A ROLE FOR MICRO RNA-146A-5P-MEDIATED REGULATION OF STROMAL INTERACTION MOLECULE 1 AND STORE-OPERATED CALCIUM ENTRY IN THE PANCREATIC-BETA CELL IN RESPONSE TO CYTOKINE-MEDIATED STRESS

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DEDICATION

I dedicate this thesis to my parents J.P and Kalpana Kanojia, my sister Jagrati, my friend Gaurav, my beloved grandparents and all my mentors for nursing me with their love, support and their dedicated partnership for success in my life.
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Store-operated Ca\(^{2+}\) entry (SOCE) is involved in the maintenance of endoplasmic reticulum (ER) Ca\(^{2+}\) levels. The SOCE involves Stromal Interaction Molecule 1 (STIM1), distributed throughout the ER, and Orai1 channels, dispersed on the plasma membrane. SOCE is activated by the depletion of ER Ca\(^{2+}\) causing STIM1 to induce ER expansion and recruits Orai1 channels thus replenishing ER Ca\(^{2+}\). We reported downregulation of STIM1 in human islets from donors with type 2 diabetes (T2D) and in INS-1 β-cells treated with cytokines, and loss of STIM1 expression impairs β-cell SOCE, ER stress, and reduced insulin secretion. However, the regulatory mechanisms of STIM1 downregulation are unknown. To test this, actinomycin D and cycloheximide chase assay was performed to define whether IL-1β treatment impacted STIM1 mRNA or protein half-life. IL-1β had no impact on mRNA or protein decay. MicroRNAs (miRNAs), a class of small non-coding RNAs can regulate gene expression post-transcriptionally by binding to complementary regions in the 3’ untranslated region (UTR) of target mRNAs, affecting mRNA stability and translatability. The objective of this study was to establish miRNA regulation of STIM1 expression and altered SOCE. To identify potential miRNA candidates, RNA-sequencing was done in human islets, treated with IL-1β and IFN-γ for 24 hrs. A total of 20 miRNAs were differentially expressed using a FC value of ≥ 1.5 and a p value of < 0.05. Of these, two miRNAs (miR-146a-5p and miR-4640-5p) were predicted by TargetScan to bind the 3’UTR of STIM1. To validate these findings, INS-1 β-cells, and human islets were treated with or without IL-1β. Only miR-146a-5p was upregulated in both systems. Consistent with inverse correlation, INS-1 β-cells transfected with miR-146a-5p mimic showed reduced STIM1 expression. To test whether miR-146a-5p inhibition preserves STIM1 expression, INS1 cells were treated with miR-146a-5p inhibitor along with IL-1β and inhibition of miR-146a-5p led to partial preservation of STIM1 expression. Future studies will test the effect of miR-146a-5p mimics and inhibitors on SOCE. The results indicate that the stress induced by IL-1β leads to induction of miR-146a-5p, which may
target STIM1 mRNA. Such studies could enable broader implementation of miRNA in β-cell dysfunction.

Mark Goebl, Ph.D., Chair
# TABLE OF CONTENTS

LIST OF TABLES ........................................................................................................... ix

LIST OF FIGURES .......................................................................................................... x

INTRODUCTION ........................................................................................................... 1
    Diabetes: the epidemic of the century ................................................................. 1
    Importance of blood glucose and its regulation .............................................. 3
    A role for calcium in normal pancreatic β cell function ............................... 5
    The unfolded protein response (UPR) and ER stress ................................ 9
    Store operated calcium entry ......................................................................... 10
    MicroRNA as stress regulators in β cells and diabetes ............................... 12

HYPOTHESIS ............................................................................................................. 17

MATERIALS AND METHODS .................................................................................... 19
    Cell culture ........................................................................................................ 19
    Human islets ..................................................................................................... 19
    Primers, antibodies and reagents .................................................................. 20
    Cell culture and islet treatment ..................................................................... 21
    Transient miRNA transfection ....................................................................... 21
    Total RNA isolation and quantitative real-time PCR (qRT-PCR) .................. 22
    Immunoblot analysis ....................................................................................... 22
    Measurements of Ca^{2+} content in the ER .................................................... 23

RESULTS AND DISCUSSION ..................................................................................... 24
    The proinflammatory cytokine IL-1β does not affect the STIM1 protein and mRNA stability in INS-1 cells .......................................................... 24
    Inflammation mediated upregulation of miR-146a-5p in cytokine treated human islets .......................................................................................... 26
    The proinflammatory cytokine IL-1β causes upregulation of miR-146a-5p in INS-1 cells .......................................................................................... 29
    The proinflammatory cytokine stress reduced β-cell STIM1 expression in INS-1 cells and human islets .................................................................. 30
    IL-1β treatment leads to impaired SOCE ......................................................... 32
    Inhibition of miR-146a-5p in presence of cytokine stress leads to partial restoration of STIM1 at the translational level in INS-1 cells .................. 35

SUMMARY AND PERSPECTIVE ................................................................................. 37

FUTURE DIRECTIONS ............................................................................................... 40

REFERENCES ............................................................................................................ 41

CURRICULUM VITAE
LIST OF TABLES

Table 1. Donor demographics of human islets used in the study ............................................19
Table 2. Quantitative RT-PCR primers ........................................................................................20
Table 3. Antibodies for western blot............................................................................................20
Table 4. Chemicals and reagents ..................................................................................................21
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.</td>
<td>Approximately 425 million adults (20-79 years) were diagnosed with diabetes in the year 2017; this number is predicted to rise by 48% in 2045.</td>
</tr>
<tr>
<td>Figure 2.</td>
<td>A schematic illustration of nutrient regulated insulin secretion, in addition to glucose, some amino acids and fatty acids may also regulate insulin secretion.</td>
</tr>
<tr>
<td>Figure 3.</td>
<td>Schematic representation of insulin maturation along the granule secretory pathway showing transcription of preproinsulin mRNA and translation to preproinsulin peptide.</td>
</tr>
<tr>
<td>Figure 4.</td>
<td>Shows several causes of ER stress in β cell, ranging from high glucose, high fatty acid intake meals to cytokine mediated stress.</td>
</tr>
<tr>
<td>Figure 5.</td>
<td>Shows a tentative activation model of SOCE reflecting a STIM1 conformational switch and the dynamic coupling between STIM1 and Orai1.</td>
</tr>
<tr>
<td>Figure 6.</td>
<td>Shows miRNA functions in pancreatic β cell.</td>
</tr>
<tr>
<td>Figure 7.</td>
<td>Shows blood miRNA changes associated with diabetes mellitus.</td>
</tr>
<tr>
<td>Figure 8.</td>
<td>STIM1 mRNA and protein stability is unaltered in INS-1 cells following IL-1β treatment.</td>
</tr>
<tr>
<td>Figure 9.</td>
<td>miR-146a-5p is upregulated under inflammatory conditions in human islets.</td>
</tr>
<tr>
<td>Figure 10.</td>
<td>IL-1β treatment results in a trend towards upregulation of miR-146a-5p expression in INS-1 cells.</td>
</tr>
<tr>
<td>Figure 11.</td>
<td>IL-1β leads to STIM1 downregulation in INS-1 cells and human islets.</td>
</tr>
<tr>
<td>Figure 12.</td>
<td>IL-1β treatment causes impaired SOCE in INS-1 cells.</td>
</tr>
<tr>
<td>Figure 13.</td>
<td>miR-146a-5p inhibition restores IL-1β mediated loss of STIM1 protein expression in INS-1 cells.</td>
</tr>
</tbody>
</table>
INTRODUCTION

Diabetes: the epidemic of the century

Diabetes mellitus (DM) is a chronic disorder of glucose homeostasis that arises from insufficient insulin production or due to the inability of the body to effectively utilize insulin. According to estimates by the International Diabetes Federation (IDF), there 425 million persons were affected by diabetes worldwide in 2017 (Figure 1). Unfortunately, by 2045 this number is estimated to reach 629 million. The prevalence of diabetes has been rising more rapidly with global modernization, changes in lifestyle and food production practices that have impacted eating habits. There are mainly two types of diabetes. According to the Center for Disease Control (CDC), 90 to 95 percent of people with diabetes in the United States have type 2, whereas 5-10 percent have type 1.

Type 1 diabetes (T1D) is an autoimmune disease where the body’s immune cells destroy the insulin producing β-cells and as a result there is minimal insulin production. Type 1 diabetics usually are diagnosed with reduced β-cell function along with diminished insulin production and require immediate treatment with insulin (American Diabetes, 2009). Type 1 diabetes is on the rise, both in incidence and prevalence, with an annual estimated increased in incidence of about 2–3% per year (DiMeglio, 2018). TID is most common among children younger than 15 years. This rise in T1D over the years cannot be explained by genetic changes alone; clearly other factors are at play.

Type 2 diabetes (T2D) is a more chronic and complex form of diabetes that involves both insulin resistance (or inability of the body to use insulin efficiently) and pancreatic β-cell dysfunction. Early during the development of insulin resistance, blood glucose levels are maintained in the normal range as a result of increased insulin output from the β cell (Davidson, 1987). Chronically, however, this compensatory response often fails as β cell mass and insulin secretion begin to decrease. Genetic studies suggest that certain “at-risk” individuals are programmed towards β cell failure in settings of insulin resistance (like obesity or pregnancy) (Ashcroft, 2012). Additionally, genetic risks contribute to systemic and local factors (i.e. elevated glucose, pro-inflammatory cytokines, and free fatty acids) that are directly toxic to β cells, causing reduced insulin secretion and β cell death and de-differentiation. Both form of diabetes can be triggered by environmental factors, including
food habits, being overweight/obese, physical inactivity, and environmental exposures (i.e. viral infection in the case of T1D) (Knip, 2012). There are also several significant diabetes-associated comorbid conditions such as retinopathy, renal failure, and cardiovascular disease that increase the burden and cost of this disease (Leon, 2015). Altogether, rising rates of diabetes and diabetes complications contribute to an increased economic burden worldwide, often in regions that are ill equipped to handle these challenges. Among high income countries, it is estimated that 79% of global healthcare expenditure was spent on diabetes (IDF). A recent World Health Organization report estimated 1.6 million deaths were directly attributed to diabetes. Therefore, there is a need for research into strategies to understand the disease better, to not only treat it but also prevent the disease onset and preserve or restore β-cell function and greatly improving outcomes for those who have the disease.
Figure 1. Approximately 425 million adults (20-79 years) were diagnosed with diabetes in the year 2017; this number is predicted to rise by 48% by 2045. (Figure adapted from IDF Diabetes Atlas-8th edition, 2017).

Importance of blood glucose and its regulation

Under normal conditions, blood glucose levels are maintained in a narrow range. Fasting blood glucose levels are maintained between 70-100mg/dL and post meal blood glucose levels are maintained between 70-140mg/dL. This crucial task involves fine tuning of a highly sophisticated network between various organs including the brain, liver, muscle and pancreas (Figure 2). Within this network, the pancreas plays a vital role as it secretes the hormone insulin, which is the main blood glucose-lowering hormone and its opponent glucagon. Any disturbances in the interplay of these hormones and peptides involved in glucose homeostasis often leads to metabolic disorders including diabetes (Roder, 2016).

The pancreas is composed of exocrine and endocrine components (Tsuchitani, 2016). The exocrine pancreas aids in digestion through secretion of digestive enzymes and
bicarbonate. The endocrine pancreas participates in regulation of blood glucose. The endocrine pancreas is present as clusters known as islets of Langerhans, which are interspersed among the exocrine pancreas, and they represent about ~50% of islet of Langerhans in humans (Da Silva Xavier, 2018). Islets are comprised of five different cell types, each releasing distinct hormones. The α-cells produce glucagon; β-cells account for the maximum of the total endocrine cluster and produce insulin, C-peptide, and amylin; γ-cells produce pancreatic polypeptide (PP), while δ-cells produce somatostatin and ε-cells produce ghrelin.

The primary and most crucial pancreatic hormone is insulin, which triggers or stimulates uptake of glucose by cells and acts to lower blood glucose. Several hormones secreted from pancreas, brain and gut, including glucagon, glucocorticoids and epinephrine, work on peripheral tissues to increase blood glucose levels through alteration of metabolic processes, including gluconeogenesis and glycogenolysis (Aronoff, 2004). The process of maintaining blood glucose levels through the balanced actions of glucagon and insulin is known as glucose homeostasis. Glucose homeostasis is achieved through the coordination multiple mechanisms, including glucose transport, glycogen synthesis, lipogenesis, protein synthesis and cellular growth (Samuel, 2016).

Blood glucose increases with food intake as ingested nutrients are absorbed into the bloodstream and then carried to the cells for production of energy. This increase in blood glucose levels during the absorptive state is detected by the β cells in the pancreas, which release insulin to stimulate glucose uptake by adipose and muscle cells (James, 2004).
A role for calcium in normal pancreatic β cell function

In pancreatic β-cells, insulin secretion is mainly induced by glucose. When blood glucose rises, glucose molecules are transported into the β cell through the glucose transporter (GLUT2 in rodents or GLUT1 in humans) (De Vos, 1995). Glucose is then phosphorylated by glucokinase and subjected to glycolysis, generating pyruvate. Pyruvate is then metabolized by pyruvate dehydrogenase and pyruvate carboxylase and pathway products enter the TCA cycle, leading to generation of ATP and an increase in the ATP/ADP ratio (Komatsu, 2013). The increase in the ATP/ADP ratio results in closure of ATP-sensitive K+ (KATP) channels (Jensen, 2008). Closure of these channels leads to plasma membrane depolarization up to a threshold potential, causing the cell to move from quiescence to initiate electrical activity and voltage dependent calcium channel (VDCC) opening. Ca$^{2+}$ influx through VDCCs leads to increased cytosolic Ca$^{2+}$, leading to fusion of insulin-containing granules to the plasma membrane and release of insulin into the bloodstream, a process termed as exocytosis (Hivelin, 2016). As the exocytosis process is
glucose dependent, it is known as glucose stimulated insulin secretion (GSIS). Although an increase in intracellular Ca\(^{2+}\) is the primary signal that triggers insulin exocytosis, there are other cell signals activated by glucose that also play roles in this process. The signaling molecules include cAMP, cGMP, inositol 1,4,5-trisphosphate (IP3), and DAG (Gromada, 1999). In addition to a central role in GSIS, calcium also functions in insulin biosynthesis by participating in the secretory pathway and by acting as essential co-factor for enzymes located in ER and Golgi (Davidson, 1987).

The insulin gene encodes the preproinsulin polypeptide. Insulin is the posttranslational product of preproinsulin and is a globular protein containing two chains, A (21 residues) and B (30 residues) that are linked by disulfide bonds (Kim, 2011). As preproinsulin is synthesized in the cytoplasm with a signal peptide, it is cotranslationally translocated into the lumen of the ER through the interaction between the signal peptide and the signal recognition particle on the ER membrane (Figure 3). Upon passage of the preproinsulin polypeptide through the channel of the ER, the signal peptide of preproinsulin is cleaved in the ER lumen (Harding, 2002). Once proinsulin is passed fully inside the ER, proinsulin undergoes protein folding whereby three disulfide bonds are formed, which are essential for stability and bioactivity. Properly folded proinsulin is then delivered to the Golgi apparatus and packaged into secretory granules (Huang, 1994). The conversion of proinsulin to insulin takes place in the secretory granules, where the C peptide is removed. Mature insulin is then released by exocytosis. Apart from acting as co-factors to the enzymes involved in secretory pathways, Ca\(^{2+}\) is also dynamically stored within ER and Golgi, and it is suggested that insulin secretion can be amplified by Ca\(^{2+}\) release from these organelle stores (Wang, 2013).
Figure 3. Schematic representation of insulin maturation along the granule secretory pathway showing transcription of preproinsulin mRNA and translation to preproinsulin peptide (adapted from Tokarz, 2018).

To support robust insulin production and secretion, calcium is compartmentalized within the cell in a specific pattern. Under normal conditions, resting cells have an intracellular Ca\(^{2+}\) level in the range of 10 - 100nM that may increase 100-fold under certain stimulated conditions. Upon Ca\(^{2+}\)-entry from the extracellular space or via release from the internal Ca\(^{2+}\)-stores, the sudden addition of Ca\(^{2+}\) to the cell interior creates a relatively large rise in the intracellular Ca\(^{2+}\) concentration, which can change the cell membrane potential (Miller, 2001).

In order to maintain low basal intracellular Ca\(^{2+}\) levels there is a tight regulation of calcium entry and extrusion (Satheesh, 2015). For this purpose, all eukaryotic cells possess intracellular Ca\(^{2+}\), with the ER being a primary organelle for Ca\(^{2+}\) stores. The ER maintains its calcium via a Ca\(^{2+}\) pump Sarco- Endoplasmic Reticulum Ca\(^{2+}\)-activated ATPase (SERCA), which is similar, but not identical to the Na\(^+\)/K\(^+\) ATPase pumps present in the surface cell membrane (Pozzan, 1994). The ER also contains several calcium associated proteins, including calsequestrin, which are considered a crucial requirement for the calcium storage function as it is a Ca\(^{2+}\) binding protein. The Ca\(^{2+}\) concentration in this
compartment is typically about 100-500 µM (Alonso, 2006), which is about 1000 - 5000 times higher than in the surrounding cytosol.

The ER could in principle diminish cytosolic Ca\(^{2+}\) signals created by the opening of Ca\(^{2+}\) channels in the surface cell membrane or by absorbing some of the Ca\(^{2+}\) entering the cell. Alternatively, the ER can function as a source of Ca\(^{2+}\) to create intracellular Ca\(^{2+}\) signals that do not depend on entry of Ca\(^{2+}\) from the outside. Calcium release from the ER into the cytosol can be created by special Ca\(^{2+}\) channels, such as ryanodine receptors. Finally, the role of other important organelles contributing to cellular Ca\(^{2+}\) include the mitochondria and Golgi, which can clear cytosolic Ca\(^{2+}\) signals by up taking of Ca\(^{2+}\) into the inner mitochondrial and Golgi space via a special Ca\(^{2+}\) transporters, such as the secretory pathway ATPase (SPCA) in Golgi (Pozzan, 1994).

The ER is a dynamic organelle that participates in the entry of nascent polypeptides in the secretory pathway, along with subsequent protein folding, assembly, and trafficking. However, the ER also participates in several other vital cellular functions such as lipid biosynthesis, Ca\(^{2+}\) storage, and cell signaling (Teske, 2011). To carry out these cellular tasks, the ER contains several specialized proteins, including protein chaperones and foldases that aid in protein folding. Together, these proteins participate in quality control mechanisms that ensure the maintenance of organelle functions in ER protein and calcium homeostasis. Calcium dysregulation, oxidative stress and perturbations in protein folding mechanisms can lead to ER stress, which can ultimately result in β cell stress and death. β cell ER stress has been shown to play a central role in the pathogenesis of both T1D and T2D (Fonseca, 2011).

Dysregulation of protein or Ca\(^{2+}\) homeostasis results in ER stress, which triggers the Unfolded Protein Response (UPR). If the UPR can relieve the ER stress, cells can restore and establish ER homeostasis. However, if the UPR fails to resolve the underlying stress, these pathways will trigger apoptosis, leading to β-cell death and diabetes.
The unfolded protein response (UPR) and ER stress

ER stress elicit an unfolded protein response (UPR) that involves translational and transcriptional changes in gene expression aimed at expanding the ER processing capacity and alleviating cellular injury. Three ER stress sensors (PERK, ATF6, and IRE1) implement the UPR (Teske, 2011). There can be several physiological, environmental, and genetic factors that contribute to ER stress in β cells. For example, hyperglycemia is results in increased ER stress. (Fonseca, 2011). In the case of T1D, proinflammatory cytokines are responsible for precipitating early β-cell dysfunction. Various studies on cell lines and isolated human islets, and islets from NOD mice, and islets from cadaveric donors with T1D show evidence for activation of the UPR, which may be involve NF-κB signaling, (Tersey, 2012). In T2D there can be various underlying mechanisms like lipotoxicity, glucotoxicity, oxidative and cytokine stress, which can also result in unresolvable ER stress leading to β-cell dysfunction (Figure 4). Saturated long-chain FFAs are potent inducers of ER stress and cause β-cell failure and cell death. In T2D, a chronic hyperglycemic state often results in detrimental metabolites for β-cells. Glucotoxicity results by accumulation of excess ROS generated by mitochondrial oxidative phosphorylation and other alternative metabolic pathways. The pancreatic β-cell is vulnerable to ROS because it has a low antioxidative stress response as β-cells do not express catalase and low levels of other antioxidant enzymes like glutathioneperoxidase (Back, 2012). Together there are multiple stress arrangements that link induction of the UPR with progression of diabetes.
Figure 4. Shows several causes of ER stress in β cell, ranging from high glucose, high fatty acid intake meals to cytokine mediated stress.

**Store operated calcium entry**

Store-operated calcium entry is a major contributor to cellular calcium signaling and to the maintenance of cellular calcium balance in mammalian cells, especially in ER. SOCE is activated in response to depletion of intracellular Ca\(^{2+}\) stores in the ER. SOCE involves a complex interaction between the stromal interaction molecule 1 (STIM1) and Orai 1 (Figure 5). STIM1 is a calcium sensor that conveys the calcium load of the endoplasmic reticulum (ER) to store-operated channels (SOCs) at the plasma membrane (Yuan, 2007). Orai 1 or Calcium release-activated calcium channel protein, which is activated by STIM1, upon ER Ca\(^{2+}\) levels depletion. Under resting conditions, STIM1 EF-hand and SAM (EF-SAM) domain situated in the ER lumen is loaded with Ca\(^{2+}\) and remains largely as a monomer. Upon Ca\(^{2+}\) depletion in the ER, dissociation of Ca\(^{2+}\) from the EF-SAM domain leads to a destabilization-coupled oligomerization. As a result, STIM1 rearranges itself at the site of plasma membrane-ER junctions. Here the activated
STIM1 multimerizes and recruits Orai1 channel that completes the formation of a pore complex. The pore complex leads to calcium influx from the extracellular space into the cytoplasm and via SERCA into the ER, thereby restoring ER Ca$^{2+}$.

**Figure 5.** Shows a tentative activation model of SOCE reflecting a STIM1 conformational switch and the dynamic coupling between STIM1 and Orai1 (Ma, 2015).

Many studies have shown that loss- and gain-of-function gene mutations in Orai1 and STIM1 in rodent models and in humans can lead to disease. These diseases are collectively known as Calcium release activated channels (CRAC) channelopathies. Loss of function of STIM1 and Orai1 are generally characterized by severe combined immunodeficiency (SCID)-like diseases, autoimmunity, muscular hypotonia, and ectodermal dysplasia, with defects in dental enamel. The autosomal dominant gain-of-function mutations in these genes result in constitutive activation of SOCE and increased intracellular Ca$^{2+}$ levels that results in several other diseases, including non-syndromic tubular aggregate myopathy (TAM) and York platelet and Stormorken syndromes. The
molecular dysfunction of STIM1 and Orai1 leading to clinical diseases, including diabetes related complications (Yang, 2018), highlights the importance of the role of STIM1 and Orai1 in the molecular regulation of SOCE in human physiology (Lacruz, 2015).

STIM1 is critical for SOCE; however, its role in the regulation of pancreatic β cell Ca\(^{2+}\) homeostasis has only recently begun to be investigated. In 2018, our lab showed that gene and protein expression of STIM1 were reduced in human islets from donors with type 2 diabetes (T2D). This result associates the loss of STIM1 expression and impaired SOCE in rodent and human models of diabetes with decreased β-cell ER Ca\(^{2+}\) levels, increased ER stress, abnormal Ca\(^{2+}\) oscillation patterns, and decreased insulin secretion. We propose that loss of STIM1 and impaired SOCE contributes to pathophysiology seen in diabetic conditions (Kono, 2018). At present, the mechanisms underlying loss of STIM1 expression in diabetes have not been elucidated. This important question is addressed in my MS thesis project.

**MicroRNA as stress regulators in β cells and diabetes**

Micro RNAs (miRNAs) are a group of small noncoding RNAs ~22 nucleotide residues in length that have emerged as drivers of gene regulation of cell development, differentiation, and survival. The miRNAs mediate gene regulation at the post-transcriptional level via its formation of RNA-induced silencing complex (RISC), which is composed minimally of an AGO protein and a miRNA molecule. The target recognition of miRNA depends on the degree of complementarity between the miRNA and the target mRNA (Catalanotto, 2016). Complementary base pairing between mRNA and the mature miRNA typically occurs at the 3’ untranslated region (UTR) of the mRNA and the seed region (nucleotides 2–7 from the 5’ end) of the miRNA. The miRNA participates in RISC formation and then guides the complex to the target mRNAs. A perfect complementary binding between the seed sequence and the 3’UTR region of the target mRNA results in mRNA degradation, whereas imperfect binding, inhibits mRNA translation. (Gebert, 2019).
Figure 6. Shows miRNA functions in pancreatic β cell. (LaPierre, 2017).

There have been over 2,000 miRNAs discovered in humans and it is estimated that they collectively regulate one third of the genes in the genome (Hammond, 2015). The significance of miRNA-based regulation of gene expression has been described for several human diseases including diabetes (Figure 6). Recent studies established a role for miRNAs in pancreatic islets, including in the regulation of β-cell secretory function, proliferation, and survival (LaPierre, 2017). In addition, many recent studies have shown altered circulating levels of different miRNAs to be linked to type 1 and type 2 diabetes, suggesting they may also serve as disease biomarkers. Figure 7 provides a list of dysregulated miRNAs most commonly associated with diabetes.

Given that STIM1 expression is reduced significantly under cytokine stress, we wished to determine whether miRNA expression is enhanced in the same model system. The preliminary RNA sequencing data obtained from the cadaveric islets of human donors,
which were treated with IL-1β and IFN-γ for 24 hours, showed a total of 20 miRNAs that be differentially expressed. Among these, one miRNA (miR-146a-5p) was validated by RT-PCR in rat INS-1 β-cells and human islets, which were treated with or without IL-1β for 24 hours.

There is evidence suggesting that miR-146a, is transcriptionally regulated by NF-κB and has a role in the regulation of the acute inflammatory responses (Mann, 2018). This miRNA has been found to be upregulated by TNF and IL-1β and in turn targets TRAF-6 and IRAK-1 expression (Assmann, 2017). miR-146 was first identified in mouse cardiac tissue in a study by LagosQuintana et al. (2002) and in humans was confirmed by Taganov et al. (2006). The primary transcripts of miR-146a-5p (pri-miR) are transcribed from two different genes, MIR146A and MIR146B. In humans, MIR146A is encoded in a larger long noncoding RNA host gene, MIR3142HG (chromosome 5q33.3), while MIR146B is found in an intergenic region of human chromosome 10 (10q24.32). The mature sequences for miR-146a and miR-146b are highly conserved among mammals, including in rat, mouse and chimpanzee (Paterson, 2017).
Figure 7. Shows blood miRNA changes associated with diabetes mellitus (Guay, 2013).

In diabetes, hyperglycemia and elevated free fatty acids often promote inflammation by contributing to increased oxidative and ER stresses. These events lead to NF-κB mediated release of a broad range of cytokines and chemokines including tumor necrosis factor (TNF) and IL-1β. IL-1β is known to further amplify the inflammation by recruitment of immune cells including macrophages, contributing to β-cell dysfunction, and loss of β-cell mass. Therefore, the elucidation of micro-RNA involved in inflammatory molecular events is essential to determine the causes of diabetes and develop new treatments for the disease (Pollack, 2016).

There are a number of recent publications on miR-146, but little is known about its function and regulation in β-cell ER Ca²⁺ homeostasis. To address the linkage between inflammation, miRNAs and intracellular calcium in β-cell, this thesis have focused on cytokine stress mediated upregulation of miR-146a-5p and a potential role for this miRNA
in the regulation of SOCE, focusing specifically on the question of whether miR-146a-5p regulates STIM1 expression. Finally, in the alter portion of this MS thesis, I examined the molecular mechanism by which miR-146a-5p causes downregulation of STIM1 under conditions associated with cytokine stress.
**HYPOTHESIS**

My central hypothesis is that pro-inflammatory cytokine stress induced upregulation of miR-146a-5p leads to STIM1 downregulation, SOCE dysregulation, disruption of ER Ca\(^{2+}\) homeostasis, and impaired insulin secretion. To test this hypothesis, I pursued the following two specific aims:

**Aim 1**: Define pathways leading to STIM1 downregulation and miR-146a-5p upregulation in rat INS-1 β-cell line and human islets.

The working hypothesis of Aim 1 was that IL-1β induced cytokine stress results in upregulation of miR-146a-5p, which leads to downregulation of STIM1. To test this hypothesis, differentially expressed miRNAs were identified from human islets treated with or without a cocktail of pro-inflammatory cytokines (IL-1β and IFN-γ) for 24 hours. Total RNA was isolated from the islets and subjected to miRNA sequencing using Ion Proton system. miRNAs with a fold change of >= 1.5 and a p-value of < 0.05 were considered for differential expression. Twenty miRNAs were found to be differentially expressed and among these, two miRNAs (miR-146a-5p and miR-4640-5p) were predicted by TargetScan database to bind to the 3’UTR of STIM1. Further validation using qRT-PCR confirmed over expression of miR-146a-5p in human islets treated with IL-1β and IFN-γ. Whereas, miR-4640-5p did not show a significant change in its expression after 24 hours of cytokine exposure. To confirm cytokine-mediated upregulation of miR-146a-5p in other model systems, I treated rat β-cell lines with IL-1β for 24 hours and through qRT-PCR analysis showed that IL-1β causes a significant upregulation of miR-146a-5p in the rat INS-1 β-cell line.

Consistent with the notion that miR-146a-5p might regulate STIM1 expression, I also performed qRT-PCR and Western blot to check for the expression of STIM1 mRNA and protein in the same samples. STIM1 mRNA and protein was found to be downregulated upon cytokine exposure. Based on this, I chose to further investigate the underlying mechanisms and functions of the cytokine-induced expression of miR-146a-5p and STIM1 downregulation.
**Aim 2**: Establish a direct regulation of STIM1 by miR-146a-5p.

The working hypothesis of Aim 2 is that cytokine stress upregulates the miR-146a-5p, which then binds to 3’UTR region of STIM1 and causes downregulation at post-transcriptional levels.

To test this hypothesis, I began by looking into whether IL-1β treatment impacted STIM1 mRNA or protein half-life directly. To this end, actinomycin D and cycloheximide chase experiments were performed. Interestingly, IL-1β had no impact on either mRNA or protein stability, suggesting an alternative mechanism of STIM1 downregulation. This led to experiments aimed at defining a direct role for miR-146a-5p in STIM1 regulation. To test this, I transfected rat INS-1 β-cell line with miR-146a-5p inhibitors with or without IL-1β treatment. Since the mechanism of miRNA action is based on the specificity of its 5′-UTR seed region, and complementary binding to the 3′-UTR sequence in the corresponding target mRNA, miRNA inhibitors and mimics commonly referred as synthetic oligomers are frequently employed, which either are complementary oligonucleotides to mature miRNAs (inhibitors/antagomir) that prevent interactions between targets and their corresponding miRNAs or have a sequence identical to a specific miRNA (mimics/agomir) that bind to their corresponding targets and regulate their expression. Methods to transfect these synthetic oligomers employ liposomes in an in-vitro setting to study the effects of over-expression or knock-out studies of a specific miRNA (Rebustini, 2016). STIM1 protein levels were analyzed using Western blot, and STIM1 protein was found to be partially preserved.
MATERIALS AND METHODS

Cell culture

INS-1 832/13 rat insulinoma cells were cultured in 11mM glucose Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 1mM sodium pyruvate, 50μM β-mercaptoethanol and, 10% FBS, penicillin and streptomycin.

Time course experiments were performed to determine the half-life of STIM1 protein and mRNA using 10µM cycloheximide or 1µg/ml actinomycin D, respectively.

Human islets

Human cadaveric islets isolated from non-diabetic donors were obtained from the Integrated Islet Distribution Program or the National Disease Research Interchange (Table 1). Upon receipt, human islets were hand-picked and allowed to recover overnight in Dulbecco’s modified essential medium (DMEM) medium containing 5.5mM glucose, 10% fetal bovine serum (FBS), 100U/ml penicillin, and 100µg/ml streptomycin

Table 1. Donor demographics of human islets used in the study

<table>
<thead>
<tr>
<th>Number</th>
<th>Gender (male/female)</th>
<th>BMI (kg/m²)</th>
<th>AGE (years)</th>
<th>Ethnicity</th>
<th>Purity (%)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>31.8</td>
<td>31</td>
<td>Hispanic/latin</td>
<td>80</td>
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<tr>
<td>2</td>
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<td>44</td>
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<td>90</td>
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<tr>
<td>3</td>
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<td>24.1</td>
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<td>80</td>
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<tr>
<td>4</td>
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<td>21.3</td>
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<td>5</td>
<td>Female</td>
<td>35.7</td>
<td>55</td>
<td>Caucasian</td>
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<tr>
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<td>25</td>
<td>Caucasian</td>
<td>95</td>
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<tr>
<td>7</td>
<td>Male</td>
<td>28.2</td>
<td>38</td>
<td>Hispanic/latin</td>
<td>90</td>
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</table>
**Primers, antibodies and reagents**

Standard polymerase chain reaction (PCR) reactions based on exponential amplification of the desired region of the DNA was used for cDNA synthesis and quantitative reverse transcription-PCR (qRT-PCR) to measure mRNA expression levels were performed using oligonucleotides synthesized by Qiagen as outlined in Table 2. Antibodies used for immunoblotting immunofluorescence experiments and are listed in Table 3. Chemicals and reagents used in this dissertation are listed in Table 4.

**Table 2. Quantitative RT-PCR primers**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Orientation</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>STIM1 (H/M/R)</td>
<td>Forward (5’-3’)</td>
<td>AGCCTCAGCCATAGTCACAG</td>
</tr>
<tr>
<td></td>
<td>Reverse (5’-3’)</td>
<td>TTCCACATCCACATCACCATTG</td>
</tr>
<tr>
<td>GAPH (human)</td>
<td>Forward (5’-3’)</td>
<td>CAGCCTCAAGATCATCAGCAA</td>
</tr>
<tr>
<td></td>
<td>Reverse (5’-3’)</td>
<td>TGTGGTCATGAGTCTTCCA</td>
</tr>
<tr>
<td>Actin (H/M/R)</td>
<td>Forward (5’-3’)</td>
<td>AGGTCATCCTATTGGCAACA</td>
</tr>
<tr>
<td></td>
<td>Reverse (5’-3’)</td>
<td>CACTTCATGATGGAATTAGTT</td>
</tr>
<tr>
<td>miR-146a-5p</td>
<td>Forward (5’-3’)</td>
<td>'UGAGAACUGAAUUCAGGGUU</td>
</tr>
</tbody>
</table>

**Table 3. Antibodies for western blotting**

<table>
<thead>
<tr>
<th>Targeted Protein</th>
<th>Host Species</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>STIM1</td>
<td>Mouse/Rat/Human</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>Actin</td>
<td>Mouse/Rat/Human</td>
<td>SigmaAldrich</td>
</tr>
</tbody>
</table>

20
### Table 4. Chemicals and reagents

<table>
<thead>
<tr>
<th>Name</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycloheximide</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Lipofectamine RNAiMAX Transfection Reagent</td>
<td>Invitrogen</td>
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<tr>
<td>M-MLV reverse transcriptase</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Hs_RNU6-2_11 miScript Primer Assay (100)</td>
<td>Qiagen</td>
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<tr>
<td>miScript II RT Kit (50)</td>
<td>Qiagen</td>
</tr>
<tr>
<td>miRNeasy Mini Kit (50)</td>
<td>Qiagen</td>
</tr>
<tr>
<td>Recombinant mouse and human interleukin-1 β (IL-1β)</td>
<td>Life Technology</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>FLIPR Calcium 6 assay</td>
<td>VWR International</td>
</tr>
<tr>
<td>STIM1 3'UTR Lenti-reporter-luciferase vector</td>
<td>ABM</td>
</tr>
</tbody>
</table>

### Cell culture and islet treatment

To mimic pro-inflammatory and diabetic conditions, INS-1 cells were treated in RPMI media containing 5 ng/mL of human IL-1β for indicated times. For human islet studies, DMEM media containing 5 ng/mL of human IL-1β, 10 ng/mL TNF-α, and 100 ng/mL IFN-γ was used. For protein and mRNA half-life studies, time-course experiments were performed using 10μM cycloheximide or 1μg/ml actinomycin D, respectively, with or without the 5 ng/mL of human IL-1β at indicated time points and total protein and RNA was isolated.

### Transient miRNA transfection

INS1 cells were transfected with miR-146a-5p mimics or inhibitors (Qiagen) or a negative control (containing scrambled sequence of the miR-146a-5p) using lipofectamine as transfection reagent. All transfections had a final oligo concentration 25nmol/ml (miR-146a-5p inhibitor). The cell media was changed at the day of transfection to media without antibiotics for 30 minutes prior to transfection. Two separate tubes, one containing the OptiMEM (Invitrogen) and Lipofectamine RNAiMAX transfection reagent and other containing the synthetic oligos, were mixed and incubated for 5 minutes at room
temperature. The contents of the two tubes were mixed in 1:1 and incubated at room temperature for 20 minutes, the transfection medium was removed after overnight incubation at 37°C in a humidified atmosphere of 5% CO2. Cells were then allowed to recover for at least another 24 hrs in INS-1 complete media. In the case of miR-146a-5p inhibitor experiments, cells were stimulated with 5 ng/mL of recombinant human IL-1β. Furthermore, the expression level of the transfected miR mimic and inhibitor was examined by total RNA isolation followed by q-PCR.

Total RNA isolation and quantitative real-time PCR (qRT-PCR)

Cultured cells or isolated islets were processed for total RNA isolation using miRNeasy mini (Qiagen, Valencia, CA) plus for miRNA and or RNeasy mini kit (Qiagen, Valencia, CA) for mRNA, according to manufacturer’s instructions. For reverse transcription, total miRNA was processed using miScript II RT Kit according to manufacturer’s instructions or in case of total mRNA by using Moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen, Grand Island, NY) on Eppendorf thermal cycler. Subsequently, qRT-PCR was performed using JumpStart™ Taq DNA Polymerase (Sigma-Aldrich, St. Louis, MO), SYBR Green I dye for mRNA and miScript SYBR green pcr kit for miRNA, on applied biosystems Quant Studio 3 machine, primers listed in Table 2.

Immunoblot analysis

Cells were lysed in 1% IGEPAL reagent supplemented with 10% glycerol, 16 mM NaCl, 25 mM HEPES, Sigma-Aldrich, St. Louis, MO), 60 mM n-octylglucoside (Research Products International Corp.), phosphatase inhibitor cocktails (PhosSTOP tablets, Roche) and protease inhibitor cocktails (EDTA-free cOmplete tablets, Roche). Protein concentration was measured using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA) and a SpectraMax M5 multiwell plate reader (Molecular Devices, Sunnyvale, CA). Equal concentrations of proteins were suspended in 10% SDS solution and heated to 70°C for 5 minutes. Protein lysates were electrophoresed and transferred to methanol activated PVDF membrane (Immobilon-FL Transfer Membrane from Millipore). Membranes were then blocked using Odyssey blocking buffer (LI-COR, Lincoln, NE) prior to incubation with
primary antibodies listed in Table 3. Subsequently, membranes were incubated with IRDye 800 or 680 fluorophore-labeled secondary antibodies from LI-COR. Protein bands were visualized using the Odyssey System (LI-COR, Lincoln, NE) and quantified with Image J software (NIH). The protein levels presented in bar graphs were normalized to actin protein levels.

**Measurements of Ca^{2+} content in the ER**

Cytosolic Ca^{2+} dynamics in INS-1 cells were measured using the FLIPR Calcium 6 Assay Kit and a FlexStation 3 plate reader (Molecular Devices, Sunnyvale, CA). To measure SOCE, INS-1 β-cells were loaded with Calcium 6 in growth medium containing 11 mM glucose for 2h. Immediately prior to Ca^{2+} imaging, cells were incubated in Ca^{2+}-free HBSS with the following composition: 138 mM NaCl, 5.3 mM KCl, 0.34 mM \( \text{Na}_2\text{HPO}_4 \), 0.44 mM \( \text{KH}_2\text{PO}_4 \), 4.17 mM \( \text{NaHCO}_3 \), and 5.5 mM glucose for 4 minutes. Baseline (F0) fluorescence was measured for a minimum of 10 seconds under Ca^{2+} free conditions and in the presence of 0.5 mM EGTA (Ca^{2+} chelator), 10 μM verapamil (L-type voltage-dependent Ca^{2+} channel [VDCC] blocker), and 200 μM diazoxide (K\text{ATP} channel opener applied to prevent VDCC activation). Next, thapsigargin (TG; a SERCA inhibitor) was used to empty ER Ca^{2+} stores, followed by supplementation with 2 mM Ca^{2+} in the media. SOCE was detected as an elevation of Calcium 6 intensity (ΔF) in response to Ca^{2+} addition, which was normalized to the basal (F0), according to the formula ΔF/F0.
RESULTS AND DISCUSSION

The proinflammatory cytokine IL-1β does not affect the STIM1 protein and mRNA stability in INS-1 cells

Previous work by our group has demonstrated that there is significant downregulation of STIM1 mRNA and protein levels under diabetic conditions (Kono, 2018). To determine whether this downregulation was secondary to alterations in either mRNA or protein stability, half-life measurements were performed under basal conditions and following treatment with the pro-inflammatory cytokine IL-1β. Under control conditions, β cell STIM1 mRNA exhibited a half-life of ~6 h, and IL-1β treatment had no significant effect on mRNA stability (Figure 1A).

Similarly, STIM1 protein in INS-1 cells exhibited a half-life of ~24 h under basal conditions (Figure 8), and IL-1β treatment had no impact on the half-life of STIM1 protein.
Figure 8. STIM1 mRNA and protein stability is unaltered in INS-1 cell following IL-1β treatment.
INS-1 cells were treated with 1µM actinomycin combined with or without 5ng/ml IL-1β for indicated times. Total RNA was isolated, and RNA was subjected to real-time qRT-PCR for quantification of STIM1 and actin transcript levels (A). INS-1 cells were treated with 10µM cycloheximide (CHX) combined with or without 5ng/ml IL-1β for the indicated times. Total protein was isolated and immunoblot was performed using antibodies against STIM1 and actin (B). Results were analyzed as amount of mRNA and protein remaining at each time point. Shown are the mean ± SEM from three independent experiments. Differences between groups were examined for significance using a two-tailed Student t test.

**Inflammation mediated upregulation of miR-146a-5p in cytokine treated human islets**

Recent studies have identified miRNAs as key regulators of β cell development, glucose-stimulated insulin secretion (GSIS) and β cell dysfunction (Hashimoto, 2017) (Osmai, 2016), (Sims, 2017). Global expression profiling performed in human and rodent islets under inflammatory stress, a common phenomenon seen in type 1 and type 2 diabetes, has demonstrated marked changes in miRNA expression patterns (Osmai, 2016). To identify potential miRNAs candidates that may play a role in cytokine-mediated STIM1 downregulation, cadaveric islets from human donors were treated with IL-1β and IFN-γ for 24 hrs to mimic in vivo diabetic conditions and subjected to RNA sequencing. These experiments were performed by Dr. Farooq Syed and Dr. Preethi Krishnan. A total of 20 miRNAs were found to be differentially expressed using a FC value of ≥ 1.5 and a P value of < 0.05. Of these, two miRNAs (miR-146a-5p and miR-4640-5p) were predicted by the TargetScan database to bind to the 3’UTR of STIM1 (Figure 9).

To further test the RNA-seq findings, cadaveric human islets from 10 donors without diabetes were obtained from the Integrated Islet Distribution Program and subjected to cytokine treatment (5 ng/mL IL-1b and 100 ng/mL IFN-γ). Total RNA was isolated and subjected to qRT-PCR for quantification of miR-146a-5p and miR-4640-5p expression levels. The data presented include the analysis of islets from 10 donors without diabetes (5 female; 5 male) and the average age (±SEM) of the donors without diabetes.
was 45.9 ± 10.3 years; the mean BMI was 25.6 ± 4.7 kg/m2. Cytokine treatment led to a significant increase in miR-146a-5p expression levels in human islets, whereas there was no significant increase in miR-4640-5p expression (Figure 10). The differences between groups were analyzed for statistically significant differences using Mann Whitney test and a p value of < 0.05 was used to indicate a significant difference between groups (*p < 0.05, ***p < 0.001).

miRNA 146a (miR-146a) is an NF-κB-dependent miRNA that has been shown to be induced in cytokine mediated inflammatory response (Taganov, 2006). However, the role of miR-146a-5p in regulation of STIM1 in β-cells has not been tested. Using human islets and INS-1 rat β cells, we sought here to define the context of β-cell miR-146a-5p upregulation and to test a potential role for miR-146a-5p in the regulation of STIM1 expression and SOCE.
Figure 9. miR-146a-5p is upregulated under inflammatory conditions in human islets.
The proinflammatory cytokine IL-1β causes upregulation of miR-146a-5p in INS-1 cells

To validate the findings of the RNA sequencing, I used the rat INS-1 832/13 β cell line. INS-1 cells were treated with or without the pro-inflammatory cytokine IL-1β for 24 h, and real-time qRT-PCR was used to quantify levels of miR-146a-5p and RNU6.

**Figure 10.** IL-1β treatment results in a trend towards upregulation of miR-146a-5p expression in INS-1 cells.

INS-1 cells were treated with or without 5ng/ml IL-1β for 24hrs (A). Total RNA was isolated, and RNA was subjected to real-time qRT-PCR for quantification of miR-146a-5p and RNU6 levels. RNU6 is small non-coding RNA that has been demonstrated to have uniform expression stability across very different biological backgrounds; RNU6 was used
to normalize qRT-PCR results. Differences between the groups were examined for significance using a two-tailed Student t test using GraphPad Prism statistics software (GraphPad Software, Inc., San Diego, CA).

The proinflammatory cytokine stress reduced β-cell STIM1 expression in INS-1 cells and human islets

To define whether STIM1 expression was impacted under in vitro diabetic stress conditions, human islets obtained from cadaveric donors without diabetes (ND donors) and rat INS-1 cells were treated with 5 ng/mL IL-1β for 24hrs. Cytokine treatment led to a significant reduction in STIM1 mRNA levels in INS-1 cells (Figure 11A), while a trend toward reduced STIM1 mRNA levels were observed in human islets (C). Next, STIM1 protein levels were measured in IL-1β-treated INS-1 cell and found to be reduced ~2-fold in response to cytokine treatment (B).
Figure 11. IL-1β leads to STIM1 downregulation in INS-1 and human islets.

INS-1 cells (A, B) and human islets (C) were treated with or without 5ng/ml IL-1β for 24hrs. Real-time qRT-PCR and immunoblot were used to quantitate STIM1 transcript and protein levels, respectively and results were normalized to actin levels in both cases. Differences between the groups were examined for significance using a two-tailed Student \( t \) test using GraphPad Prism statistics software (GraphPad Software, Inc., San Diego, CA). Results were displayed as the mean ± SEM; a p value of <0.05 was used to indicate a significant difference between groups.
IL-1β treatment leads to impaired SOCE

Defective Ca\(^{2+}\) handling is a key mechanism underlying pancreatic β-cell endoplasmic reticulum (ER) dysfunction. We found that under cytokine-mediated stress, human and rodent β-cells led to upregulation of miR-146a-5p with a simultaneous decrease in STIM1 expression. To define the impact of cytokine treatment on SOCE, INS-1 cell lines were cultured in a 96 well plate and treated with IL-1β for 24hrs. SOCE was measured according to the strategy outlined in Material and Methods using a FLIPR Calcium 6 assay.
Figure 12. IL-1β treatment causes impaired SOCE in INS-1 cells.
INS-1 cells (A, B) were treated with or without 5ng/ml IL-1β for 12hrs and 24hrs. INS-1 cells were loaded with Calcium 6, and Ca²⁺ imaging was performed in the presence of 5.5 mmol/L glucose, 200 mmol/L diazoxide, and 10 mmol/L verapamil (Dz + V). (A) and (B) show significant reduction in SOCE after treatment IL-1β at both 12hrs (p-value <0.0001) and 24hrs (p-value <0.0001) compared to untreated control cells. Results were displayed as the mean ± SEM; and results were analyzed using two-tailed Student t test GraphPad Prism statistics software. (*p < 0.05, ***p < 0.001, ****p<0.0001).
Inhibition of miR-146a-5p in presence of cytokine stress leads to partial restoration of STIM1 at the translational level in INS-1 cells

To test whether inhibiting miR-146a-5p can preserve STIM1 expression, INS-1 cells were treated with IL-1β combined with or without the miR-146a-5p inhibitor (Qiagen) for 24hrs, followed by an immunoblot analysis of isolated proteins using antibodies against STIM1 and actin.

**Figure 13.** miR-146a-5p inhibition restores IL-1β-mediated loss of STIM1 protein expression in INS-1 cells.

miR-146a-5p inhibitor (Qiagen) for 24hrs, followed by an immunoblot analysis of isolated proteins using antibodies against STIM1 and actin.

INS-1 cells (A) were transfected with 25nM of miR-146a-5p inhibitor, one day before 5ng/ml of IL-1β treatment for 24hrs along with control (cells not transfected with
miR-146a-5p inhibitor), partial restoration of the STIM1 protein was observed in inhibitor treated group. Differences between the groups were examined for significance using one-way ANOVA using GraphPad Prism statistics software (GraphPad Software, Inc., San Diego, CA). Results were displayed as the mean ± SEM; a p value of <0.05 was used to indicate a significant difference between groups.
SUMMARY AND PERSPECTIVE

Our previously published study showed that loss of STIM1 in pancreatic β-cells leads to impairment of glucose-stimulated Ca\(^{2+}\) oscillations and reduced insulin secretion while simultaneously increasing susceptibility to ER stress and cell death in response to proinflammatory conditions. However, the downstream pathways that regulate STIM1 expression and altered endoplasmic reticulum Ca\(^{2+}\) in the β cell have not been fully defined.

To establish a pathophysiological role of downregulated STIM1 in pancreatic β cell, a functional analysis of SOCE was carried out following the cytokine treatment in INS-1 cell (Figure 12a and 12b), which showed a significant decrease in SOCE, indicating an imbalance in intracellular calcium levels, which can lead to development of ER stress and β-cell dysfunction.

The overall goal of this project was to determine the molecular pathway that are involved in STIM1 downregulation under inflammatory stress conditions.

To address this, we first determined the half-life of STIM1 protein and mRNA under basal and pro-inflammatory conditions. Data from INS-1 cells demonstrated that treatment with the cytokine IL-1β had no significant effect on STIM1 mRNA and protein half-life (Figure 8a and 8b). These data are suggestive that loss of STIM1 protein expression could be secondary to a reduction in translation, which is a mechanism of action compatible with miRNA-mediated regulation.

Many studies have reported abnormal β cell microRNA (miRNA) expression in diabetes and have been shown to act as critical regulators of β cell differentiation, development, death and function, and have also been identified as mediators of the complex β cell response to inflammatory stress (Bernardo, 2012)

A comprehensive review has recently summarized miRNAs in β-cell biology, insulin resistance, and diabetes related complications (Fernandez-Valverde et al., 2011). However, miRNAs involved in inflammatory processes during both form of diabetes have not been reviewed yet.

To determine the differentially expressed microRNAs in response to cytokine mediated environment, human islets collected from non-diabetic cadaveric donors were treated with or without cytokine for 24hrs (Figure 9) followed by total RNA isolation and
cDNA library preparation, which was subjected to small RNA sequencing. Out of 20 differentially expressed microRNAs, only two microRNA ie miR-146a-5p and miR-4640-5p were predicted to target STIM1 mRNA using TargetScan, which is a bioinformatics tool that predicts biological targets of microRNAs by searching for the presence of sites that match the seed region of each miRNA. Based on this analysis, of these two microRNAs, only miR-146a-5p upregulation was validated through qRT-PCR validation.

Cardozo et al previously described a role for pro-inflammatory cytokines and NFκB dependent upregulation of inducible nitric oxide synthase (iNOS) as a precipitant of ER stress and loss of β cell function in models of diabetes (Kharroubi, 2004).

We hypothesized that NFκB dependent miR-146a-5p is involved in STIM1 expression, at posttranslational mechanism.

To test this hypothesis, we used rat insulinoma cell line, INS-1, a commonly used cell line model for diabetes and human islets obtained from human cadaveric donors.

To validate the relevancy of our findings, rodent INS-1 cell line (Figure 10) were treated with and without IL-1β and showed an increase in trend of miR-146a-5p upregulation consistent with our RNA sequencing analysis. We next examined the changes in the level of the STIM1 mRNA and protein following the proinflammatory cytokine treatment in INS-1 and human Islets (Figure 11a-c). As predicted, there was a significant decrease in both STIM1 mRNA and protein in INS-1 and STIM1 transcript levels in human islets.

Finally, to confirm a more direct role of miR-146a-5p mediated effect on STIM1 protein levels, immunoblots for STIM1 protein were performed on INS-1 cells after transfection with an inhibitor of miR-146a-5p. This analysis demonstrated partial restoration in the levels of STIM1 protein (Figure 13). Although the above data are suggestive of a direct role, future experiments are needed to confirm this, future work will use in vitro β cell-specific models to study the effects of miR-146a-5p over-expression along with the state-of-the-art techniques like biotin labelled miRNA pull-down assay and luciferase assay.

There are several limitations to our study, several artifacts have been reported involving transient miRNA transfection experiments, such as transfection of miRNA mimics/inhibitor at high concentrations may alter gene expression in a non-specific
manner, while at low concentrations may failed to efficiently suppress target gene expression. The non-specific effects of miRNA mimics/inhibitor may be caused by the supraphysiological levels of mature miRNAs and the accumulation of mutated guide strands. (Søkilde et al., 2015). In addition, nuclear factor-kB has been shown to directly bind and modulate STIM1 gene promoter activity in other cell types (DebRoy, 2014), we and others have previously identified a deleterious effect of nuclear factor-kB–mediated nitric oxide signaling on ER calcium regulation (Tong, 2015) which can lower the STIM1 expression. Notwithstanding these other possible regulatory mechanisms of STIM1 expression, our results suggest a novel miRNA mode of regulation in STIM1 expression and SOCE in the beta cell. Thus, providing an important insight into the role of inflammation-induced elevations in β-cell miR-146a-5p during the development of diabetes and may function as early markers of diabetic homeostasis changes or may offer new therapeutic targets.
FUTURE DIRECTIONS

Taken together, the data shows that miR-146a-5p might be playing a role in STIM1 regulation. However, the mechanism for this regulation is not yet clear. To establish a direct molecular interaction between STIM1 and miR-146a-5p and using INS-1 β cells as model system, a micro-RNA pulldown assay will be performed followed by a luciferase assay.

A sensitive and specific biochemical method to identify miR-146a-5p targets is pulldown assay. To check STIM1 mRNA being targeted by miR-146a-5p, INS-1 cells will be transfected with biotinylated miR-146a-5p mimic, and mRNAs significantly enriched in the biotinylated-miRNA will be pulled down with streptavidin, followed by a total RNA isolation and qRT-PCR to check STIM1 mRNA enrichment. This then will be followed by a luciferase assay to see a direct interaction between miR-146a-5p 5’ UTR seed region and 3’UTR STIM1 mRNA region. Luciferase assay is commonly used for studying miRNA-mediated, post-transcriptional regulation of target genes. This is achieved by a luciferase gene construct containing the predicted miRNA targeting sequence from the target gene (often located in the 3’-UTR) (Jin, 2013).

I will next test ER stress along with SOCE functioning through RT-PCR using ER stress marker, spliced XBP1 mRNA, as spliced XBP1 mRNA is induced by activated IRE1 and ATF6, a signaling pathway that are activated in response to ER stress, followed by a functional analysis of SOCE by measuring cytosolic and ER Ca²⁺ levels using Calcium 6 assay, in miR-146a-5p transfected INS-1 β-cell line.

Thus, I am hopeful this study will enable broader understanding of the role of miRNAs in inducing β-cell dysfunction in diabetes and provide new insights into the regulatory networks of noncoding small micro-RNAs. This work has the potential to provide novel insight into the molecular processes that drive the development of diabetes. These studies may reveal potential microRNAs that could have translational impact, either as biomarkers of disease or as targets of pharmacological interventions for the treatment of metabolic diseases.
REFERENCES


CURRICULUM VITAE
Sukrati Kanojia

Education:
Indiana University, Indianapolis, Indiana
2017-2019
• Completed MS degree in the Department of Biochemistry and Molecular Biology.
• Thesis title: miRNA-146a-5p mediated Regulation of Stromal Interaction Molecule 1 and Store-Operated Calcium Entry in the Pancreatic-β cell in Response to Cytokine-Mediated Stress.

Nagpur University, Nagpur, Maharashtra, India
2011-2013
• Completed MSc degree in Biotechnology.
• Thesis title: In vitro and In vivo studies on the effect of Nitric Oxide in intracellular survival of Staphylococcus aureus at Indian School of Science, Bangalore, Karnataka, India.

St. Aloysius College, Jabalpur, Madhya Pradesh, India
2008-2013
• Completed BSc degree in Biotechnology.

St. Joseph’s Convent School, Jabalpur, Madhya Pradesh, India
2006-2008
• Completed academic honors diploma, graduating with distinction.

Employment:
Academic Experience:
Indian Institute of Science (IISc), Bengaluru, India
Dec 2012-June 2013: Project Trainee
Performing experiments for various ongoing research projects at the laboratory, handling animal models, laboratory management to improve workflow and maintaining biosafety requirements of the laboratory under the institutional guidelines.

Identified and tested selective media and enriched media formulations for Staphylococcus aureus (ATCC 25923)
Cultured and maintained RAW264.7, U937 and HaCat cell lines
Performed Intracellular survival assay and Nitrite assay.

**Biotech Consortium India Limited (BCIL), Bhopal, Madhya Pradesh, India**
**October 2013-April 2014: Research Intern**
- Developed and validated viral nucleic acid extraction protocols from diverse clinical samples.
- Developed and optimized PCR-based detection assay for oncogenic HPV infection.
- Performed restriction length polymorphism for genotyping of HPV.

*Industrial Experiences:*
**3BBlack Bio Biotech India Limited, Bhopal, India**
**May 2014-May 2015: Research Associate**
- Cloned gene constructs for expression of recombinant proteins with affinity peptide tags
- Developed protocol for in-house large-scale production and purification of Taq DNA polymerase enzyme.
- Running Gradient, Nested and q-PCR for primer validation and optimization.
- R&D involved in NASBA Techniques.
- Designing and Optimization of Molecular biology kit-based diagnosis of Mycobacterium tuberculosis, Human papillomavirus.
- Running Cellular and biochemical assays like ELISA and Line Probe assay (LiPA).
- Presented data from experiments at weekly-organized project team meetings
- Coordinated with scientists and reported to the project team lead for validation.
- Handling of diverse human clinical samples and their processing.
Indiana University School of Medicine, Indianapolis, Indiana

October 2017-May 2019: Research Technician

- Cultured and maintained Rat Insulinoma cell lines (INS-1).
- Assessed protein expression levels through semi-quantitative protein-based and quantitative mRNA-based assays
- Performed immunohistochemistry staining of mouse pancreatic tissues using markers for oxidative stress (4-HNE) and β-cell marker Insulin antibody (INS)
- Performed cell transfection and nucleic acid isolation, purification and quantification
- Optimized Immunoassays, in vitro assays and protein quantification, assays performance and troubleshooting (western blot, SDS-PAGE, gel electrophoresis, immunofluorescence)
- Conducted cytotoxicity assays on murine cell lines,
- Cellular association and apoptosis signaling via cell viability assays
- Developed protocol to establish the interaction of micro-RNA and their target messenger-RNA through pull-down assay by bioconjugation of biotin labelled micro-RNAs.
- Managing multiple projects simultaneously.
- Assisted my supervisor and contributed to the research data for publications.
- Presented data from experiments at weekly-organized lab meetings

Technical Skills:

- Protein expression techniques
- Performing a variety of biochemical in vitro assays
- Evaluating compounds in cell viability assays using rodent cell lines
- Recombinant DNA technology
- qPCR
- Genotyping
- Agarose Gel Electrophoresis/ SDA-PAGE/ Western Blotting
Software Knowledge:
- GraphPad Prism
- Adobe Photoshop & Illustrator
- Endnote
- BLAST
- MS Office: Word, Excel, PowerPoint

IUSM Biochemistry & Molecular Biology MS Courses

Fall 2017:
- G715 Biochemical Basis of Metabolic Disease
- B890 Biochemistry Seminar
- G505 Responsible Conduct of Research
- G828 Concepts in Biotechnology
- B854 Introduction to Research

Spring 2018:
- B890 Seminar in Biochemistry
- G817 Molecular basis and cell structure biology
- G848 Bioinformatics
- G828: Concepts in Biotechnology
- B855 Research in Biochemistry

Fall 2018:
- G716 Molecular Biology: A
- G855 Research in Biochemistry

Spring 2019:
- B855 Research in Biochemistry: