGen, no. SPA-860), Herp (1:1000, Biomol Internation, no. Pw9705), FLAG (1:1000, Sigma, no. F3165), KDEL (1:500, StessGen, SPA-827).

2.4 Short interfering RNA (siRNA)-mediated knock-down

To knock-down Jagn1 mRNA in INS-1 832/13 cells, reverse transfection was performed using lipofectamine RNAiMAX reagent (Invitrogen) and was performed according to the manufacturer’s instructions. Briefly, 12 pmol of either Jagn1 siRNA (Invitrogen) or Luciferase siRNA (Invitrogen) was diluted in 200 μl of Opti-MEM reduced serum in each well and incubated for 5 minutes at room temperature. After incubation, 2 μl of lipofectamine RNAiMAX reagent was added to each well and was incubated for 30 minutes at room temperature. During this time, cells were trypsinized, resuspended in RPMI media and counted to obtain a final concentration of 500,000 cells/ml. RPMI media (1 ml) was added to each well after the incubation period, to achieve a final concentration 10 nM siRNA. Plates were then incubated at 37°C and 5% CO₂ for 72 hours. Luciferase siRNA was used as a control. Three different Jagn1 siRNA’s were tested (#2, 3, 4). Jagn1 siRNA #3 was used for all experiments. Knock-down was confirmed by qPCR analysis using Jagn1 specific primers.
2.5 RNA Isolation

Following treatments, cells were washed once with PBS. 1ml of TRIzol reagent (Invitrogen) was then added to each well and incubated at room temperature for 5 minutes. After the incubation period lysates were collected into Eppendorf tubes and 200 µl of chloroform was added, mixed and kept at room temperature for 2 minutes. Samples were then centrifuged at \( \geq 10,400 \) rpm at 4°C for 15 minutes and RNA was isolated according to the RNeasy RNA isolation kit protocol provided by the manufacturer (Qiagen). RNA concentration was measured using a Nanodrop machine.

2.6 Reverse Transcription-PCR (RT-PCR)

To examine Jagn1 mRNA expression in rat and mouse cells and tissues, RT-PCR was performed using Jagn1 primers directed against sequences derived from the rat and mouse genomes. Following total RNA isolation, 500 ng of RNA was combined with reagents provided by the Qiagen OneStep RT-PCR kit and the following reaction conditions were used to carry out the polymerase chain reaction: 50°C (30 min); 95°C (15 min); 30 cycles of 94°C (30 s), 53°C (30 s), 72°C (1.5 min); 72°C (10 min). The cDNA products were then resolved on a 1.5% agarose gel and visualized using ethidium bromide staining.

Primer sequences used for rat samples (INS-1 832/13 cells and rat liver):

*Jagn1 Forward* 5’ aaa cto cag atg gcg tct cgg gca ggc cca 3’

*Jagn1 Reverse* 5’ aaa gtc gac tca ttt acg ttt ctt ctc ctg tgt gct gg 3’

Primer sequences used for mouse samples (tissue samples):

*Jagn1 Forward* 5’ gcg tct cgg gca ggc ccg cga 3’

*Jagn1 Reverse* 5’ ttt ccg ttt ctc ctg tgt gct gg 3’

2.7 Real time, Quantitative-PCR (qPCR) analysis

To quantify mRNA expression levels under various conditions, a qPCR was performed. First, a high capacity RT-PCR was done following RNA isolation using 1.95 µg of RNA from each sample and reagents from the HC-RT-PCR kit (Applied Biosystems). The reaction was performed under the following reaction conditions: 25°C (10 min); 37°C (120 min); 85°C (5
The cDNA products were then used for quantitative real-time PCR analysis using the TaqMan gene expression system (Applied Biosystems). Serial dilutions of control cDNA samples were used to generate a standard curve. Final reaction mixtures consisted of 10 µl of cDNA, 1.25 µl of TaqMan gene expression primers (20x), 1.25 µl of double-distilled water and 12.5 µl of TaqMan universal PCR master mix (2x). The reaction was then run on an ABI Prism 7900HT Sequence Detection system (Applied Biosystems) under the following conditions: 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C and 1 min at 60°C. The standard curve and corresponding values for each sample were determined with the SDS 2.1 software of the ABI Prism 7900HT instrument. Samples were run in duplicates and obtained values were normalized to the expression of β-actin mRNA and presented as a mean ± SE of at least three independent experiments.

The following gene-specific primers were used (Applied Biosystems):

- β-actin specific primers (rat β-actin, 435291E), Jagn1 (Rn01421134_m1), Ins2 (Rn01774648_g1)

### 2.8 Immunofluorescence Staining

Cells were grown in twelve-well plates on glass coverslips and treatments were applied as described in the figure legends. Following treatments, cells were washed twice with PBS, then fixed with 3% paraformaldehyde (PFA) diluted in PBS for 20 min at room temperature. Cells were then washed twice with PBS, followed by incubation with 100 mM glycine (in PBS) for 15 minutes at room temperature to remove any remaining PFA. Samples were washed again once with PBS and then permeabilized with 0.1% Triton X-100/0.05% BSA or 25 µg/ml digitonin in PBS for 15 min at room temperature. Cells were subsequently washed three times with PBS and incubated in blocking buffer consisting of 2% non-fat dry milk/2% BSA in PBS for 1 hour at room temperature. Primary antibody in blocking solution was then added and samples were incubated for one hour at room temperature. Subsequently, cells were then washed three times in PBS for 5 minutes each and then incubated with a secondary antibody in blocking solution for one hour in the dark. Following this incubation, cells were washed again in PBS, 3 times for 5 minutes each in the dark. Coverslips were then mounted onto glass slides using Fluoromount G mounting medium (Electron Microscopy Science, Inc., Hatfield, PA). Slides were left to dry
overnight in the dark before imaging with either confocal or epifluorescence microscopy. For GFP detection, the protocol was stopped after fixation with 3% PFA, washed three times with PBS and mounted onto glass slides. Following microscopy imaging, images were pseudo-coloured for presentation using ImageJ software.

The following primary antibodies were used:
α-myc/9E10 (1:1000, Sigma, no. M4439), α-myc (1:400, polyclonal, Cell signaling, no. 2272S), TFG (1:1000, obtained from Dr. Anjon Audhya, University of Wisconsin, Madison, Wisconsin), COPI/CM1A10 (1:500, obtained from Dr. J. Rothman, Yale University, New Haven, CT86), Insulin (1:300, Santa Cruz, no. 9168).

The following secondary antibodies were used:
Oregon Green 488 (1:1000, life technologies, no.011038 and no. 011033), Alexa Fluor 647 (1:1000, Obtained from Dr. C. Yip, University of Toronto)

2.9 Glucose stimulated insulin secretion (GSIS) and Rat Insulin Radioimmunoassay (RIA)

To measure insulin secretion levels in response to glucose stimulation, INS-1 832/13 cells were first seeded into a 12-well plate (500 000 cells/well) and transfected with control (Luc) or Jagn1 siRNA as described above. The cells were then washed twice in Krebs-Ringer Bicarbonate Buffer (KRBH) consisting of 128.8 mM NaCl, 4.8 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2.5 mM CaCl2, 5 mM NaHCO3, 10 mM HEPES, pH 7.4 and supplemented with 0.1% BSA. The cells were then incubated in 1 ml/well KRBH/0.1% BSA for one hour at 37°C and 5% CO2. Subsequently, the cells were incubated in KRBH/0.1% BSA containing 2.8 mM or 16.7 mM glucose for 1 hour, again at 37°C and 5% CO2. Once the incubation period was complete, the plates were placed on ice and 700 µl of media was collected into eppendorf tubes, centrifuged for 5 minutes at 5, 300 rpm at 4°C. The supernatant was collected (500 µl) and stored at -80°C. The remaining cells were then lysed as described in section 2.2. To measure the amount of secreted insulin, a rat insulin RIA was used (Linco Research Inc.) according to the manufacturer’s instructions. Secreted insulin was expressed as the amount of insulin released (nanograms) per milligram of total protein content in each condition. Assays were performed in duplicates for each condition and four independent experiments were analyzed.
Insulin content was also measured using the rat insulin RIA. However, lysates were first diluted $10^4$ in KRBH media.

2.10 Insulin Pulse-Chase Assay and Immunoprecipitation

INS-1 832/13 cells were seeded in two 12-well plates with 500,000 cells/well and transfected with control (Luc) or Jagn1 siRNA as described above. Cells were then washed once with PBS and incubated in 0.5 ml of labeling media consisting of $^{35}$S-Meth/Cys (100 $\mu$Ci/0.5 ml, Perkin Elmer, neg022T001MC) in Methionine/Cysteine-free DMEM media at 37°C/5% CO$_2$ for 20 minutes. Following incubation, Plate #1 was placed on ice while plate #2 was washed once with 1x PBS and incubated with regular (cold) DMEM, serum-free media for 30 minutes. Following this incubation, plate #1 and #2 were washed once with PBS and lysed. Protein concentrations were determined and immunoprecipitation was performed, in which equal amounts of protein from each treatment were incubated with 5 $\mu$g of Insulin antibody (Santa Cruz), overnight at 4°C with rotation. The next day, samples were incubated with Protein A Dynabeads (25 $\mu$l, Invitrogen) for three hours at 4°C, with rotation. Samples were then placed on a magnetic stand for 1 minute, supernatant was removed and pellets were washed three times with 500 $\mu$l of lysis buffer (without PMSF) at 4°C, with rotation for 5 minutes each. Pellets were then washed again with 500 $\mu$l of PBS supplemented with 0.1% Triton X-100 for 2 minutes (4°C, with rotation). Samples were then placed on a magnetic stand, SN was discarded and 20 $\mu$l of 2x NuPage sample buffer (supplemented with 10% $\beta$-mercaptoethanol) was added to each sample. Tubes were vortexed, heated at 95°C for 5 minutes, vortexed again, then centrifuged and placed on the magnetic stand. The supernatant was then collected and run on a NuPage gel. The gel was then immersed in Commassie blue solution for 15-30 minutes with shaking at room temperature. Following staining, the gel was washed with distilled water and incubated in Commassie de-staining solution for one hour, with shaking at room temperature and then left overnight at 4°C (without shaking). The next day de-staining solution was removed and the gel was washed with distilled water and immersed in distilled water supplemented with 10% glycerol for 15 minutes with shaking, at room temperature. The gel was then dried and dehydrated at 52°C for 3h. Once the gel was dried it was exposed to a Phosphor screen (Amersham Biosciences) for approximately 5 days and then imaged using a Phosphor imager (Storm 840, Molecular Dynamics). Three independent experiments were performed. Bands were
then quantified using ImageQuant software and expressed as a fold change relative to the control (Luc siRNA, t = 0).

The immunoprecipitation experiments described in section 3.10 were done in order to test for Jagn1-VKOR interaction and were performed as described above. However, AD293 cells were initially co-transfected with myc-Jagn1 and FLAG-VKOR and once samples were obtained after the final wash and mixed with sample buffer, proteins were resolved by western blot analysis.

2.11 Electron Microscopy

INS-1 832/13 cells were seeded in a 12-well plate (500 000 cells/well) in duplicates and transfected with Luc or Jagn1 siRNA for 72h. Following this treatment, cells were washed twice with PBS, collected into eppendorf tubes and centrifuged. The supernatant was discarded and the pellet was fixed with 2% glutaraldehyde for 30 minutes at room temperature. The samples were prepared for transmission electron microscopy (TEM) by the electron microscopy facility at Mount Sinai Hospital (Toronto). Briefly, pellets were rinsed in 0.1 M sodium cacodylate buffer (pH 7.3) for 2 h, rinsed with the same buffer for 10 minutes and post-fixed for 1.5 hours in 1% OsO4. The pellet was then washed with the same buffer for 10 minutes and dehydrated through a graded ethanol series up to 100% ethanol. Subsequently, cells were embedded in Spurr resin in an oven at 65°C overnight. Thin sections (~100 nm) were cut on an RMC MT6000 ultramicrotome. The sections were placed on copper grids and stained with uranyl acetate for 20 minutes and lead citrate for 10 min. The grids were examined in a FEI Tecnai 20 transmission electron microscope and images were captured on a Gatan Dualview digital camera. ER luminal area and cytoplasmic area was then measured in each image using ImageJ software and mean ER luminal space was normalized to overall cytoplasmic area.

2.12 XBP-1 Splicing Assay

Total RNA was first isolated from samples as described previously, and then an RT-PCR was performed to amplify XBP-1 cDNA using the QIAGEN Onestep RT-PCR kit (no. 210212). The RT-PCR was conducted using primers that flank the intron excised by IRE1 exonuclease activity as described previously. The reaction was carried out under the following conditions: 50 °C (30 min); 95°C (15 min); 30 cycles of 94°C (1 min), 62°C (1 min), 72 °C (1 min); 72 °C (10 min).
min). The PCR products were then resolved on a 3% agarose gel and imaged using ethidium bromide staining.

*XBP-1 forward primer: 5’ aac cag agt agc agc aca gac tgc 3’
*XBP-1 reverse primer: 5’ tcc ttc tgg gta gac ctc tgg gag 3’

2.13 Cloning and Generation of Myc- and GFP-tagged Jagn1 constructs

To generate an N-terminal myc-tagged Jagn1 construct, an RT-PCR was first performed using total RNA isolated from INS-1 832/13 cells and Jagn1 primers containing 5’ *PstI* and 3’ *SalI* restriction sites. The cDNA was then run on a 1.5% agarose gel (visualized with ethidium bromide) and extracted using the Qiagen gel extraction kit according to the manufacturer’s instructions. The extracted DNA, as well as the pCMV-Tag3B (Agilent Technologies, no. 211173) plasmid was then incubated with *PstI* and *SalI* restriction enzymes at 37°C for 2 hours. Following this incubation, the plasmid was treated with 1 µl of CIP at 37°C for 30 minutes. Both the Jagn1 insert and the plasmid products were then run on an agarose gel and extracted using the Qiagen gel extraction kit. The insert and plasmid were then ligated together by adding 10 µl of ligase, 8 µl of the Jagn1 insert and 2 µl of the pCMV-Tag3B plasmid (total 20 µl) and incubated at 16°C for 30 minutes. The same incubation period was done for the negative control sample consisting of 8 µl of distilled water and 2 µl of the plasmid. To amplify the plasmids, transformation into DH5α bacteria was performed. Briefly, the entire plasmid product (20 µl) was added to 50 µl of DH5α and incubated on ice for 30 minutes, heat shocked at 45°C for 45 seconds and then placed back on ice for 5 minutes. S.O.C media (900 µl, Invitrogen) was then added to each sample and incubated at 37°C for one hour with shaking. The bacteria were then grown on kanamycin plates (50 µg/ml) to select for the transformed DH5α, given that the plasmids contain a kanamycin resistance gene. The plates were then kept overnight in incubation at 37°C. The following morning, plates were placed at 4°C, until later that afternoon at which point 5-6 colonies were picked and grown in 2.5 ml of LB media supplemented with 50 µg/ml kanamycin and incubated overnight at 37°C, with shaking. The next day plasmids were isolated from the bacteria using the miniprep DNA purification kit (Promega). DNA concentration was measured using the Nanodrop machine and was stored at -20°C. The same protocol was used for generating the GFP-Jagn1 construct. However, a pEGFP-c1 vector (BD Biosciences, no. 076-1)
was used. Plasmids were sequenced (AGCT Corp., Toronto, ON) to confirm the presence of a Jagn1 insert and that the insert was in frame. A FLAG-VKOR construct was also generated, however, cloning was performed by Liling Zhang (Lab Technician).

**Primers used for cloning into pCMV-Tag3b:**

*Jagn1 Forward* 5’ aaa ctg cag atg gcg tct cgg gca ggc cca 3’

*Jagn1 Reverse* 5’ aaa gtc gac tca ttt acg ttt ctt ctc tgt gct gg 3’

**Primers used for cloning into pEGFP-c1:**

*Jagn1 Forward* 5’ aaa aga tct atg gcg tct cgg gca ggc 3’

*Jagn1 Reverse* 5’ aaa gtc gac tca ttt acg ttt ctt ctc tgt gct gg 3’

**2.14 Site-directed mutagenesis**

Using the myc-Jagn1 (in pCMV-Tag3B plasmid) a missense mutation (aspartic acid replaced with an asparagine residue at position 16, D16N) was introduced into the Jagn1 sequence using the QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene). In order to introduce this mutation the following primers were used: *Forward:* 5’ ggc acc gac ggc agc aac ttt cag cac cgg g 3’; *Reverse:* 5’ c ccg gtg ctg aaa gtt gct gcc gtc ggt gcc 3’. Amplification was performed according to the manufacturer’s instructions. Briefly, the following reaction conditions were used: 95°C for 30 sec; 18 cycles of 95°C for 30 sec, 55°C for 1 min, 68°C for 4 min. Samples were then incubated on ice for 2 min. After cooling, samples were digested with *DpnI* enzyme for 1 hour at 37°C and the plasmid was then transformed into XL10-Gold Ultracompetent cells (Stratagene). Successful mutagenesis was then confirmed by sequencing analysis (AGCTcorp.).

**2.15 Transient Transfection**

INS-1 832/13, HeLa and AD293 cells were transiently transfected with N-terminal tagged Jagn1 constructs for immunofluorescence imaging, immunoprecipitation and overexpression experiments. First cells were seeded in a 12-well plate (500 000 cells/well) and incubated overnight at 37°C and 5% CO₂ to allow the cells to recover. The following day 4 μl of Lipofectamine reagent and 2 μg of DNA were each diluted in 100 μl of Opti-MEM medium (Invitrogen) separately and incubated at room temperature for 5 minutes. The Opti-MEM/Lipofectamine and Opti-Mem/DNA solutions were then combined and incubated at room
temperature for 20 minutes. Subsequently, the Lipofectamine-DNA complexes (200 µl) were added to the media and cells were incubated with the DNA at 37°C and 5% CO₂ for 24-48h.

2.16 Cell death detection assay (ELISA)

For the data presented in figure A1.2 in the appendix, the cell death detection kit ELISA plus (Roche Diagnostics) was used to measure the effect of IRE1 inhibition on ER stress-mediated apoptosis. C96Y cells were seeded in a 12-well plate (500 000 cells/well) and treated as described in the figure legend. Treatments were done in duplicates. Following treatment conditions, cells were lysed and oligonucleosomes in the cytoplasm (indicative of apoptosis-associated DNA degradation) were quantified according to the manufacturer’s instructions. Results were normalized to the control (untreated C96Y cells). Five independent experiments were performed.

2.17 Data Analysis

Results are presented as mean ± SE. Statistical significance between two experimental conditions was analyzed using a two-sample t-test assuming equal variance. p ≤ 0.05 was considered statistically significant.
CHAPTER 3: ANALYSIS OF JAGN1 EXPRESSION IN PANCREATIC β-CELLS AND ISLETS AND ITS ROLE IN INSULIN BIOSYNTHESIS AND SECRETION

(Tissue and islet isolation was performed by Liling Zhang, Confocal imaging was performed by Pamuditha Silva, ATF6 overexpression samples were prepared by Tanya Odisho and TEM imaging was performed by the Mount Sinai electron microscopy facility)
3.1 Introduction

The ER stress response is a key component in maintaining cellular homeostasis. However, numerous studies over the years have shown that under chronic ER stress, the UPR is likely contributing to pancreatic β-cell dysfunction and apoptosis, leading to the pathogenesis of diabetes. UPR activation results in a complex interplay of gene induction and repression to create an environment to allow for the cell to return to homeostasis. Understanding how the pancreatic β-cell responds to ER stress is important in finding strategies to maintain β-cell function and prevent apoptosis.

Jagn1 was identified in a SILAC screen performed following mutant proinsulin-induced ER stress in pancreatic β-cells. Jagn1 has never been studied in mammalian cells. Based on findings observed in D. melanogaster, I hypothesized that Jagn1 would play a role in insulin biosynthesis and secretion in pancreatic β-cells. This chapter describes the results of various experiments performed in an attempt to decipher the role Jagn1 plays in β-cells.

3.2 Results

3.2.1 Jagn1 is expressed in pancreatic β-cells and islets and is induced under ER stress conditions

A SILAC proteomic analysis comparing protein expression in control cells versus cells expressing mutant proinsulin (C96Y)-tagged with GFP identified Jagn1 as being increased in the latter condition (results not shown). Therefore, my first aim was to confirm that Jagn1 was in fact expressed in pancreatic β-cells (rat INS-1 832/13 insulinoma β-cell lines as well as in primary mouse islets) and that it is induced in response to ER stress. To do this, an RT-PCR was performed using total RNA isolated from INS-1 832/13 cells, as well as from primary mouse islets with Jagn1 specific primers. As shown in Fig. 3.1, Jagn1 mRNA is expressed in both the cell line and mouse islets. Jagn1 mRNA expression was also detected in rat as well as several other mouse tissues and cells including brain, islet, liver, adipose, muscle and dendritic cells. It is important to note however, that Jagn1 is highly expressed in mouse islets considering the amount of total islet RNA used in the PCR reaction was less (Fig. 3.1C, lower panel).
To determine the effect of ER stress on Jagn1 mRNA expression, total RNA was also isolated from INS-1 832/13 cells treated with the pharmacological ER stressors: tunicamycin and thapsigargin. There was no indication of enhanced Jagn1 expression at the mRNA level following ER stress induction by RT-PCR (Fig. 3.1A, B). However, by quantitative PCR, increased expression of Jagn1 mRNA was observed in INS-1 832/13 cells, as well as mouse islets treated with tunicamycin (Fig. 3.2). Similar results were also seen with the more physiologically relevant diet-induced ER stress. Although not statistically significant, mouse islets isolated from mice fed a 45% high fat diet for 12 weeks showed a trend towards increased Jagn1 mRNA expression (Fig. 3.2C). Overall, these results show that Jagn1 mRNA is expressed in insulinoma cell lines and rodent islets and that mRNA is increased by pharmacological and potentially physiological ER stress.

Figure 3.1 Jagn1 is expressed in INS-1 832/13 insulinoma cells, primary mouse islets and in various mouse tissues.

An RT-PCR was performed using Jagn1 specific primers for the following samples: (A) Total RNA was isolated from rat INS-1 832/13 cells treated with 2 µg/ml Tunicamycin (Tm) for 16 hours or 1 µM thapsigargin (Tg) for 6 hours to induce ER stress or from rat liver tissue. RT-PCR was also done using INS-1 832/13 total RNA but lacking one of the primers ((−) cont) (B) Independent experiment performed as in A. (C) Total RNA was isolated from the following mouse tissues: brain, islets, liver, adipose, muscle, dendritic cells (DC) and DC treated with lipopolysaccharides (LPS). Lower panel represents rRNA within these tissues, detected by ethidium bromide, as a measure of RNA abundance and integrity (1 µg loaded for all samples except 100 ng of islet RNA was loaded).
3.2.2 Analysis of UPR pathways that may regulate Jagn1 expression

As previously stated and shown in figure 1.3, the UPR consists of three main arms: PERK, IRE1 and ATF6, each of which is activated under ER stress conditions. There is a lot of redundancy in terms of the specific target genes that are regulated by each pathway. However, a particular UPR pathway may be the major regulator of a particular target gene. Experiments were performed to determine which pathway of the UPR may be regulating the expression of Jagn1 under ER stress conditions. First, the contribution of the IRE1 pathway was assessed. This was
done using an inhibitor of IRE1 activity, 4µ8c (4-methyl umbelliferone 8-carbaldehyde)\textsuperscript{88}. This inhibitor is a non-competitive inhibitor of the endoribonuclease domain of IRE1. It forms a stable Schiff base with a lysine side chain within a hydrophobic pocket of this RNase domain and inhibits its flexibility and therefore, functionality\textsuperscript{88}. As a result, XBP1 mRNA is not cleaved and a functional XBP1 transcription factor cannot be made. Therefore, XBP1 cannot act to induce its target genes. After treating INS-1 832/13 cells with Tm for 16h in order to induce ER stress, Jagn1 mRNA levels increased as previously shown (Fig. 3.3A). However, when cells were treated with Tm in combination with the IRE1 inhibitor (4µ8c), Jagn1 levels were surprisingly further increased compared to Tm-treated cells only. Jagn1 expression was not affected by 4µ8c alone, indicating that 4µ8c has no effect on Jagn1 mRNA levels in non-stressed cells (Fig. 3.3A). At the concentration of the inhibitor used, IRE1 endoribonuclease activity, as measured by its ability to splice XBP1 mRNA, is completely prevented (Results not shown)\textsuperscript{89}.

These results were further verified when using an alternative IRE1 inhibitor, MKC-3946, which inhibits IRE1 endoribonuclease activity in a similar manner to 4u8c\textsuperscript{90}. Jagn1 expression was enhanced when INS-1 832/13 cells were treated with Tm and MKC-3946 as well as in C96Y cells treated with Dox and MKC-3946, in comparison to Tm and Dox-only treated cells (Fig. 3.3B, C). MKC-3946 alone had no effect on Jagn1 expression. As will be discussed later, the increased Jagn1 mRNA expression observed, when inhibiting IRE1 endoribonuclease activity, is likely due to the fact that IRE1 RIDD activity is also blocked.

The ability of ATF6α and/or ATF6β to regulate Jagn1 expression was also assessed. An adenovirus expressing either the active form of ATF6α or ATF6β (samples obtained from Tanya Odisho) was used to test the effect of active ATF6 overexpression on Jagn1 mRNA levels. Overexpression of ATF6α had no effect on Jagn1 expression compared to control cells. However, ATF6β overexpression in INS-1 832/13 cells caused a significant decrease in Jagn1 mRNA (Fig. 3.3D).
3.2.3 Analysis of commercial antibodies to detect Jagn1 protein

In an attempt to detect Jagn1 at the protein level, two different commercial antibodies were obtained. Lysates were made from INS-1 832/13 and HeLa cells. Samples were resolved by
SDS-Page and immuno-blotted with a Jagn1 antibody obtained from Abcam. However, no signal around the expected molecular weight of 20 kDa was observed (Fig. 3.4A Lanes 1, 3, 5, 7). I then attempted to detect overexpressed Jagn1. In order to do so, N-terminal myc-tagged and GFP-tagged Jagn1 constructs were generated as described in the Materials and Methods. After transfecting INS-1 832/13 and HeLa cells with the myc-Jagn1 plasmid for 24h, immunoblot analysis was performed using the Abcam Jagn1 antibody. Again, there was no signal at the expected molecular weight (20kDa) (Fig. 3.4A Lanes 2, 4, 6, 8). To confirm the presence of the overexpressed version of Jagn1, the same membrane was reused to detect myc-Jagn1 using a myc-specific antibody. Myc-Jagn1 was detected in the transfected cells, confirming the inability of the Jagn1 antibody to detect neither the endogenous nor overexpressed Jagn1 (Fig. 3.4C Lanes 2, 4, 6, 8).

Another antibody against Jagn1 from Santa Cruz Biotechnology Inc. was also tested. Initially, it seemed there may have been a signal at 20 kDa detecting endogenously expressed Jagn1 (Fig. 3.4B Lane 1). However, in the samples containing overexpressed Jagn1 (Fig. 3.4B Lanes 2 and 4) the signal at ~20 kDa decreased compared to non-transfected cells and therefore, is unlikely to be Jagn1.

These same antibodies were tested again using lysates generated from AD293 cells transfected with either myc-Jagn1 or GFP-Jagn1 vectors for 24h. This cell line was chosen because better transfection efficiency was obtained using these cells. To confirm GFP-Jagn1 was expressed following transfection, a GFP-specific antibody was used in a western blot analysis. The GFP-Jagn1 was detected at its expected molecular weight of ~48 kDa (Fig. 3.4D). Unfortunately, both of these antibodies were again proven to be ineffective in detecting overexpressed Jagn1 as can be seen in Fig. 3.4E, F, given there is no signal detected at ~20kDa. Therefore, these commercial antibodies are not useful for Jagn1 protein detection.
Figure 3.4 Analysis of Jagn1 antibodies

Commercial antibodies specific to Jagn1 were obtained and tested for their ability to detect Jagn1 protein. (A) Total cell lysates (10 μg and 20 μg of protein) from INS-1 832/13 and HeLa cells either untreated (lanes 1, 3, 5, 7) or transfected with myc-Jagn1 for 24h (lanes 2, 4, 6, 8). Jagn1 specific antibody obtained from Abcam was used. (B) Total cell lysates (15 μg of protein) from INS-1 832/13 and HeLa cell lines either untreated (lanes 1 and 3) or transfected with myc-Jagn1 for 24h (lanes 2 and 4). Jagn1 specific antibody obtained from Santa Cruz was used. (C) Myc-specific antibody was used on the same gel as in A to confirm transfection of overexpressed myc-Jagn1. (D) AD293 cell lysates (10μg of protein) were used and were either untreated (lane 1) or transfected with GFP-Jagn1 (lane 2) or myc-Jagn1 (lane 3). GFP- specific antibody was used to confirm GFP-Jagn1 overexpression. (E, F) Same treatments as in D, however, endogenous Jagn1 nor overexpressed Jagn1 could be detected by commercial Jagn1 antibody obtained from Abcam (E) nor from Santa Cruz (F).
3.2.4 Jagn1 is localized to the ER, but likely cycles between the ER and Golgi

As mentioned, an N-terminal myc-tagged Jagn1 construct was generated. This construct was used to determine the localization of Jagn1, considering there is no antibody available to detect the endogenous protein. After transfecting INS-1 832/13 cells with the myc-Jagn1 construct for 24 h, cells were fixed, permeabilized with Triton X-100 and immunofluorescence (IF) was performed using an anti-myc antibody. Following IF staining, cells were imaged with epifluorescence microscopy, which revealed a punctate expression pattern throughout the cytoplasm, but was excluded from the nucleus (Fig. 3.5A). Jagn1 has four transmembrane domains therefore its N-terminus can be located on the cytoplasmic or luminal side of the ER, although previous work on the Drosophila protein suggests the N-terminus is in the cytoplasm. To confirm its orientation, digitonin was used to permeabilize the cells. Triton X-100 is a detergent known to permeabilize both intracellular and extracellular membranes, while digitonin permeabilizes only the plasma membrane, leaving intracellular membranes intact. As a result, if fluorescence is still observed following digitonin permeabilization and IF staining for anti-myc, then this would suggest that the N-terminus is located on the cytoplasmic side of the ER. As can be seen in Fig. 3.5B, fluorescence was observed with digitonin permeabilization, thereby confirming N-terminal cytoplasmic localization.

It is also important to note that when comparing Triton X-treated and digitonin-treated cells, there is a change in the expression pattern of myc-Jagn1. Fewer punctate structures and more reticular ER-like staining in the digitonin permeabilized cells compared to Triton X permeabilized cells is observed. This is likely due to the fact that digitonin maintains intracellular membranes and therefore is likely to be more representative of a Jagn1 expression pattern. The prominent punctate structures seen in Triton X-treated cells could be artifact of ER membrane permeabilization and consequent membrane clumping therefore, resulting in myc-Jagn1 aggregation.
The presence of punctate structures in conjunction with the dilysine motif at the C-terminus of the Jagn1 protein could be suggestive of Jagn1 localizing at ER exit sites. Therefore, co-immunofluorescence experiments were performed in INS-1 832/13 and HeLa cells with myc-Jagn1 and an ER exit site marker, TFG (TRK-fused gene) and analyzed by confocal microscopy (Fig. 3.6A). TFG proteins form a matrix around ER exit sites and assist in COPII vesicle formation. Some co-localization was observed between the two proteins as well as regions where the two were in very close proximity. These regions of close proximity may still be

**Figure 3.5** Myc-tagged Jagn1 is present in punctate structures throughout the cytoplasm and has an ER-like reticular localization.

(A) INS-1 832/13 cells were transfected with myc-Jagn1. After 24h, cells were fixed, permeabilized with Triton-X 100 and immunofluorescence staining was performed with a myc-specific antibody. Cells were then imaged using epifluorescence microscopy. (B) INS-1 832/13 and HeLa cells were transfected with myc-Jagn1 constructs. After 24h, cells were fixed, permeabilized with digitonin (upper panels) or Triton-X 100 (lower panels) and immunofluorescence staining was performed with a myc-specific antibody. Cells were then imaged with confocal microscopy.
indicative of Jagn1 localized to ER exit sites because TFG is not located directly within the exit sites but forms a matrix surrounding these regions.

Co-immunofluorescence experiments were also performed with myc-Jagn1 and COPI retrograde transport vesicles in INS-1 832/13 and AD293 cells. These experiments were performed using a myc-specific antibody and an antibody directed at the COP1 coat protein complex (CM1A10)\(^{86}\). Some co-localization was also observed (Fig. 3.6B), suggesting that Jagn1 likely exits the ER, but is recycled back via interactions between the dilysine motif and COPI coat proteins.

Jagn1 co-localization with calnexin, an ER membrane protein, was also attempted. Unfortunately, calnexin staining was not effective and therefore no conclusions could be made regarding Jagn1 co-localizing with calnexin (data not shown).
Figure 3.6 Jagn1 is also localized to ER exit sites and COPI retrograde transport vesicles. 

(A) Myc-Jagn1 construct was transfected into INS-1 832/13 (upper panel) and HeLa (lower panel) cells. After 24h, cells were fixed, permeablized with digitonin and co-immunofluorescence staining was performed using an anti-myc antibody and TFG-specific antibody. Cells were then imaged using confocal microscopy. (B) myc-Jagn1 constructs were transfected into INS-1 832/13 and AD293 cells for 24h. Cells were then fixed, permeablized with digitonin and co-immunofluorescence staining was performed using an anti-myc antibody and a COPI-specific antibody. Cells were imaged using confocal microscopy.

3.2.4.1 GFP-tagged Jagn1 expression has a more characteristic ER-like staining pattern

We also examined localization of N-terminal GFP-tagged Jagn1. GFP-Jagn1 was transfected into INS-1 832/13 as well as AD293 cells, fixed and imaged with either epifluorescence or confocal microscopy (Fig. 3.7A and B, respectively). A highly reticular staining pattern, more characteristic of ER-like staining in both cell types was observed with almost no punctate structures. It is possible that the larger GFP tag may be preventing Jagn1 from exiting the ER and therefore no longer giving rise to a punctate expression pattern that was commonly observed with myc-Jagn1.

Figure 3.7 GFP-Jagn1 localization is characteristic of ER.

An N-terminal GFP-Jagn1 construct was transfected into INS-1 832/13 (A) and AD293 cells (B). (A) Following 24h of transfection cells were fixed and imaged with epifluorescence microscopy. (B) Following 24h of transfection, cells were either fixed and unpermeablized or were fixed, then permeablized with digitonin. Samples were then imaged with confocal microscopy.
3.2.5 Jagn1 knock-down in INS-1 832/13 cells causes an increase in insulin secretion and insulin content

Jagn1 is expressed in insulinoma cell lines, is increased in response to ER stress and is localized primarily in the ER. To determine what role Jagn1 has in pancreatic β-cells, Jagn1 expression was knocked down using Jagn1-specific siRNA. Several siRNAs were tested. However, the most effective one caused an approximate 60% knock-down of Jagn1 mRNA expression after 72 h of siRNA treatment (Fig. 3.8A). Unfortunately, since an antibody to detect Jagn1 protein is unavailable, Jagn1 knock-down efficiency could only be tested at the mRNA level using qPCR analysis. Given localization in the ER and a role in protein secretion in Drosophila oocytes, I hypothesized that Jagn1 knock-down would alter the β-cell’s ability to synthesize and secrete insulin. To test this hypothesis, INS-1 832/13 cells were transfected with either control or Jagn1 siRNA for three days then treated with low or high glucose concentrations for one hour. Media was then collected and the amount of insulin secreted was measured using a rat insulin radioimmunoassay (RIA). Interestingly, as seen in Fig. 3.8B, cells treated with Jagn1 siRNA had enhanced insulin secretion compared to control cells under both basal (2.8 mM) and stimulating (16.7 mM) glucose concentrations. This suggests that Jagn1 may be playing a role in either regulating insulin secretion or insulin production.
Figure 3.8 Knock-down of Jagn1 using siRNA in INS-1 832/13 cells enhances insulin secretion.

(A) INS-1 832/13 cells were transfected with Jagn1 siRNA (10 nM) or control siRNA (directed to firefly luciferase) for 72h. Total RNA was then isolated and qPCR analysis was performed for Jagn1 (N = 3). (B) INS-1 832/13 cells were transfected with 10 nM Jagn1 siRNA or control siRNA for 72 h. The cells were then treated with low (2.8mM) or high (16.7mM) glucose concentrations for one hour. Media was isolated and a rat insulin RIA was performed to measure insulin levels. Results are expressed as secreted insulin levels normalized to total protein content (Left) and as secreted insulin normalized to control siRNA transfected cells at basal (2.8mM) glucose (right) (N = 4). * = p ≤ 0.05
Cellular insulin content was also measured in the insulin secretion experiments shown in Fig. 3.9. This was to determine whether the increased insulin secretion observed in Jagn1 knock-down cells is due to an increase in insulin production or whether it is due to an increase in the kinetics, or rate of insulin secretion. A clear trend towards increased insulin content in Jagn1 knock-down cells in both low and high glucose concentrations was observed (Fig. 3.9A). Therefore, this suggests that cells expressing lower levels of Jagn1 have enhanced insulin production and consequently an increase in insulin secretion. Further supporting this finding, steady-state proinsulin levels were also increased in Jagn1 knock-down INS-1 832/13 cells as monitored by western blot analysis (Fig. 3.9B).

The expression of the INS2 gene at the mRNA level was also assessed in INS-1 832/13 cells in order to determine if these changes observed are due to transcriptional or post-transcriptional mechanisms. A qPCR analysis revealed no change in INS2 mRNA levels in Jagn1 knock-down cells versus control siRNA treated cells (Fig. 3.9C). Collectively, this data suggests that the observed increase in insulin levels in Jagn1 knock-down cells is likely due to an increase in insulin biosynthesis.
3.2.6 Jagn1 knock-down in INS-1 832/13 cells causes an increase proinsulin biosynthesis but does not affect proinsulin transport

Once an insulin mRNA transcript is translated into the preproinsulin molecule, the signal sequence is cleaved, generating proinsulin within the lumen of the ER. The proinsulin molecule is then transported from the ER to the Golgi where it is packaged into secretory granules and where mature insulin is produced by the action of prohormone convertases. Based on previous data shown above, I hypothesized that proinsulin biosynthesis would be enhanced in INS-1
832/13 cells treated with Jagn1 siRNA. A proinsulin pulse-chase analysis was performed to assess proinsulin biosynthesis and transport in control and Jagn1 siRNA-treated cells. INS-1 832/13 cells were treated with radiolabelled amino acids for 20 minutes and then chased with unlabelled media for zero or 30 minutes. Interestingly, higher levels of proinsulin were observed at both chase times of zero and 30 minutes in Jagn1 knock-down cells compared to control siRNA-treated cells (Fig. 3.10A, B). Additionally, as expected, proinsulin levels decreased after the 30 minute chase, as proinsulin is processed into mature insulin along the secretory pathway. There was no apparent affect on the rate of proinsulin transport in Jagn1 knock-down cells compared to control samples (Fig. 3.10B, C).

Combined, my data shows that a decrease in Jagn1 expression enhances proinsulin biosynthesis and as a result, causes an increase in insulin synthesis and secretion. Therefore, this suggests that Jagn1 plays a role in down-regulating proinsulin biosynthesis, but does not seem to affect the rate at which proinsulin is transported through the secretory pathway.
Jagn1 was shown to play a key role in ER morphology in the oocyte of Drosophila flies. Mutants expressing a non-functional Jagn1 had reduced ER clustering compared to wildtype oocytes and did not develop a swollen ER luminal space during times of high protein trafficking\(^8\). As a result, I hypothesized that Jagn1 may play a role in regulating ER morphology in pancreatic β-cells as well. INS-1 832/13 cells were treated with either control siRNA or Jagn1 siRNA for 72 h, then fixed and imaged using transmission electron microscopy. In Jagn1 knock-down cells the ER was more extensive and more abundant compared to control cells (Fig. 3.11A). ER area was measured relative to total cytoplasmic area and showed an approximate 30% increase in ER abundance in Jagn1 knock-down cells (Fig. 3.11B). The protein levels of two resident ER proteins, calnexin and PDI, were also measured by western blot analysis to determine if chaperone proteins are also increased (Fig. 3.11C). There were no differences in protein levels of calnexin nor PDI in Jagn1 knock-down cells compared to the control. However, because the increase in ER content was only 30%, such small differences may be more difficult to detect with western blot analysis.
Figure 3.11 Treatment of INS-1 832/13 cells with Jagn1 siRNA increases ER abundance.

(A) Electron microscopy (EM) images of ER in INS-1 832/13 cells treated with 10 nM of either control Luciferase siRNA (left) or Jagn1 siRNA (right) for 72 h. (B) EM images were analyzed to measure total ER luminal area relative to total cytoplasmic area. (N = 7) for control cells, (N = 10) for Jagn1 knock-down cells. (C) Western blot analysis of PDI and calnexin protein levels in either control or Jagn1 knock-down cells. 10 μg of protein loaded (N = 3). * = p ≤ 0.05
3.2.8 Knock-down of Jagn1 mRNA does not induce ER stress

Considering the enhanced proinsulin biosynthesis and insulin secretion in Jagn1 knock-down cells, I hypothesized that INS-1 832/13 cells treated with Jagn1 siRNA might have higher levels of ER stress compared to control-treated cells. However, levels of sXBP1 mRNA, Grp78 and Grp94 protein were not increased compared to that of control cells (Fig. 3.12). Therefore, the loss of Jagn1 expression does not induce measureable ER stress in pancreatic β-cells.

3.2.9 Overexpression of myc-Jagn1 decreases proinsulin and insulin protein levels and does not induce ER stress

I have shown that knock-down of Jagn1 results in an increase in proinsulin biosynthesis, insulin content and insulin secretion. Therefore, I hypothesized that the opposite would occur when Jagn1 is overexpressed i.e. a decrease in insulin protein levels. To test this hypothesis, INS-1 832/13 cells were transfected with Myc-Jagn1 for 24 h, protein lysates were prepared and

![Figure 3.12 Analysis of ER stress markers in INS-1832/13 cells treated with Jagn1 siRNA.](image)

(A) Total RNA was isolated from INS-1 832/13 cells transfected with 10 nM Jagn1 or control siRNA for 72h. Cells treated with 2 μg/ml Tm (16h) were used as a positive control for ER stress. An RT-PCR was performed to monitor spliced and unspliced XBP1 mRNA (N = 3). (B) Protein lysates were made from INS-1 832/13 cells treated with either 10 nM control (GFP) or Jagn1 siRNA for 72h. A western blot analysis was then performed using a KDEL specific antibody and anti-tubulin antibody as a loading control (N = 3).
an anti-insulin western blot analysis was performed to detect proinsulin. The western blot analysis revealed lower levels of proinsulin protein expression in the cells transfected with myc-Jagn1 compared to untransfected cells (Fig. 3.13A, B).

Bip/GRP78 protein levels were not affected by myc-Jagn1 overexpression, indicating that Jagn1 overexpression does not cause ER stress (Fig. 3.13C).

![Western Blot Analysis](image)

**Figure 3.13** The overexpression of Jagn1 decreases proinsulin levels and does not induce ER stress.

(A) Protein lysates were generated from INS-1 832/13 cells after transfection with myc-Jagn1 for 24 h. Western blot analysis was performed for the indicated proteins (N = 3). (B) Proinsulin levels were quantified and normalized to tubulin protein levels. (C) Using the same membrane as in A, a western blot analysis was performed using a Grp78 (BiP)-specific antibody (N = 3). * = p < 0.05

The effect of myc-Jagn1 overexpression on insulin protein levels was also determined by immunofluorescence staining. Insulin staining in cells that were transfected with myc-Jagn1 was lower than in untransfected cells (Fig. 3.14A, C). Interestingly, insulin levels were unaffected in cells transfected with a plasmid expressing the mutant Jagn1^{D16N} (Fig. 3.14B, C). This is the
same semi-lethal mutation identified by Lee and Cooley that disrupts oocyte development in Drosophila flies.\textsuperscript{81}

**Figure 3.14** Effect of overexpression of wildtype and mutant Jagn1 in INS-1 832/13 cells on insulin levels.

(A) INS-1 832/13 cells were transfected with myc-Jagn1 for 24h. Cells were fixed and IF was performed using myc antibody and insulin antibody. Samples were imaged using confocal microscopy (60x magnification). (N = 3). (B) Same experimental plan as in A, except cells were transfected with myc-Jagn1\textsuperscript{D16N} (N = 3). (C) Mean fluorescence signal was measured for insulin protein expression in untransfected cells and in cells transfected with either wildtype or mutant Jagn1. (D) Mean fluorescence signal of Myc-Jagn1 expression in transfected and untransfected cells. ** = p \leq 0.005
3.3 Discussion

ER stress has been shown to play a key role in the loss of pancreatic β-cell function and mass, and contributes to the pathogenesis of type II diabetes \(^{29,92,93}\). Pancreatic β-cells respond to ER stress by activating the UPR. The UPR consists of a series of signal cascades that when activated, upregulate numerous target genes encoding proteins which are involved in various mechanisms to alleviate the stress, such as protein folding and the ERAD pathway. Another important stress-alleviating mechanism of the ER stress response is the transiently reduced protein load on the ER due to PERK-mediated inhibition of global translation. Therefore, the ways in which a cell responds to ER stress and the mechanisms the cell undergoes to relieve this stress occur at multiple levels. The profile of genes induced by the UPR can be characteristic of a certain cell-type and given that pancreatic β-cells are highly specialized insulin producing cells, they have a highly developed ER and likely a unique profile of ER stress-responsive genes.

To study the UPR in β-cells we used a mutant insulin-expressing cell line and performed a SILAC proteomic analysis. We identified Jagn1 to be one of these proteins which is increased in the presence of ER stress induced by mutant proinsulin expression and accumulation within the ER. Jagn1 was initially identified and studied in the oocyte of \(D.\ melanogaster\) \(^{81}\), although the gene is also present in various multicellular eukaryotes. Jagn1 has not been studied in mammalian cells. Therefore, the main objective of this study was to determine the role of Jagn1 in ER stress-prone pancreatic β-cells.

I first determined that Jagn1 is expressed in insulinoma β-cell lines and in rat and mouse islets. I also found that Jagn1 is expressed at the mRNA level in various mouse tissues, including: brain, liver, adipose, muscle and dendritic cells. Although, Jagn1 was particularly abundant in islets, its ubiquitous expression suggests it has a role in most, if not all tissues and mammalian cells. Unfortunately, no useful Jagn1-specific antibody is available, so I was unable to show Jagn1 expression at the protein level.

I validated that Jagn1 expression is ER stress responsive. Using the pharmacological ER stress inducer, tunicamycin (inhibits N-linked glycosylation \(^{94}\)), an increase in Jagn1 mRNA expression was observed with qPCR analysis in INS-1 832/13 cells and in mouse islets treated with Tm (Fig. 3.2A, B). Given that high fat diet-induced obese mice have been shown to have increased ER stress in their pancreatic islets \(^{54}\), we also observed increased Jagn1 expression in
islets isolated from mice on a 45% HFD, although this did not reach statistical significance. As mentioned above, we initially identified Jagn1 in a SILAC screen for proteins increased in response to ER stress caused by mutant proinsulin expression, thus these findings validate Jagn1 as an ER stress-inducible gene.

As mentioned in the introduction, each arm of the UPR, PERK, IRE1 and ATF6, is known to regulate a particular set of target genes. Although there is a lot of redundancy among the various genes that are targeted by each arm, some genes are primarily regulated by one pathway over the other. I sought out to identify which of the three pathways of the UPR was regulating Jagn1 expression under ER stress conditions. INS-1 832/13 cells treated with Tm alone had increased Jagn1 expression, as expected. However, when cells were treated with Tm in combination with the IRE1 inhibitor, 4μ8c (inhibitor of IRE1 RNase activity), Jagn1 mRNA levels were even further enhanced. These results were also validated in INS-1 832/13 cells treated with Tm and an alternate IRE1-specific inhibitor, MKC-3946, as well as in C96Y cells treated with Dox in combination with MKC-3946 (Fig. 3.3A, B, C). This was an unexpected result as we predicted either no change or a decrease in target gene expression following IRE1 inhibition. However, the reason we see an increase in Jagn1 mRNA expression, is likely explained by the fact that regulated IRE-1 dependent decay (RIDD) activity is blocked by these inhibitors. Under irresolvable or chronic ER stress conditions the IRE1 RNase domain can become hyper-activated and start promiscuously cleaving non-XBP1 mRNA transcripts within its vicinity. Commonly, transcripts encoding ER-localized proteins are within this region and are therefore more susceptible to degradation by RIDD activity. This is likely the case with Jagn1 mRNA – in the presence of IRE1 hyper-activity, which is likely with 16 h Tm treatment or 24 h mutant proinsulin accumulation, Jagn1 mRNA is non-specifically cleaved by the RNase domain of IRE1. However, when this function of IRE1 is blocked, Jagn1 can no longer be degraded and therefore its expression is seen to be further enhanced under these conditions. Thus, IRE1/XBP1 is unlikely to mediate Jagn1 induction in response to ER stress.

I next analyzed the role of ATF6 in regulating Jagn1 transcription. The effect of overexpression of active forms of ATF6α or ATF6β on Jagn1 mRNA levels in INS-1 832/13 cells was tested. Overexpression of ATF6αp50 had no effect on Jagn1 mRNA levels, whereas the overexpression of ATF6βp60 significantly decreased Jagn1 mRNA expression (Fig. 3.3D). This was interesting, yet initially surprising and apparently contradictory. ATF6β is active in the
presence of ER stress, yet Jagn1 mRNA levels are increased by ER stress. However, given the kinetics of ATF6β induction in response to ER stress, we propose a model of ATF6β regulation of Jagn1 (discussed later, Fig. 3.3.2).

To analyze the localization of Jagn1, N-terminal tagged myc- and GFP-Jagn1 constructs were generated, given that there is no available antibody that is effective in detecting endogenous or overexpressed Jagn1. N-terminal tags were chosen because blocking the dilysine motif with a C-terminal tag has been shown to alter the localization of the protein. This was seen in the study done by Lee and Cooley where they generated a Jagn1-GFP C-terminal tagged construct. Jagn1-GFP was expressed both at the plasma membrane as well as the ER, whereas the construct expressing a Venus tag fused in between the first and second transmembrane domains of Jagn1 only localized to the ER. This is believed to be due to the loss of ER retention since the dilysine motif was blocked by the GFP tag\textsuperscript{81}. When INS-1 832/13 and HeLa cells were transfected with myc-Jagn1, IF staining revealed an ER-like expression pattern consisting of punctate structures present throughout the cytoplasm and was excluded from the nucleus. I also compared the expression pattern in cells permeablized with Triton X-100 or digitonin (Fig. 3.5). Fewer punctate structures were observed and more characteristic of ER-like staining was evident in cells treated with digitonin compared to Triton X-100. This is likely due to the fact that digitonin only permeablizes the plasma membrane, unlike Triton X-100, which also permeablizes intracellular membranes. The prominent punctate structures seen in TX-100-treated cells could be aggregated protein, not representative of normal cellular localization. Thus, digitonin was used for the remainder of the experiments.

The presence of punctate structures in myc-Jagn1 transfected cells is characteristic of an ER exit site expression pattern\textsuperscript{98,99}. Co-localization experiments with myc-Jagn1 and the ER exit site marker, TFG, confirmed that some punctate structures co-localized with TFG (Fig. 3.6A). Additional co-localization experiments with myc-Jagn1 and COPI retrograde transport vesicles revealed that some of the punctate structures also co-localized with COPI vesicles (Fig. 3.6B). Thus, Jagn1 is localized primarily to the ER, but likely cycles between the ER and Golgi. The C-terminal dilysine motif recognized by the COPI coat likely allows for efficient retrograde transport of Jagn1 that escapes the ER.
An N-terminal GFP-tagged Jagn1 construct was also generated to analyze Jagn1 localization. However, when transfected cells were imaged, using either epifluorescence or confocal microscopy, a more reticular and ER-like pattern, with fewer punctate structures was observed (Fig. 3.7). It is possible that the loss of punctate expression is a result of the large 27 kDa GFP-tag, as it may be preventing the protein from exiting the ER and therefore, it cannot be localized to ER exit sites and COPI transport vesicles. The bulkiness of the GFP tag can affect the localization of some proteins\textsuperscript{100}.

It has previously been shown that Jagn1 is important for protein trafficking and secretion in the oocyte of Drosophila flies, particularly during conditions in which a high degree of protein trafficking occurs\textsuperscript{81}. Thus, I hypothesized that Jagn1 would play an important role in insulin biosynthesis and secretion in pancreatic \(\beta\)-cells. I monitored glucose-stimulated insulin secretion (GSIS) in INS-1 832/13 cells following Jagn1 knock-down with siRNA. Knock-down efficiency, which could only be monitored at the mRNA level, was 60%. Interestingly, enhanced insulin secretion in cells treated with Jagn1 siRNA following both low and high glucose concentrations was observed as there was an obvious trend with higher levels of secreted insulin in the knock-down samples (Fig. 3.8). The increase in secreted insulin was also supported by an increase in insulin content in Jagn1 knock-down cells compared to control cells (Fig. 3.9A), thereby suggesting an increase in insulin production rather than enhanced insulin secretion. Considering an increase in insulin levels and secretion was observed at basal glucose (2.8mM glucose) in Jagn1 knock-down cells, this suggests that steady-state levels would be affected in these cells. Indeed, proinsulin steady-state levels were higher in INS-1 832/13 cells treated with Jagn1 siRNA compared to the control (Fig. 3.9B) suggesting that proinsulin biosynthesis is enhanced, rather than an enhancement in insulin processing and maturation. However, there were no changes in steady-state INS2 mRNA expression levels in Jagn1 knock-down versus control cells (Fig. 3.9C). This indicates that the changes observed in proinsulin levels must be a result of post-transcriptional mechanisms. These results were further validated by a pulse-chase analysis on proinsulin biosynthesis and processing, which revealed, again, higher levels of proinsulin biosynthesis in Jagn1 knock-down cells, but there was no apparent affect on proinsulin transport or processing of proinsulin into mature insulin (Fig. 3.10). Although the changes observed were generally small, had Jagn1 knock-down efficiency been greater than 60\%, the results may have been more robust.
There are many ways in which insulin biosynthesis may be regulated post-transcriptionally, some of which include: alternative splicing and mRNA maturation as well as mRNA transport, storage and translation\textsuperscript{101}. Insulin mRNA has a long half life of approximately 29-77 hours and its stability can be attributed to its interaction with an RNA-binding protein (RBP) known as PTB (polypyrimidine tract-binding protein). Insulin translation has also shown to be regulated by a highly conserved nucleotide sequence in the 5'UTR, as well as through the action of a stem loop within this region. However, the factors that interact with these regions and that are involved in regulating translation are unknown\textsuperscript{101, 102}. Another RBP, known as HuD, has been identified as a negative regulator of insulin mRNA translation by interacting with the 5'UTR. However, upon glucose stimulation, HuD dissociates from the mRNA and allows for protein translation to ensue\textsuperscript{102}.

There are many possible mechanisms through which Jagn1 may be regulating insulin biosynthesis, whether it’s involved in regulating insulin mRNA stability or protein translation is unknown. It would be interesting to identify interacting proteins with Jagn1 in order to help elucidate the various possible mechanisms in which it may be regulating insulin biosynthesis in pancreatic β-cells (discussed in section 3.4).

I have shown that in INS-1 832/13 cells treated with Jagn1 siRNA have enhanced proinsulin biosynthesis and insulin secretion. As a result, this increased protein load on the ER could be inducing an ER stressed environment. However, when sXBP1 levels were measured in Jagn1 knock-down cells there were no differences compared to control cells (Fig. 3.12A). Similarly, there was no difference in Grp78 or Grp94 expression levels in Jagn1 knock-down versus control cells (Fig. 3.12B). However, the changes observed in proinsulin biosynthesis were small and therefore the effects on ER stress activation may be small as well. In which case, small changes in sXBP-1 or chaperone protein levels would be more difficult to detect with these types of experiments used. Therefore, the presence of ER stress and activation of the UPR cannot be ruled out.

When Jagn1 mRNA expression is knocked-down, it results in an increase in proinsulin biosynthesis and insulin secretion. Therefore, I hypothesized that the opposite would be true as well – if Jagn1 was overexpressed it would cause a decrease in insulin protein levels. This is what was observed in a western blot analysis (Fig. 3.13A, B) and immunofluorescence staining
that was performed following myc-Jagn1 transfection in INS-1 832/13 cells (Fig. 3.14A, C). Interestingly, overexpression of mutant Jagn1 resulted in no difference in insulin levels compared to untransfected cells (Fig. 3.14B, D). Since the mutation was introduced within the highly conserved N-terminal region, it implicates the importance of this region in its function. This may be the site of interaction with other proteins and allows for Jagn1 to carry out its activity.

In summary, I have shown that a decrease in Jagn1 causes an increase in proinsulin translation, while an increase in Jagn1 causes the opposite effect (Fig. 3.3.1). Given that Jagn1 is induced in response to ER stress, I have developed a model of Jagn1 function in β-cells (Fig. 3.3.2). In the presence of ER stress Jagn1 expression is enhanced, resulting in reduced proinsulin translation. This is beneficial for the cell as it would reduce the protein folding load on the ER, aiding in alleviating the stress. However, once the ER stress has been resolved and homeostasis is restored, Jagn1 is down-regulated by the late-activated ATF6β transcription factor and thereby allows insulin synthesis to continue normally.

It should also be noted that Jagn1 was shown to be expressed in various other tissues other than pancreatic β-cells and islets, and therefore its effects are likely not insulin-specific. The role of Jagn1 may be on generic protein synthesis i.e. it may play a role in disulphide bond formation or is involved in mechanisms regulating translation efficiency. The purpose of its role presents curiosity given that the PERK pathway already attenuates translation in the presence of ER stress. However, it is possible that Jagn1 is down-regulating the levels of proteins, via other unknown post-translational mechanisms. It could be acting to “assist” in the effects on PERK-mediated translation inhibition, by proposing the same effect, however, at the post-translational level. An alternative possible explanation for Jagn1 activation in ER stress may rely on the fact that the effect of PERK inhibition on global translation is short lived\textsuperscript{103} and Jagn1 may be prolonging this effect once PERK has been deactivated. Therefore, future studies should include experiments determining the time frame in which Jagn1 is induced during ER stress and when it is restored to normal levels.
In response to ER stress Jagn1 is induced which results in reduced proinsulin translation. ATF6β is also induced in response to ER stress, however, as a late event\(^6\) and in addition to regulating UPR genes, serves to down-regulate Jagn1 expression as ER homeostasis is restored. This then allows for proinsulin translation to proceed normally.

In the study done by Lee and Cooley, they had also shown that Jagn1 plays a key role in ER reorganization and ER morphology. They had observed that oocytes expressing mutant Jagn1 had reduced ER clustering in the subcortex and did not develop ER luminal swelling, unlike cells expressing the wildtype Jagn1\(^8\). Therefore, I hypothesized that Jagn1 would play an important role in regulating ER morphology in pancreatic β-cells. Using transmission electron microscopy (TEM) and ER luminal area analysis, an approximate 30% increase in ER abundance in Jagn1 knock-down cells was observed compared to control siRNA treated cells (Fig. 3.11). There are many secretory cell types, such as plasma cells and pancreatic acinar cells, in which they adapt their ER content in order to accommodate for an increased protein load\(^1\). This could be the case in INS-1 832/13 cells treated with Jagn1 siRNA – in these cells I have shown that proinsulin
biosynthesis is enhanced and therefore, the cells could simply be adapting to this response by increasing their ER abundance. However, the mechanism in which ER biogenesis is regulated is not well known. Two studies done by Sriburi et al. identified sXBP-1 to be involved in regulating ER abundance. They had shown that fibroblasts overexpressing sXBP1 had enhanced synthesis of phosphatidylcholine (PtdCho), a major component of the ER membrane. This was due to increased activity of two enzymes involved in the PtdCho synthesis pathway, CCT and CPT1. Fibroblasts overexpressing sXBP-1 also had elevated levels of ER proteins and a robust ER expansion. They had demonstrated that the increase in ER abundance in these cells overexpressing sXBP1 was due to the increase in PtdCho synthesis and the enhanced ER protein expression. Another study done by Wright et al. had observed that yeast overexpressing HMG-CoA reductase, an enzyme involved in sterol biosynthesis, had enhanced ER biogenesis. They discovered various genes involved in chromatin structure and transcriptional regulation to play key roles in this HMG-CoA reductase-induced ER biogenesis. Therefore, whether the increase in ER abundance in Jagn1 knock-down cells is merely a compensatory response to enhanced ER activity and protein load, or whether Jagn1 is involved in regulating one of the various components or pathways involved in ER biogenesis is not known. Further experiments would be required to assess the context of its role in this process.

3.4 Analysis of Jagn1 interacting proteins

3.4.1 Introduction

To better understand the mechanisms by which Jagn1 functions at the molecular level, identification of interacting proteins is required. Given that Jagn1 is a membrane protein, the best method to identify interacting proteins would be through a membrane yeast two hybrid (MYTH) screen. The MYTH system is specific for identifying interactors of membrane proteins in an in vivo model system in which S. cerevisiae is used as the host organism. This is different from the classic yeast two hybrid screens which requires an interaction of proteins within the nucleus and thus, could not work for integral membrane proteins. The method of this screen involves the “split ubiquitin system”. Ubiquitin is normally used as a tag to target proteins to be degraded by the 26S proteasome. However, within this system ubiquitin is split into two separate stable subunits, an N-terminal fragment (Nub) and a C-terminal fragment (Cub). The Cub fragment is
fused to the membrane protein of interest, also known as the “bait,” as well as a reporter molecule, such as a transcription factor (TF). The N\textsubscript{ub} fragment is fused to the “prey” genes which consists of a library of various genes or transcripts generated from either genomic or cDNA derived from a particular species and tissue. When the C\textsubscript{ub} and N\textsubscript{ub} subunits are in close proximity they are able to re-associate and generate a pseudoubiquitin molecule. Deubiquitinating enzymes recognize and remove this pseudoubiquitin molecule, allowing the TF to be released. The TF can then enter the nucleus to induce the expression of reporter genes and consequently allowing for selection\textsuperscript{107}.

A recent study performed a MYTH screen to identify interacting proteins of vitamin K expoxide reductase (VKOR), an enzyme that has shown to be involved in disulphide bond formation within the ER. In this screen a human adult liver cDNA library was used to generate “prey” proteins. As a result of this screen, Jagn1 was one of many proteins identified to interact with VKOR\textsuperscript{108}. Given that I have shown Jagn1 plays a role in regulating proinsulin biosynthesis and that VKOR has been shown to be involved in disulphide bond formation within the ER\textsuperscript{109}, the two proteins may be interacting with one another to regulate insulin production. This is what led us to validate this interaction between Jagn1 and VKOR.

3.4.2 Results: Analysis of Jagn1 and VKOR interaction

As mentioned above, Jagn1 was identified in a MYTH screen performed to identify interacting proteins of VKOR, but this interaction was not further validated\textsuperscript{108}. In an attempt to validate this interaction, a FLAG-tagged VKOR construct was made and immunoprecipitation experiments were performed with myc-Jagn1 following co-transfection in INS-1 832/13 cells. Unfortunately, experiments with both anti-myc and anti-FLAG immunoprecipitation suggested there was no interaction between VKOR and Jagn1. This can be seen as there was no detection of FLAG-Jagn1 (Fig. 3.15A) or myc-Jagn1 (Fig. 3.15B) in the pellet fractions, but they were detected in the supernatant fractions.
It is known that proinsulin biosynthesis is regulated at the post-translational level. Our lab has previously shown that upon PDI knock-down in β-cells, proinsulin biosynthesis and secretion was actually enhanced\textsuperscript{110}. Given that disulphide bond formation within these cells was unexpectedly unimpaired, it suggests other factors are present to allow for oxidation and disulphide bond formation to occur. This is what led us to further inquire about a Jagn1/VKOR interaction considering that VKOR has been shown to play a role in disulphide bond formation within the ER\textsuperscript{109}. However, we could not demonstrate a VKOR/Jagn1 interaction making it

\textbf{3.4.3 Discussion}

AD293 cells were transfected with myc-Jagn1 and FLAG-VKOR for 24h. Cells were then lysed and immunoprecipitation was performed using myc antibody (A) or FLAG antibody (B). Mouse IgG antibody was used as a control. The supernatant was collected and proteins were resolved in a 10% SDS gel and immuno-detected with either FLAG antibody (A) or myc antibody (B). (C) Represents immunoprecipitation test to confirm efficiency of immunoprecipitation protocol used. AD293 cells were transfected with myc-Jagn1 for 24h, then lysed. Both immunoprecipitation and immuno-detection was performed using a myc-antibody.

\begin{figure}
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Immunoprecipitation reveals no interaction between Jagn1 and VKOR}
\end{figure}
unlikely that Jagn1 functions by modulating VKOR function. Therefore, performing a MYTH screen (as described above) in which Jagn1 is used as the “bait” protein would be essential in determining interacting proteins of Jagn1 and identifying how Jagn1 may be carrying out its function at the molecular level. This is a current on-going experiment that is being performed by another member in our lab (Tanya Odisho). In this screen a split ubiquitin system is being used in which Jagn1 acts as the “bait” protein containing a C-terminal ubiquitin tag. Interacting proteins are then screened using a pancreatic islet cDNA library. Proteins identified in the MYTH screen would then need to be validated via immunoprecipitation experiments.

3.5 Overall Summary and Future Directions

In summary, I have shown that Jagn1 is expressed in pancreatic β-cell lines, as well as in mouse islets and is ubiquitously expressed in numerous mouse tissues. I have also shown that Jagn1 mRNA expression levels are enhanced under ER stress conditions that were either induced pharmacologically with tunicamycin or pathologically mediated by high fat diet or mutant proinsulin expression and accumulation. I have attempted to identify which arm of the UPR is predominantly responsible for regulating Jagn1 transcription in the presence of ER stress. Blocking IRE1 endoribonuclease activity, and consequently production of active XBP1 transcription factor, revealed that the IRE1 pathway is not responsible for regulating Jagn1 expression, but rather was shown to further enhance Jagn1 mRNA abundance when its activity is inhibited. This was an unexpected result but is likely due to the inhibition of RIDD activity and therefore, enhances Jagn1 mRNA stability. The effect of ATF6α and ATF6β overexpression on Jagn1 expression was also assessed and found that overexpressed active ATF6α had no effect on Jagn1 mRNA levels, whereas active ATF6β markedly reduced Jagn1 expression. ATF6β may serve to down-regulate Jagn1 levels to restore proinsulin translation when ER stress has been relieved (Fig. 3.3.2). The role of the PERK pathway of the UPR on Jagn1 expression is unknown and future experiments should be performed to analyze the contribution PERK may have on Jagn1 induction in the presence of ER stress.

The effect of Jagn1 knock-down on insulin biosynthesis and secretion was also analyzed. Following Jagn1 siRNA treatment for 72 h, INS-1 832/13 cells were measured for their GSIS response. It was observed that Jagn1 knock-down cells had enhanced insulin secretion both with
and without glucose stimulation. This was further supported by an increase in insulin content as well as higher levels of steady-state proinsulin in Jagn1 knock-down cells compared to control siRNA-treated samples. A pulse-chase analysis also revealed increased rates of proinsulin biosynthesis in Jagn1 knock-down cells, but there was no apparent effect on proinsulin transport and processing into mature insulin in the knock-down cells compared to control. We also observed that the opposite was true in which Jagn1 overexpression led to a decrease in insulin protein levels. To determine if these observed changes in insulin biosynthesis are mediated by transcriptional or post-transcriptional mechanisms, a qPCR analysis on \textit{Ins2} mRNA was performed. No difference in \textit{Ins2} mRNA expression levels was observed in Jagn1 knock-down cells, thus the effects on proinsulin biosynthesis are most likely. Therefore, post-transcriptional mechanisms are at play here. An approximate 30% increase in ER abundance was also observed in Jagn1 knock-down cells which could be a compensatory response to the increase in insulin production. However, the mechanism by which Jagn1 may be regulating proinsulin biosynthesis is not known.

Future work needs to identify interacting proteins so the molecular mechanisms by which Jagn1 affects proinsulin translation can be elucidated. Based on recent findings in the literature, Jagn1 was identified on a screen performed to discover interacting proteins with VKOR$^{108}$. However, my immunoprecipitation experiments revealed no interaction in cells. Therefore, it would be useful to perform a MYTH screen using Jagn1 as bait. Such experiments are currently on-going and future work would require validation experiments with any interacting proteins identified.

It would also be interesting to see if the findings observed in these \textit{in vitro} cell culture studies are also observed \textit{in vivo}. Therefore, future experiments should analyze insulin biosynthesis in Jagn1 β-cell knockout mice particularly under ER stress conditions. I hypothesize, that mice deficient in Jagn1 expression in pancreatic β-cells under a HFD would have increased proinsulin translation and therefore, enhanced insulin biosynthesis and secretion. This might make the mice more susceptible to diabetes development or may make the β-cells more susceptible to ER stress-induced apoptosis.
APPENDIX 1: EXAMINING THE ROLE OF IRE1 IN THE UNFOLDED PROTEIN RESPONSE IN PANCREATIC β-CELLS

The results reported in this Appendix are part of a manuscript in press in BMC Cell Biology.

“IRE1 inhibition perturbs the unfolded protein response in a pancreatic β-cell line expressing mutant proinsulin, but does not sensitize the cells to apoptosis”

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This appendix briefly reviews my contribution towards a project performed by several lab members. A manuscript for this project is currently in press (BMC Cell Biology)\textsuperscript{89} and is titled “IRE1 inhibition significantly perturbs the unfolded protein response to mutant proinsulin expression in pancreatic β-cells, but does not sensitize the cells to apoptosis”. In this appendix I will provide a brief introduction, the results of my contribution and the major findings from this project.

A1.1 Introduction

PERK, IRE1 and ATF6 are three ER stress response sensors that mediate the unfolded protein response when activated under ER stress conditions. Each of these pathways is activated in attempt to counteract the stress, and does so by inducing the transcription of various target genes which can encode pro-survival or pro-apoptotic proteins\textsuperscript{29, 55}. The specific subset of genes induced in the presence of ER stress can be cell-type specific and can be specifically induced by a particular UPR pathway\textsuperscript{111}. The Volchuk lab has used a mutant proinsulin-expressing cell line to identify the UPR in this model of ER stress\textsuperscript{80}. All three UPR pathways are activated in this system and the recent development of specific inhibition of the UPR pathways has allowed for analysis of pathways that contribute to the global UPR.

IRE1 is the most conserved UPR signaling pathway and its cytoprotective role is present throughout all eukaryotes\textsuperscript{112} and its activity can be inhibited by two structurally distinct inhibitors 4μ8c and MKC-3946. By inhibiting the endoribunclease domain of IRE1, a functional XBPI transcription factor cannot be generated and therefore downstream targets will not be induced. Using these inhibitors allowed us to determine which genes and proteins are IRE1-dependent and thus identifying particular pathways that are reliant on IRE1 activity.
A1.2 Results

A1.2.1 Inhibiting the IRE1 pathway does not affect ERAD activity.

The role of IRE1 in the ERAD pathway was analyzed by examining the effect of IRE1 inhibition on mutant proinsulin expression. Insulin 2 C96Y-GFP cells were treated with Dox for 24 h to induce mutant proinsulin expression as well as with or without the IRE1 inhibitor, 4µ8c. Cyclohexamide was then added to prevent any new protein synthesis for zero, three and six hours. I found that the inhibitor had no effect on mutant proinsulin degradation compared to cells without 4µ8c treatment (Fig. A1.1A, B). This was also supported by the results observed when analyzing the effect of IRE1 inhibition on Herp protein expression. Herp is an integral ER membrane protein and plays a role in the ERAD pathway by recruiting ubiquitin to misfolded proteins. It was seen that Herp protein levels were significantly induced by mutant proinsulin expression, and that this was unaffected by the 4µ8c inhibitor (Fig. A1.1C). This result is consistent with Herp mRNA analysis, which were still significantly increased in the presence of the inhibitor (results not shown).
A1.2.2 IRE1 inhibition does not sensitize C96Y cells to apoptosis

The effect of IRE1 inhibition on mutant proinsulin-induced apoptosis was also assessed. When cells are treated with the 4μ8c inhibitor, IRE1 activity is blocked and expression of a functional XBP1 transcription factor is prevented (results not shown). As a result, the UPR is compromised and its ability to relieve the stress might be hindered. Therefore, we hypothesized that inhibiting IRE1 activity would sensitize the cells to apoptosis. Using an ELISA apoptosis kit, the level of apoptosis was measured in mutant proinsulin-expressing cells with or without the IRE1 inhibitor. In Fig. A1.2, it can be seen that although mutant proinsulin expression induced apoptosis, the inhibitor had no significant effect in these cells.
A1.3 Discussion

In this study the effect of IRE1 pathway inhibition on the UPR in a cellular model system of mutant proinsulin-induced ER stress was examined. Various outputs of the UPR in response to ER stress were assessed, such as changes in global gene expression via qPCR validation, mutant proinsulin degradation and apoptosis. My role focused on analyzing mutant proinsulin degradation by the ERAD pathway as well as cellular induced apoptosis in cells lacking IRE1 activity. It is well known that improperly folded proteins in the ER are degraded by the ERAD system, mediated by UPR activation, to prevent aggregation and accumulation\textsuperscript{115}. It has also been shown mutant proinsulin is degraded by the ERAD pathway\textsuperscript{115} and that Herp is required for this process given that its knock-down was shown to increase mutant proinsulin levels\textsuperscript{80}. Herp has been shown to be induced in the presence of ER stress\textsuperscript{116}, however, the contribution IRE1 makes towards Herp expression as well as ERAD function is not known. Therefore, I analyzed whether mutant proinsulin degradation was altered following IRE1 inhibition. As expected, mutant proinsulin was decreased over time, but there was no difference in degradation kinetics in 4μg\textsuperscript{8c}-treated cells compared to cells treated with Dox alone (Fig. A1.1A, B). This finding is also supported by the fact that Herp protein levels, which were increased in response to mutant proinsulin, were not significantly affected by the IRE1 inhibitor (Fig. A1.1C). We also show that Herp mRNA expression is still induced in the presence of 4μg\textsuperscript{8c} treatment, as well as other genes encoding ERAD components such as HRD1 and EDEM1 (data not shown). Combined, these results indicate that the IRE1 pathway is not essential for ERAD activity.

Apoptotic levels were also examined in C96Y cells treated with Dox either with or without the IRE1 inhibitor. An ELISA apoptosis detection kit was used which measures cytoplasmic oligonucleosomes. Although mutant proinsulin expression induced apoptosis, there was no significant difference in apoptotic levels in cells treated with or without 4μg\textsuperscript{8c}. Initially, it was hypothesized that inhibiting IRE1 activity would make the cells more susceptible to apoptosis. This was because without IRE1 activation, and subsequent XBP1 activity, it was assumed that induction of pro-survival genes or genes encoding proteins involved in aiding to resolve the stress would be hindered and would therefore further sensitize the cells to apoptosis. However, this was not the case and this result is consistent with other findings observed from this project (data not shown). For example, TRB3, a pro-apoptotic gene whose expression is known to be
significantly enhanced in the presence of ER stress and a downstream target of CHOP\textsuperscript{117}, was seen to require IRE1 activation for its maximal induction. The same effect was seen with TXNIP mRNA expression, another known ER stress inducible pro-apoptotic gene\textsuperscript{118}. Therefore, without maximal induction of various pro-apoptotic genes, it is likely that ER stress-mediated cell death was not further enhanced.

In summary, IRE1 is not required for ERAD-mediated mutant proinsulin degradation and its inhibition does not further sensitize cells to apoptosis.
References


