MICRORNA-191: REGULATOR OF PANCREATIC BETA CELL STRESS RESPONSE

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A Thesis

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Master of Science

in

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by

Juan C. Araujo Sariñana 2012

Fall 2012

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ABSTRACT

MICRORNA-191: REGULATOR OF PANCREATIC BETA CELL STRESS RESPONSE

by

Juan C. Araujo Sariñana

Master of Science in Biology

California State University, Chico

Fall 2012

Diabetes is one of the United States' worst epidemics; the dangers associated with the disease can be both fatal and varied. The constant presence of high concentrations of glucose and free fatty acids (FFA) becomes a source of stress for cells, which results in programmed cell death (apoptosis). Our lab has identified a microRNA (miRNA), miR-191, that may be involved in the stress response of pancreatic beta cells. miRNAs have been recognized as key regulators of the translation of messenger RNA (mRNA). miRNAs are involved in virtually all cellular processes, have the ability to target multiple genes, and play important roles in diseases such as cancer and diabetes. Previous research suggested that miR-191 is under the control of the pancreatic and duodenal homeobox 1 transcription factor (PDX-1) and of the neurogenic differentiation 1 transcription factor (NeuroD1), both of which are regulated in the presence of glucose. I have also observed

that miR-191 is up-regulated when rat insulinoma cells (INS-1) are treated with glucose. Bioinformatic analysis suggests possible roles for miR-191 in regulating apoptotic genes. Inhibiting miR-191 in a UV-induced stress assay in insulinoma (INS-1) cells results in decreased cell death. Decreased cell death was also observed when miR-191 was knocked down in samples that were treated with 30 mM glucose and 0.5 mM palmitate. It is not yet clear how miR-191 is involved in the beta cell stress response but based on data presented here it appears that miR-191 is promoting apoptosis in response to stressful conditions.

CHAPTER I

INTRODUCTION

MicroRNA

MicroRNAs (miRNA) are a class of genes that have been characterized as regulators of messenger RNA (mRNA). The first miRNA was discovered in *Caenorhabditis elegans* during late 1993 when a group of researchers noticed that the gene lin-4 produced two small RNA molecules that regulate larval development by repressing the translation of the mRNA produced by the lin-14 gene (Lee et al. 1993). It was initially thought that this biological phenomenon was specific to *C. elegans*, but since the discovery of lin-4 over 20,000 miRNAs have been characterized through random cloning and sequencing or computational prediction in various organisms ranging from worms to primates and plants (miRBase 19, http://www.mirbase.org/; (Griffith-Jones et al. 2008). Since their discovery much effort has gone towards understanding the biogenesis and mechanism of action of miRNAs as well as dysregulations in miRNAs that have been observed in many diseases.

The complex biogenesis of miRNAs begins in the nucleus with the transcription of the miRNA gene by RNA polymerase II (Lee et al. 2004, Cai et al. 2004). This generates a long primary miRNA (pri-miRNA) transcript which contains the mature

miRNA sequence within its stem-loop structure, as well as cap structures and a poly(A) tail (Lee et al. 2004, Cai et al. 2004). The pri-miRNA is cleaved while still inside the nucleus by Drosha, an endonuclease, with the help of the DiGeorge syndrome critical region gene 8 (DGCR8), the resulting transcript is a ~70-nucleotide hairpin known as precursor miRNA (pre-miRNA) (Lee et al. 2002, Han et al. 2004). Following cleavage by the Drosha-DGCR8 complex the pre-miRNA is exported to the cytoplasm where the final steps of miRNA maturation take place.

The exportation of pre-miRNA is performed by nuclear export receptor exportin-5 (Yi et al. 2003, Bohnsack et al. 2004). Once in the cytoplasm the pre-miRNA undergoes a final cleavage step by Dicer, a second endonuclease, yielding a ~20-nucleotide double stranded miRNA (Ketting et al. 2001, Knight et al. 2001). This miRNA duplex contains a sense and antisense copy of the miRNA (denoted miRNAs/miRNAas or miRNA/miRNA*).

In order for this mature miRNA to become fully functional one of the two strands, the ‘guide’ strand, must be loaded into a ribonucleoprotein complex, known as the RNA induced silencing complex (RISC). It is not fully understood how the selection of strands works, but the prevalent view is that the strand that has its 5’ end at the thermodynamically less stable end of the miRNA duplex is preferentially loaded into the RISC as the guide strand (Siomi and Siomi 2009).

The RISC is made up of a variety of proteins; the main component is a member of the Argonaute (Ago) protein family. The current model for RISC loading involves the incorporation of the miRNA/miRNA* duplex into Ago and strand separation within the Ago protein (Kawamata and Tomari 2010). The loading of the miRNA/miRNA*

complex into Ago is still not fully understood, but it is generally accepted that it can be similar to the mechanisms seen in small interfering RNA (siRNA).

Loading of a siRNA duplex into Ago2 has been characterized to a great extent in *Drosophila*, where it has been shown that Ago2 alone cannot accept siRNA duplexes. A RISC loading complex (RLC) composing of Dicer-2 and R2D2 is required to load and unwind the siRNA duplex (Pham et al. 2004). This has not proven to be the case with human Ago or fly Ago1, where it has been shown *in vitro* that absence of Dicer does not impair activity of siRNAs (Kawamata et al. 2009, Martinez et al. 2002).

There is some evidence for a Dicer independent mechanism for the unwinding duplexes, which might apply to miRNAs (Matranga et al. 2005). This mechanism relies on mismatches in the miRNA/miRNA* pairing to unwind the RNA strand and analysis of miRNA/miRNA* duplexes have shown that mismatches in the central region promote unwinding (Kawamata et al. 2009).

The manner in which miRNAs target mRNA has been widely studied and there is much that we now know about the mechanism of action of miRNAs. Since the discovery of lin-4 it was hypothesized that lin-4 might bind to complementary repeats in the 3' untranslated region (3' UTR) of the lin-14 mRNA (Lee et al. 1993). This prediction was not only correct, but also turned out to be the general mechanism for miRNA-mediated mRNA silencing (Wightman et al. 1993, Filipowicz et al. 2008).

Nearly all miRNAs act to repress their target mRNA via imperfect binding to a site in the 3' UTR, although they have been shown to work in the 5' UTR and it is thought that there may be miRNA recognition elements (MREs) in the coding region of mRNAs. The most important factor for miRNA target recognition is the nucleotides at

position 2-8 of the miRNA, also known as the ‘seed region’ (Doench and Sharp 2004). Perfect pairing of the seed region to the mRNA is sufficient for translational repression, but less than perfect binding of the seed regions requires stabilizing binding in the 3’ end of the miRNA (Brennecke et al. 2005). The ability of miRNAs to repress a mRNA with imperfect binding is part of what makes miRNAs so versatile, a single miRNA can target multiple mRNAs.

miRNAs can block translation by destabilizing the mRNA, leading to mRNA degradation, or by blocking the initiation of translation (Filipowicz et al. 2008). The degradation of mRNA requires near perfect binding of the miRNA to the target mRNA and is only achieved by Ago2. Blocking of translation is more typical and can be achieved by Ago1, Ago3, or Ago4, and does not require perfect binding to the mRNA.

Since their discovery miRNAs have been shown to be involved in virtually every aspect of cellular activity. It is proposed that over 50% of human genes are under the regulation of miRNAs; from differentiation and development, to metabolism and apoptosis (Kawamata and Tomari 2010). One would be hard pressed to find a cellular process that is not affected by a miRNA.

The importance of miRNAs is highlighted even more by the patterns of dysregulation (overexpressed and underexpressed) that are observed in the molecular pathophysiology of many diseases. Most notably, miRNAs have been identified as being involved in different cancerous tissues as either tumor suppressors or oncogenes (Shenouda and Alahari 2009). Examples of this include let-7 and miR155 (Guzman-Villanueva et al. 2012, Huang et al. 2011). In the case of let-7, its downregulation leads to aberrant cell growth and metastasis in lung cancer, while overexpression of miR-155

suppresses apoptosis in the bone marrow of acute myeloid leukemia patients (Johnson et al. 2005, Sasson et al. 2008, Isken et al. 2008).

Because of their involvement in a myriad of diseases, miRNAs have been subject to various studies that hope to restore or antagonize the function of miRNAs (Bader et al. 2010). There are many challenges in the development of miRNA therapies such as stability of the miRNA, off-target effects, delivery, and immunological responses to the introduction of double stranded RNA into cells. In 2008 the pharmaceutical company Santaris began clinical trial with a locked nucleic acid (LNA) based oligo directed against miR-122 for the treatment of hepatitis C (Wahid et al. 2010). The company has reported that the results of the trial have been encouraging and phase 2 trials are planned. Other possible miRNA therapies are currently being investigated by other companies, many of these possible therapies are for cancers but there are also studies being conducted for HIV/AIDS, heart failure, inflammatory bowel disease, pulmonary arterial hypertension, and herpes (Wahid et al. 2010).

micro RNA 191

miR-191 was first identified by a group of German researchers as a part of a large scale cloning experiment from spleen and kidney tissues. Later studies have confirmed the expression of miR-191 in a wide variety of tissues (Lagos-Quintana 2003, Volinia et al. 2006). miR-191 is located on chromosome 3 in humans at position 49,058,051-49,058,142, it is located within a cis-antisense gene pair. Interest in miR-191 has been on the rise since it was discovered that it is differentially expressed in cancerous tissues. One of the first indications of a role for miR-191 in cancer development came from a large

scale miRnome study where it was observed that miR-191 was overexpressed in colon, lung, pancreas, prostate, and stomach cancer tissues (Kim et al. 2004). Though the majority of cancer studies have found miR-191 to promote cell proliferation, others have found it to be linked to cell death.

Colon cancer studies have shown that miR-191 is overexpressed 1.4 fold in colorectal cancer tissues vs. normal tissues (Xi et al. 2006a). Another study in colorectal cancer found a lower expression of miR-191 in human colorectal cancer (HCT-116) cells with null p53 when compared to HCT-116 cells with wild type p53 (Xi et al. 2006b). Other than this observation, the relationship between miR-191 and p53 remains unknown.

miR-191 has been shown to be down regulated in the lungs of rats that had been exposed to cigarette smoke for 28 days, which is consistent with the previous finding that miR-191 downregulation in lung cancer tissues results in cell proliferation (Izzotti et al. 2009, Cheng et al. 2005). The role of miR-191 in lung cancer is still up for debate however due to a study that found that overexpression of miR-191 along with other miRNAs is not enough to promote cell proliferation in lung cells (Patnaik et al. 2010).

The over expression of miR-191 that was first described in pancreatic cancer tissues has been observed by a subsequent study in pancreatic ductal carcinoma where they also that inhibition of miR-191 diminished anchorage independent growth in PK-9 and Su86.86 pancreatic cell lines (Kent et al. 2009).

Overexpression of miR-191 has also been observed in breast cancer tissues and in hepatocellular carcinoma (HCC) [38-41](Iorio et al. 2005, Mar-Aguilar et al. 2012, Elyakim et al. 2010, He et al. 2011). The role of miR-191 in HCC has been better

documented than in other cancers, as it has been shown that inhibition of miR-191 results reduced proliferation and increases apoptosis in HCC cells lines (Elyakim et al. 2010). In the same study it was also shown that miR-191 could potentially be regulating TGF-B and MAPKK, two signaling pathways with big roles in proliferation, differentiation, adhesion and cell survival. The regulation of miR-191 expression has also been studied in HCC and it has been found that overexpression is a result of hypomethylation of the miR-191 locus (He et al. 2011) .

The functions of miR-191 have also been well characterized in gastric carcinoma (Shi et al. 2011). Like previous studies, this study showed that miR-191 promoted cell growth in a gastric adenocarcinoma cell line. They also showed that inhibition of miR-191 led to increased cell death, pointing to a clear role of miR-191 in the proliferation of cancerous cells. Moreover, this study identified a target gene of miR-191, NDST1, by analyzing expression levels and by cloning the 3' UTR of NDST1 into a GFP reporter plasmid. NDST1 deficiency has been shown to result in increased proliferation.

Other potential targets of miR-191 have been identified in red blood cell differentiation. Two genes involved in the final steps of red blood cell differentiation, Riok3 and Mxi1, are usually upregulated during these final stages of red blood cell differentiation while miR-191 is downregulated (Zhang et al. 2011). The two genes were found to be downregulated if miR-191 was overexpressed, this indicates that they might be under the control of miR-191. The most recent studies that have found targets for miR-191 indicate that miR-191plays a role in triggering keratinocyte senescence (Lena et al. 2012). Here it was shown that the upregulation of miR-191 in human keratinocytes leads to senescence, and that miR-191 affects senescence by targeting SATB1 and CDK6.

miR-191 dysregulation has been implicated in other diseases besides cancer. There is evidence of lowered levels of miR-191 in the circulating plasma of type 2 diabetes patients (Zampetaki et al. 2010). Lowered miR-191 expression has also been seen in T-regulatory cells of type 1 diabetes patients (Hezova et al. 2010). Differential expression of circulating miR-191 has also been observed in inflammatory bowels disease, where it was shown that mIR-191 has a significantly higher expression rate when compared to healthy samples.

Previous research suggests that miR-191 might be under the control of the pancreatic and duodenal homeobox 1 transcription factor (PDX-1) and of the neurogenic differentiation 1 transcription factor (NeuroD1) (Keller et al. 2007) in pancreatic beta cells. PDX-1 and NeuroD1 are highly important transcription factors in pancreatic beta cells, playing vital roles in beta cell development and maturation. PDX-1 is also a key component in beta cell function as it has been shown to be activated by increased glucose levels as well as being necessary for the expression of the insulin gene (Mosley et al. 2004). If miR-191 is under the regulation of PDX-1 means that mIR-191 could potentially be playing an important role in pancreatic beta cells.

Pancreatic Beta Cell Physiology

Pancreatic beta cells are crucial for the maintenance of blood glucose homeostasis. Their primary function is the production and secretion of insulin in response to increased concentrations of glucose in the bloodstream, usually the result of having a meal. Once insulin is released into the bloodstream it stimulates glucose-dependent tissues to absorb glucose to use as fuel or store as fat (Rorsman 1997).

The secretion of insulin is dependent on the metabolic processes of pancreatic beta cells that convert glucose into Adenosine Triphosphate (ATP). This increase of intracellular ATP after glucose absorption causes ATP-sensitive K^+-channels to close [49]. This change triggers a membrane depolarization event that in turn leads to Ca^{2+} influx via voltage gated Ca^{2+} channels. It is this increase of intracellular Ca^{2+} that signals the exocytosis of insulin granules (Wollheim and Sharp 1981).

Free fatty acids (FFA) have also been shown to play a part in the secretion of insulin. Medium and long-chained FFAs are known to amplify insulin secretion by binding to the G-protein coupled receptor (GPCR) GPR40 (Gromada 2006). The binding of FFAs to GPR40 leads to inositol triphosphate (IP_3) production, which stimulates the release of Ca^{2+} from endoplasmic reticulum, thus enhancing the exocytosis of insulin granules (Fujiwara et al. 2005). GPR40 also mediates an increase in cAMP which leads to enhanced excitability of the beta cell by inhibiting the voltage gated K+-channels that cause the cell to repolarize (Gromada 2006).

Type 2 Diabetes

Diabetes mellitus is currently one of the worst epidemics in the United States. According to statistics released by the Center for Disease Control there is an estimated 25 million cases of diabetes mellitus in the US, type 2 diabetes accounts for about 90% of these cases. There are a variety of factors that have been correlated to this metabolic disorder. Various polymorphisms have been shown to increase an individual's predisposition to diabetes, but obesity is regarded as one of the main culprits of this disease (Prentki and Nolan 2006).

Diabetes mellitus is characterized by the chronic presence of high blood glucose concentrations (hyperglycemia), usually the result of a loss in pancreatic beta cell function. Hyperglycemia is usually accompanied by a high concentration of free fatty acids (FFA) known as dyslipidemia. Together hyperglycemia and dyslipidemia lead to the loss of function in pancreatic beta cells and eventually lead to pancreatic beta cell death.

Glucolipotoxicity

The detrimental effects that hyperglycemia and dyslipidemia cause for pancreatic beta cells are commonly known as glucolipotoxicity. The molecular mechanisms of glucolipotoxicity are still being studied but it is generally thought that ceramide formation, endoplasmic reticulum (ER) stress, and the formation of oxidative compounds are the main pathways that lead to pancreatic beta cell dysfunction and death.

Ceramide is a lipid that is composed of a sphingosine and fatty acid. It was once thought that the function of ceramide was purely structural since it is found in the plasma membrane. We now know that ceramide can actually act as a signaling molecule and has been shown to be a part of various cellular processes, including cell death (Basu and Kolesnick 1998). Increased ceramide formation is a result of the inhibition of the enzyme carinitine-palmitoyl tranferase-1 (CPT-1) by increased levels of malonyl-CoA (Vincent et al. 2010). CPT-1 normally transports fatty acids into the mitochondria for beta-oxidation. The inhibition of this enzyme stops this process and leads to the build up of long-chain acyl-CoA esters (LC-CoA) in the cytoplasm (Vincent et al. 2010). The accumulated LC-

CoA serves as a substrate for esterification pathways which lead to the formation of ceramide as well as phosphatidic acid, diglycerides, triglycerides and phospholipids.

Accumulation of ceramide via this pathway has been shown to inhibit the protein kinase (PKB)/Akt pathway (Powell et al. 2003). The inhibition of the PKB signaling pathway ultimately leads to decreased cAMP response element-binding (CREB), a transcription factor which promotes the expression the anti-apoptotic gene B-cell lymphoma 2 (Bcl2) (Du and Montminy 1998, Kim et al. 2008). Bcl2 is known to regulate cysteine-aspartic acid protease 3 (Caspase 3), one of the effector caspases involved in the late stages of apoptosis. Therefore, the inhibition of the PKB pathway as a result of increased ceramide formation clears the way for apoptosis to take effect.

One of the characteristics of pre-type 2 diabetes is the overcompensation response of pancreatic beta cells in order to keep up with the hyperglycemic conditions and maintain homeostasis. This overcompensation involves an increase in the expression rate of insulin and other secretory related genes . Eventually the ER becomes overloaded with newly translated proteins and the protein folding machinery cannot keep up, leading to the accumulation of misfolded and unfolded proteins (Cunha et al. 2008). The accumulation of misfolded/unfolded proteins triggers a response called the unfolded protein response (UPR).

The UPR works through three pathways that are regulated by three ER associated proteins: protein kindase R-like ER kinase (PERK), inositol requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) (Karunakaran et al. 2012). ATF6 contributes to the impairment of insulin secretion by downregulating the expression of the insulin gene through the small heterodimer partner (SHP). IRE1 has also been shown to inhibit

insulin secretion by activating the c-Jun N-terminal Kinsase (JNK) (Karunakaran et al. 2012). JNK is also involved in the downregulation of Bcl2 which further contributes to the apoptotic pathway. The same is the case with CCAAT/enhancer binding protein (CHOP), which is activated by PERK (Karunakaran et al. 2012).

ER stress further contributes to the development of the apoptotic pathways via the depletion of ER $Ca2^{+}$ stores into the cytoplasm (Jeffrey et al. 2008). The depletion of ER $Ca2^{+}$ stores has been shown to be induced by palmitate and leads to cytochrome c (Cyto C) being released from the mitochondria into the cytoplasm (Jeffrey et al. 2008). Cyto C then goes on to activate caspase 9 which eventually leads to the activation of caspase 3 and cell death.

High glucose concentrations have previously been shown to induce the formation of reactive oxygen species (ROS). Mechanisms by which ROS are formed include the advanced glycation endproducts/receptor for advanced glycation endproducts (AGE/RAGE) pathway and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) activity. Free fatty acids can also contribute to the formation of oxidative stress by inducing the expression of nitric oxide synthase (iNOS). Oxidative compounds such as superoxide (O_2^{*}), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH^{*}) can react with DNA bases, causing damage to the original DNA sequence. The damage to the DNA This damage can sometimes be repaired but chronic exposure to oxidative stress will result the activation of the apoptotic pathways.

This study will aim to find a function for miR-191 in pancreatic beta cells. Previous research has suggested that miR-191 has a role in cell proliferation, as its been shown to be overexpressed in a number of cancerous tissues. miR-191 has also been

suggested as a regulatory target of transcription factors PDX-1 and NeuroD1. These two transcription factors are important for pancreatic beta cell growth and function leading me to believe that miR-191's function in pancreatic beta cell may also be important for beta cell growth or function. If miR-191 is in fact a regulatory target of PDX-1 then it is also possible that miR-191 expression may be affected by glucose concentrations, as PDX-1 activity is affected by glucose. I will gain more information about the possible role of miR-191 by investigating the predicted targets of miR-191. This will hopefully lead me to generate new hypothesis about the function of miR-191 in pancreatic beta cells.

CHAPTER II

MATERIALS AND METHODS

Bioinformatic Analysis of miR-191 Locus

The genomic region of the *Rattus novegicus* miR-191 gene was analyzed using the University of California Santa Cruz (UCSC) genome browser. Potential PDX-1 (TAAT) and NeuroD1 (CANNTG) binding sites were considered to be those that showed evolutionary conservation between rats, mice, and humans.

Bioinformatic Analysis of miR-191 Predicted Targets

Predicted gene targets of miR-191 were obtained using the mirWalk database (http://www.ma.uni-heidelberg.de/apps/zmf/mirwalk/). The predicted targets were analyzed using the Gene Ontology for Functional Analysis (GOFFA) tool found in ArrayTrack (http://www.fda.gov/ScienceResearch/BioinformaticsTools/Arraytrack/default.htm). Gene ontology terms were obtained from the GOFFA database and were used for predicting possible roles of mir-191.

Beta Cell Culture

Rat insulinoma INS-1 cells were cultured in 10 cm dishes with 10 mL of RPMI 1640 (supplemented with 10% fetal bovine serum (FBS), 100 ug/ml penicillin, 100 ug/ml

streptomycin, 1 mM sodium-pyruvate, and 50μM beta mercaptoethanol) at 37°C in a humidified chamber containing 5% CO_2. Media was replaced every 2 days. Cells were split once 80%-90% confluency was reached by trypsinizing and diluting 1:3 into new culture plates.

miR-191 Glucose Response

INS-1 cells were plated into four 35 mm tissue culture plates at a concentration of $1x10^6$ cells per plate. Growth media was replaced by low glucose (2 mM) media one day after plating. After 24 hours of treatment with 2 mM glucose media, glucose was added to the plates at concentrations of 11 mM 30 mM, and 60 mM. After 1 hour RNA was extracted. RNA extraction was carried out using 1 mL of TRIzol reagent (Invitrogen) per 3.5 cm^2 cell culture plate. RNA was extracted according to the manufacturer's instructions. RNA yield and quality were calculated by obtaining the absorbance values at 260nm (A260) and 280nm (A280) wavelengths of the RNA sample using a Biotek Synergy HT 96-well plate spectrophotometer. RNA yield was calculated as: A260 x dilution factor x 0.04 μg/μl. Complementary DNA (cDNA) was synthesized from extracted RNA using Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase (Promega, Madison, WI) with random primers and following manufacturer's instructions. A sample without reverse transcriptase was used as a negative control.

Real Time Polymerase Chain Reaction

Real-time polymerase chain reaction (PCR) was carried out on cDNA using an Eppendorf RealPlex^2 Mastercycler real-time PCR machine under the following

parameters: 1 cycle of 95^{o}C for 10 minutes, 40 cycles alternating between 95^{o}C and 60^{0}C for 30 seconds each. Gene specific primers for the insulin gene (F:CTACACACCCAAGTCCCGTCGTG, R:CAACCTCCAGTGCCAAGGTCTGA), 18S ribosomal RNA (F:CGGACACGGACAGGATTGACAGA, R:ACCACCCACGGAATCGAGAAAGA), mir-375 (F:CCTCGCACAAACCGGACCT, R: GCCTCACGCGAGCCGAAC), and miR-191 pre-cursor (F:AGCTGTTGTCTCCAGAGCATTCCA, R:AGCAGGGAACGAAATCCAAGTGC) were used. Samples were tracked for fluorescence using SYBR green reagent (Thermo-Fisher). Serial dilutions of purified genomic DNA were prepared and run with the appropriate primers to generate standard curves for each real time PCR run.

Hairpin Inhibitor Transfection

miRIDIAN hairpin inhibitors were used to knockdown the function of miR-191 (Dharmacon). A negative control inhibitor that is specific to a miRNA found only in *C. elegans* was also purchased and used as a control. Inhibitors were transfected at a concentration of 50 nM using TransIT reagent (Mirus). Samples were transfected for 3 days after which they were used for various experiments.

Taqman miRNA Assay

Taqman miRNA assay was used to confirm the results of transfection with Miridian hairpin inhibitors. RNA was extracted from cells transfected with miR-191 inhibitors and negative control inhibitors. This RNA was used to make cDNA using a miR-191 specific primer. The cDNA generated was used to run qPCR with a miR-191 probe in the

following conditions: 1 cycle at 95^{o}C for 30 seconds, 40 cycles alternating between 95^{o}C for 15 seconds and 65^{o}C for 1 minute.

UV-induced stress response

INS-1 cells were plated in four 3.5 cm plates at a density of 5x10^{5} cells per plate. After 16 hours cells were transfected with miRIDIAN miRNA haripin inhibitors, two plates were transfected with an inhibitor specific to miR-191 and two plates were transfected with a negative control inhibitor. After 3 days cells were exposed to UV light using a transluminator (UVP, Inc.), two plates were used as controls (0 sec) and the other two plates were exposed to UV light for 10 seconds to induce stress. Cell viability was measured using trypan blue.

Glucolipotoxicity Assay

INS-1 cells were plated in a 96-well tissue culture plate at a density of 20,000 cells per well. Normal growth media was exchanged for high glucose and lipid media (RPMI 1640, 1%BSA, 1% FBS, 1 mM sodium pyruvate, 1x Pen/Strep, 50 uM β-mercaptoethanol, 30 mM glucose, 0.5 mM sodium palmitate) 24 hours after plating. Cells were incubated under glucolipotoxic conditions for 72 hours. Cells where assayed for viability at the end of the 72 hours incubation using trypan blue

Trypan Blue

Cells that were treated with ultraviolet light or under glucolipotoxic conditions were trypsinized and collected for staining with 0.4% trypan blue. Samples were diluted

1:5 in PBS before staining. Using a hematocytometer dead and live cells were counted to determine the percentage of dead cells, three replicate counts were taken per sample.

Apoptotic DNA Isolation

Samples were treated with DNA fragmentation lysis buffer (0.1% Triton X-100, 5 mM Tris-HCl pH 8, 20 mM EDTA) to extract DNA. High molecular weight DNA was selectively precipitated by the addition of 2.5% PEG 8000 and 1 M NaCl. Large DNA fragments were removed by centrifuging samples for 10 minutes at 16,000 g. The supernatant was removed and small molecular weight DNA was precipitated using ethanol. Samples were ran on a 1.5% agarose gel and visualized with ethidium bromide

MTS Cell Viability Assay

INS-1 cells were plated in a 96-well tissue culture plate at a density of 20,000 cells per well. Cell viability was measured by adding 20 uL of MTS reagent, samples were incubated for 2 hours at 37^{o}C. Absorbance measurements were taken at 510 nm using Synergy HT plate reader.

CHAPTER III

RESULTS

miR-191 is Expressed in Pancreatic Beta Cells

In order to determine whether miR-191 was expressed in pancreatic beta cells I first determined its expression in rat insulinoma cells (INS-1). Using cDNA that was synthesized from RNA extracted from INS-1 cells I performed real time reverse transcriptase polymerase chain reaction (qRT-PCR). Figure 1 shows the relative expression of the insulin gene, pre-miR-375 and pre-miR-191. The insulin gene and pre-miR-375 are both known to be expressed in INS-1 cells. When comparing pre-mir-191 expression levels to these two genes and to the negative control I can conclude that pre-mir-191 is expressed in INS-1 cells and therefore the mature miRNA is produced as well.

miR-191 Locus

Previous research has already identified mir-191 as a potential target for the transcription factors PDX-1 and NeuroD1 [46]. Further examination of the miR-191 locus led us to discover that there are two potential PDX-1 binding sites (TAAT boxes) and three potential NeuroD1 binding sites (E-boxes) (Figure 2). Both of these transcription factors have been shown to be of high importance for the development and function of pancreatic beta cells. These sites show conservation between humans, rats and

mice. Though we do not know yet if PDX-1 and NeuroD1 associate with these sites, their presence and conservation add weight to the idea of miR-191 being regulated by these two important transcription factors.

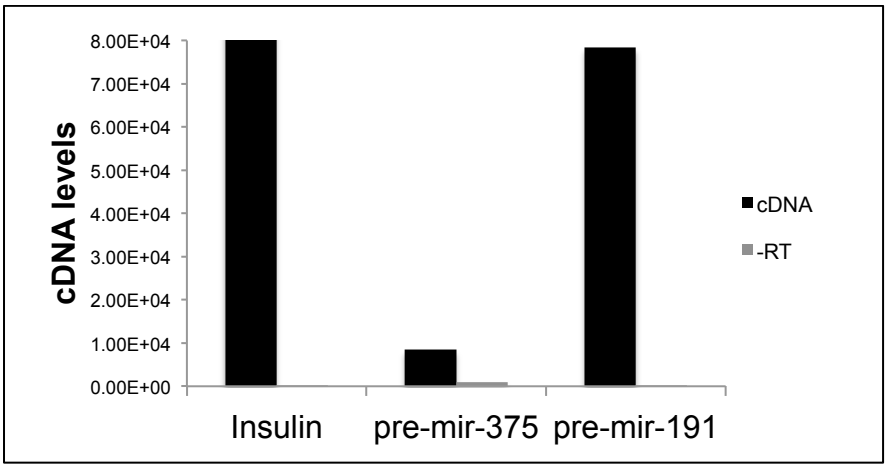

Figure 1 – miR-191 is expressed in pancreatic beta cells. RNA was isolated from INS-1 cells to make cDNA. Samples were used to run qPCR with primers for the pre-miRNA of miR-191. Primers for mir-375 and the insulin gene were used as positive controls. All samples show clear expression when comparing the cDNA samples to the –RT negative control samples. (n=1)

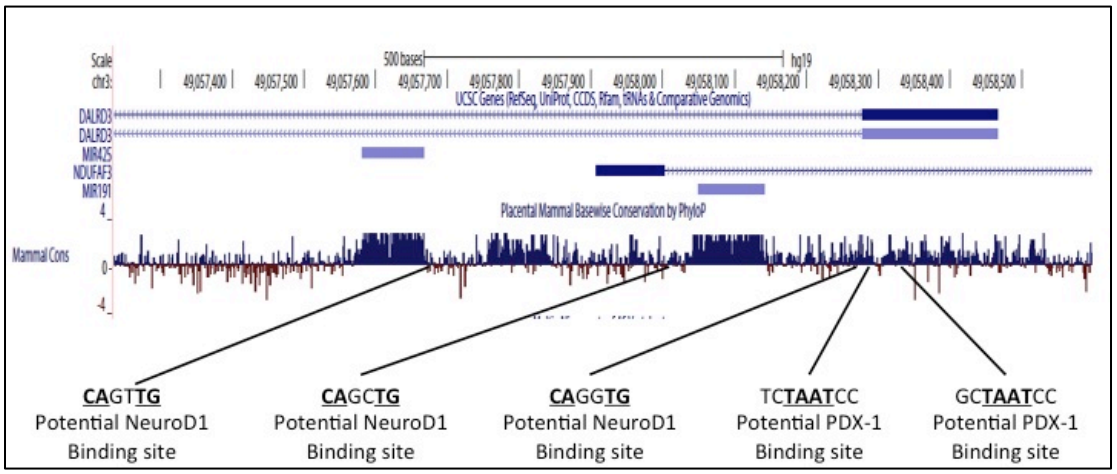

Figure 2 – miR-191 loci. miR-191 and miR-425 are shown in this excerpt the UCSC genome browser. miR-191 and miR-425 are found clustered together in chromosome 3 of the human genome, in the introns of two other genes. There are two potential PDX-1 binding sites (TAAT) and three potential NeuroD1 binding sites (CANNTG).

mir-191 is upregulated in the presence of glucose

Because miR-191 may be potentially under the control of PDX-1 and NeuroD1, I decided to test whether miR-191 demonstrated a response to glucose stimulation. I performed a glucose dose response on the expression of miR-191 at concentrations varying form 2mM glucose to 60 mM glucose. After one hour total RNA was harvest and cDNA was generated with random primers. After performing qPCR with the generated cDNA I observed an increase in pre-cursor miR-191 levels (Figure 3).

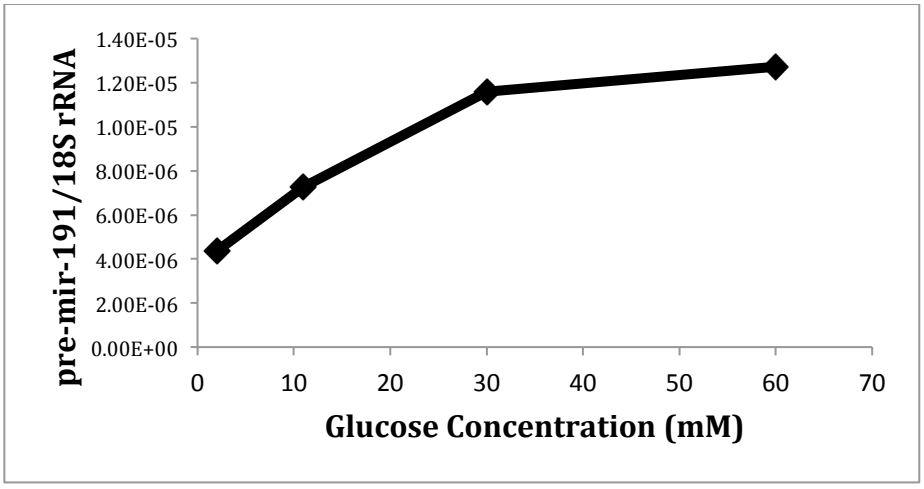

Figure 3 – miR-191 is up-regulated in the presence of glucose. INS-1 cells were plated in 35 mm plates, cells were starved overnight (2 mM glucose) then treated with glucose concentrations ranging form 2 mM to 60 mM. RNA was isolated from the samples and was used to make cDNA. Samples were used to run qPCR with primers for the precursor to miR-191. Samples were normalized to 18S rRNA. (n=3)

I investigated the expression of miR-191 in response to glucose by performing a time course experiment with 2 mM and 30 mM glucose (Figure 4). After incubating with 2 and 30 mM glucose, RNA was extracted and cDNA was generated for time points at 1, 4, 8, and 16 hours. The results of the time course experiment show higher expression in

the samples that were cultured in 30 mM glucose when compared to 2 mM glucose. Although expression levels decrease over the course of the experiment, this decrease is observed in both samples and could be due to the 24 hour starvation treatment.

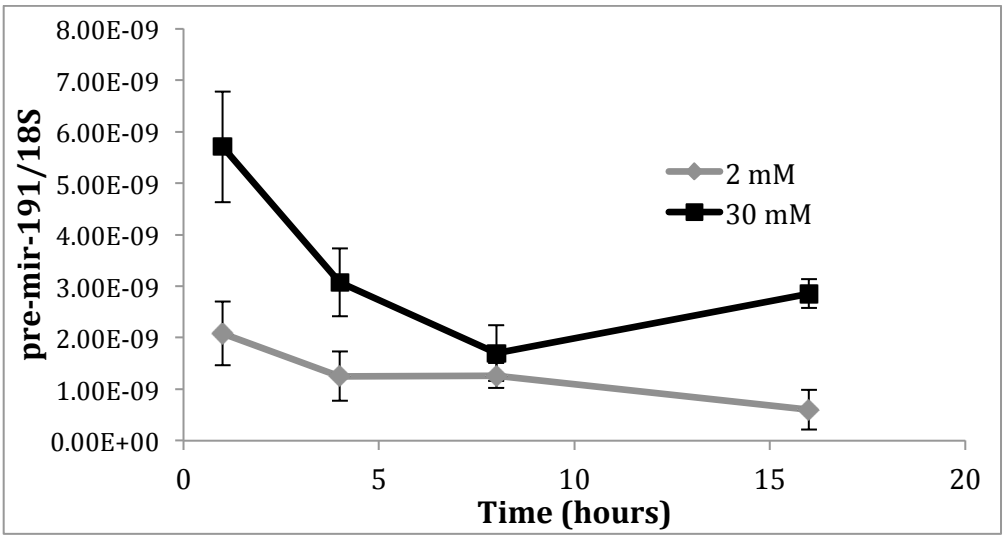

Figure 4 – Glucose time course reponse on pre-miR-191 expression. INS-1 cells were plated in 35 mm plates, they were starved overnight (2mM glucose) then treated with either 2 mM or 30 mM glucose for 1, 4, 8, and 16 hours. RNA was extracted from the samples and used to generate cDNA. Samples were used to run qPCR with primers for the precursor to miR-191. Samples were normalized to 18S RNA. (n=3)

Potential Targets of miR-191

In order to gain insight into what role miR-191 may be playing in the cellular processes of pancreatic beta cells, I investigated the predicted gene targets of miR-191. To do this, I used miRwalk, a miRNA target prediction database. miRwalk is a database with a prediction algorithm of its own, but it also incorporates data from four other target prediction databases (miRwalk, miRanda, miRDB, TargetScan). This list of 25 predicted target genes analyzed using the Gene Ontology for Functional Analysis (GOFFA) tool

found in ArrayTrack. The results from this database gave us an idea of the functions of each gene, and about the possible roles of mir-191 in pancreatic beta cells.

Table 1 shows all of the genes predicted by miRwalk, miRanda, miRDB, and TargetScan. Of these 25 genes, 5 returned gene ontology terms corresponding with apoptosis and cell cycle functions. Because of the previous studies done on miR-191 that show possible roles of miR-191 in cancer, we decided to explore the possibility of miR-191 playing a role in apoptosis.

Ultra Violet Light Induced Apoptosis

In order to assay the involvement of miR-191 in apoptosis I used a classic way to induce cell death. I exposed INS-1 cells to UV light as it is a potent inducer of apoptosis, activates the p53 pathway, and causes some of the same DNA damage as glucolipotoxicity. A time course was first performed to determine the optimal length of exposure (Figure 5). Cells were exposed to UV light by placing the tissue culture plates directly onto a UV transluminator for the allocated amount of time. 10 seconds was chosen to perform our subsequent experiments since it resulted in approximately 50% cell death.

I confirmed that UV exposure was indeed causing apoptosis by performing a DNA fragmentation assay. As cells undergo apoptosis their DNA is cleaved at the nucleosomes, causing a ladder-like pattern when the fragmented DNA is run on a 1.5% agarose gel. Figure 6 shows this laddering pattern on the lanes where the DNA of cells exposed to UV light were ran. Thus, this assay confirms that our cells are undergoing apoptosis.

Table 1 – List of predicted gene targets of miR-191. Genes shown are predicted across four different target prediction databases. Expression in pancreatic tissue was determined by looking at the UniGene database available from NCBI (http://www.ncbi.nlm.nih.gov/unigene). Gene ontology terms were obtained by running the gene list through the Gene Ontology for Functional Analysis tool of ArrayTrack.

Gene Name	Expression in Pancreas	GO Terms
CEBPB	Yes	Apoptosis
DAPK1	Yes	
MAGEH1	Yes	
NOTCH2	Yes	
RNF216	Yes	
CEBPB	Yes	Cell Cycle
SPO11	No	
NOTCH2	Yes	
CREBBP	Yes	Regulation of Transcription
ZBTB34	Yes	
MYNN	Yes	
TAF5	No	
ZNF154	No	
LAMC3	Yes	Structural Molecule Activity
FOPNL	Yes	Microtubule Anchoring
RDH19	Yes	Oxidoreductase Activity
EHHADH	No	Fatty Acid beta-oxydation
CERS6	Yes	Sphingolipid biosynthesis process
TMOD2	Yes	Tropomyosin binding
SLC25A24	Yes	Metal ion binding
SKIDA1	No	Nucleotide binding
TET1	No	DNA methylation
ARHGAP39	Yes	GTPase activator
BRMS1L	No	Regulation of growth
NEURL4	Yes	Protein binding
B4GALT6	Yes	Carbohydrate metabolic process
AMMECR1L	Yes	Molecular Function

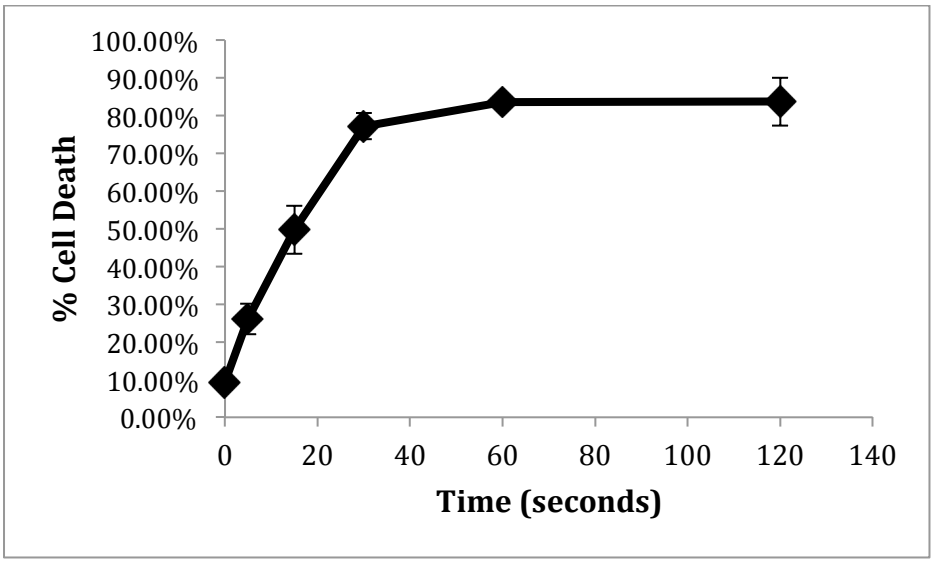

Figure 5 – UV cell death time course. INS-1 cells were exposed to UV light for 0, 5, 15, 30, 60, and 120 seconds. Cell death was measured by staining with 0.4% trypan blue after 16 hours of UV exposure. (n=3)

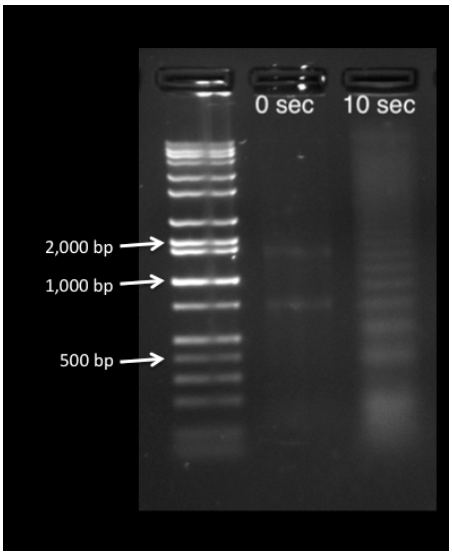

Figure 6 – DNA fragmentation in cells exposed to UV light. Samples of INS-1 cells that were exposed to 0 and 10 seconds of UV light were treated with DNA Fragmentation Lysis Buffer. High molecular weight DNA was selectively precipitated and removed by centrifugation. Small molecular weight DNA was ethanol precipitated and ran on 1.5% agarose gel.

Having determined the conditions for our UV induced apoptosis assay I proceeded to determine if knocking down miR-191 function had any effect on the cell death rate. Using miRIDIAN small hairpin inhibitors, I was able to effectively lower the level of mature miR-191 present in INS-1 cells. The inhibition of miR-191 was confirmed using a Taqman microRNA assay specific for miR-191 (Figure 7). We tested four different concentrations of the miR-191 inhibitor but used 50 nM for subsequent studies as it showed a significant decrease in mature miR-191 with the lowest variation between replicates. As a control we used a negative control mirIDIAN small hairpin inhibitor that is based on a miRNA that is only found in *C. elegans*.

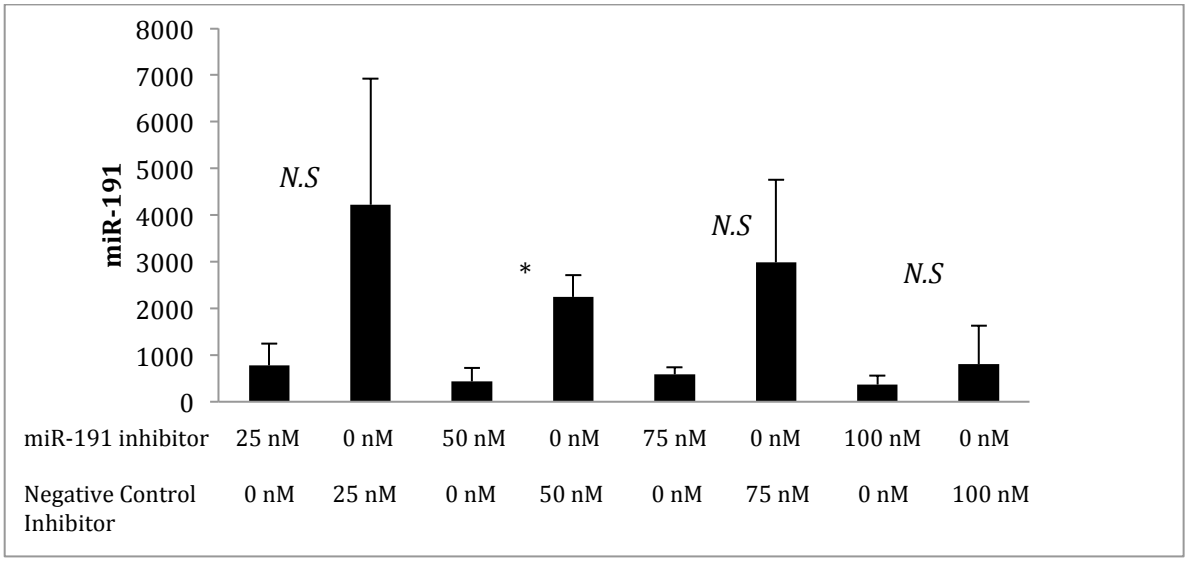

Figure 7 – miR-191 detection is inhibited by small hairpin inhibitors. Cells were transfected with miR-191 inhibitor and negative control inhibitor at concentrations varying between 25 nM and 100 nM. After three days RNA was extracted and used to make cDNA with a miR-191 specific Taqman primer. qPCR was run using the Taqman miR-191 probe. (n=2, *= $p<.05$, N.S= No Significance)

Using the UV induced apoptosis assay with the inhibition of miR-191 function, we were able to observe a change in the amount of cell death on cells that were transfected with inhibitor compared to the control. In the cells that were exposed to UV light, the cells that were transfected with miR-191 inhibitor showed 21% less cell death than in the samples transfected with negative control inhibitor (Figure 8). This suggests that miR-191 is somehow involved in promoting apoptosis in response to UV stress.

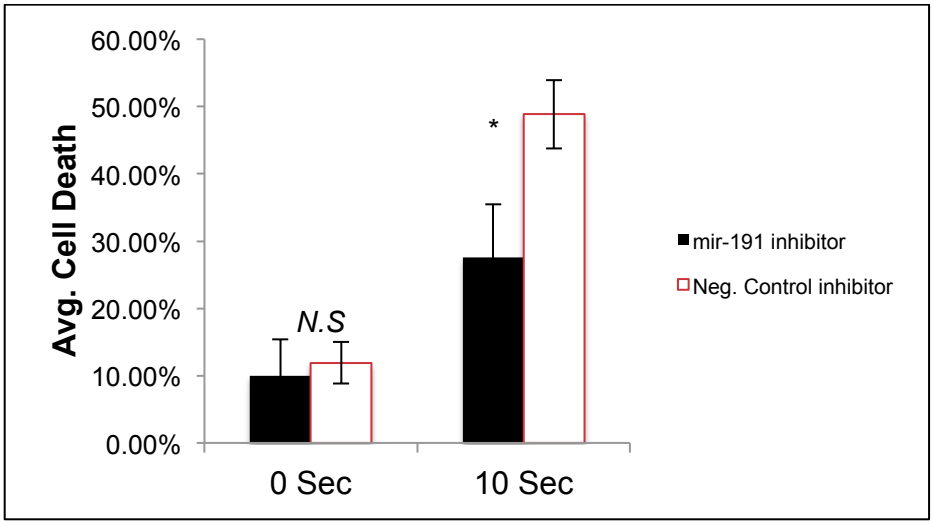

Figure 8 - miR-191 shows pro-apoptotic function in response to UV stress. INS-1 cells were transfected with a miRIDIAN miRNA inhibitor specific to miR191 and a miRIDIAN miRNA negative control inhibitor. After 3 days of transfection the cells were exposed to UV light on a transluminator for 10 seconds and 0 seconds as a control. Cells were stained with 0.4% trypan blue to count for viable cells. (n=9, *= p<.05, N.S= No Significance)

Glucolipotoxicity Induced Stress

While UV light invokes similar stress response to that of glucolipotoxicity in beta cells, I next determined whether miR-191 plays a role in glucolipotoxicity-mediated cell death. Because miR-191 was upregulated in the presence of 30 mM glucose I

hypothesized that miR-191 might regulate cell death caused by high glucose. I assayed the viability of cells that were cultured in 11 mM glucose and 30 mM glucose, but found that culturing the cells with glucose alone did not reduce their viability (Figure 9, Lanes 1 and 3). Instead we coupled high glucose levels with palmitate, a fatty acid that is known to cause cell death. High glucose and high fatty acid level are more commonly known together as glucolipotoxicity and are typical of the type 2 diabetes pathophysiology. When coupled together, high glucose and high palimitate concentrations were able to significantly decrease cell viability (Figure 9, Lane 4).

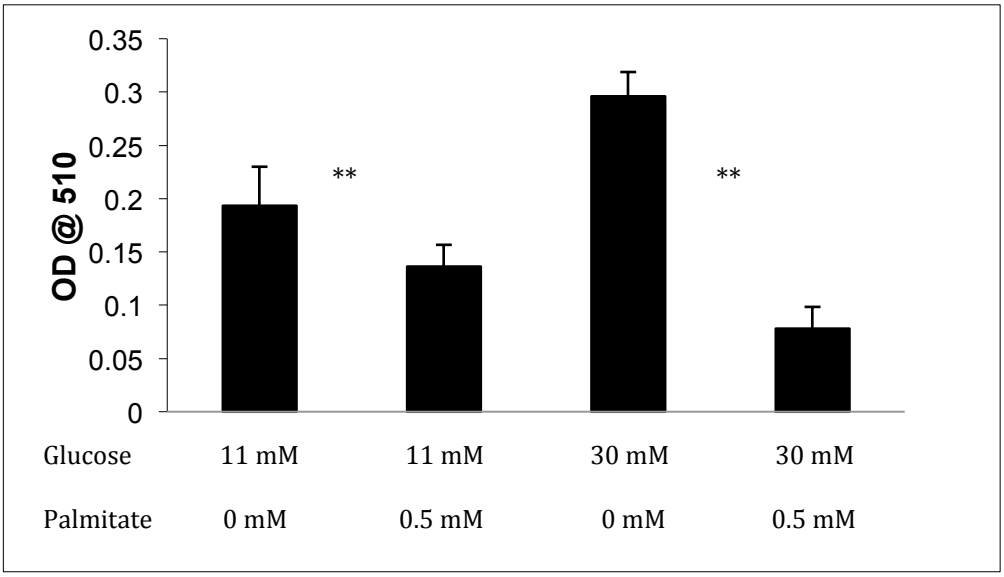

Figure 9 – Glucolipotoxic stress decreases cell vialbility. INS-1 cells were plated on a 96-well plate and exposed to glucolipotixic conditions for 72 hours (11 and 30 mM glucose, +/- .5 mM palmitate). After 72 hours cell viability was measured using MTS reagent, color change as a result of metabolism was measured by absorbance at 510 nm. (n=3, **= p<.01)

Before testing the effect of miR-191 on glucolipotoxic induced cell death I measured miR-191 expression level in response to palmitate. Cells were incubated with

11 mM glucose and 0.5 mM palmitate for a course of 24 hours, after which I extracted RNA and ran qPCR. In general, the response of miR-191 expression increases over time in response to palmitate (Figure 10).

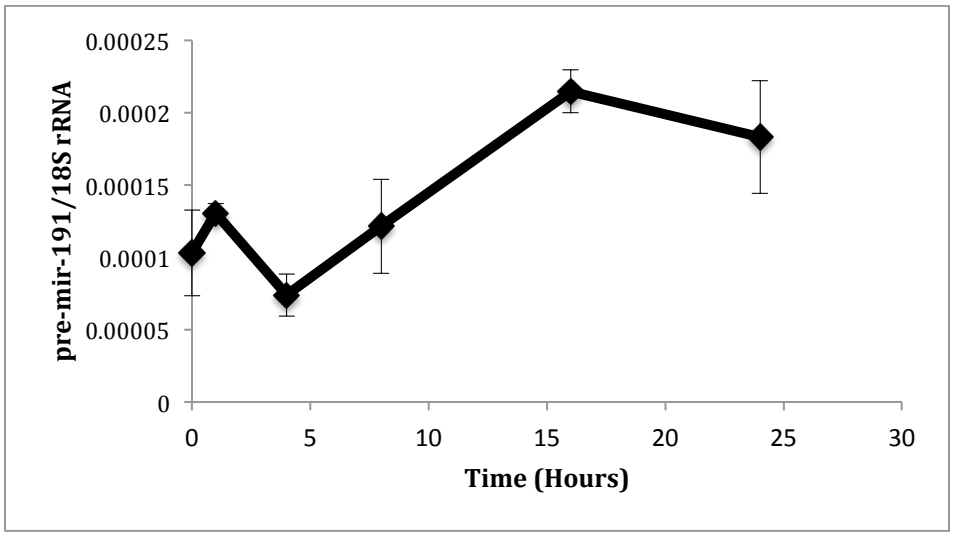

Figure 10 – Palmitate increases expression of miR-191. INS-1 cells were cultured in 35 mm plates with media containing 11 mM glucose and .5 mM palmitate for a course of 24 hours. RNA was extracted from the samples and cDNA was generated for use in qPCR. Samples were normalized to 18S rRNA. (n=3)

Next, I tested if miR-191 had an effect on the cell death rate induced by glucolipotoxicity in a similar fashion as with UV induced cell death. First, a time course experiment was performed to determine the optimal conditions for testing glucolipotoxic induced cell death (Figure 11). Cells were treated with 11 mM glucose and either with or without palmitate. The percentage of dead cells was measured each day for 4 days by counting cells stained with 0.4% trypan blue. We determined that cell death reached saturation by day 2 and proceeded to test cell death after one day of incubation.

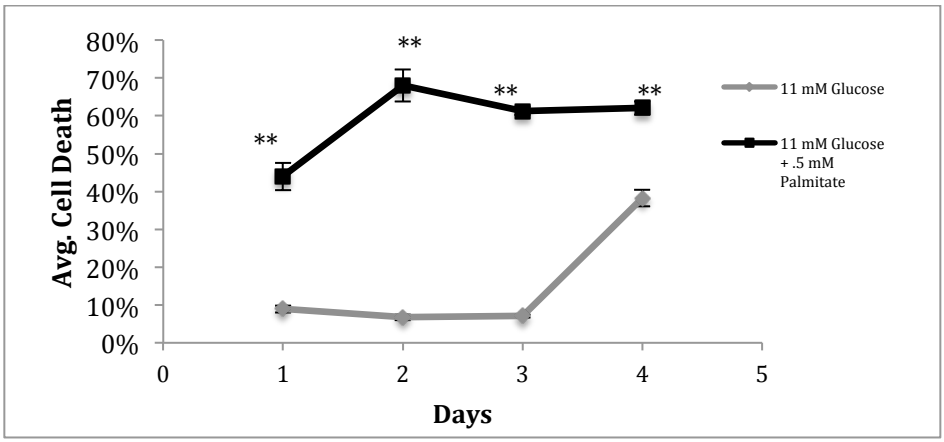

Figure 11 – Lipotoxicity cell death time course. INS-1 cells were plated on 35 mm plates. They were treated with media containing 11 mM glucose and 0.5 mM palmitate, control samples were treated with media containing only 11 mM glucose. Cell death was measured by staining with 0.4% trypan blue and counting cells with a hemocytometer. Data was collected every 24 hours for a total of 4 days. (n=3, **= p<.01)

Having determined the conditions for glucolipotoxic incubation, I proceeded to knock down the function of miR-191. Transfecting cells with 50 nM of miR-191 inhibitor resulted in decreased cell death when compared to either a negative control inhibitor or an mirR-375 inhibitor. This demonstrates that the results were specific to miR-191 knockdown. Figure 12 shows the significant decrease in cell death when samples were incubated with high concentrations of palmitate, high concentrations of glucose, and high concentrations of both palmitate and glucose together. Cells cultured with 11 mM glucose and 0.5 uM palmitate resulted in a 26% decrease in cell death when miR-191 was inhibited. Samples with 30 mM glucose alone and 30 mM glucose with 0.5 mM palmitate showed a decrease of 14% and 23% respectively, when compared to the negative control inhibitor.

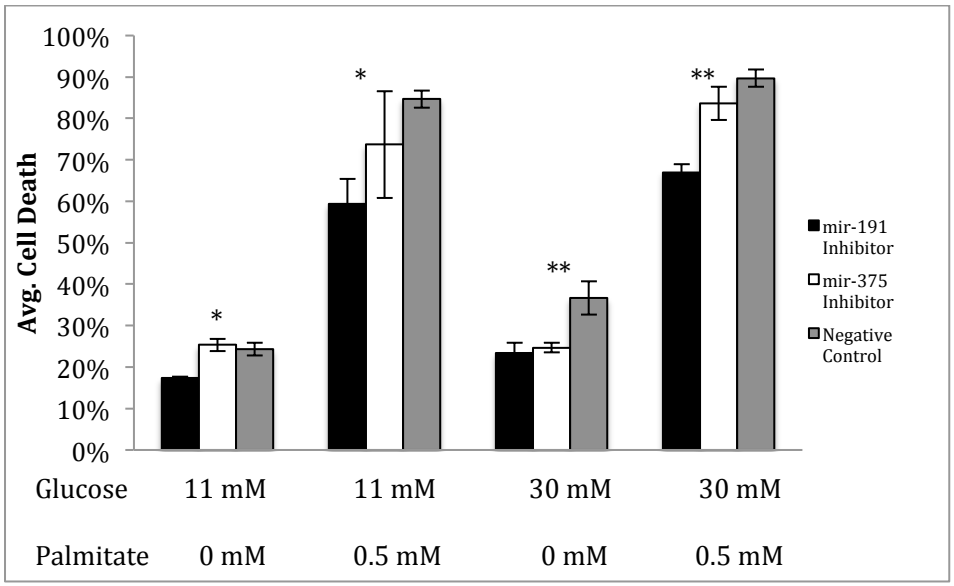

Figure 12 - Glucolipotoxicity and inhibition of mir-191. INS-1 cells were plated in 35 mm plates and transfected with inhibitors for miR-191 and miR-375 as well as with a negative control inhibitor. After 2 days cells were split and treated with media containing 11 mM or 30 mM glucose and with or without .5 mM palmitate. Cell death was measured 24 hours later by staining with 0.4% trypan blue. (n=9, *=p<.05, **=p<.01)

CHAPTER IV

DISCUSSION

The objective of my study was to identify a function for miR-191 in pancreatic beta cells. I have been able to show that the levels of pre-mir-191 increase in response to glucose and palmitate, and that inhibiting the function of miR-191 results in a decreased percentage of cell death when apoptosis is induced (Figure 13). The results I have found are contrary to the functions that I had first hypothesized for miR-191 (growth and function). This data demonstrates that miR-191 is somehow involved in the process of cell death in response to stressful conditions.

The increased expression of pre-miR-191 in response to increased glucose concentrations corresponds well with the prediction of miR-191 being under the control of PDX-1 and NeuroD1. It indicates that the increased expression of miR-191 could be due to PDX-1 being activated by glucose. I still do not have hard evidence for the relationship of miR-191 with PDX-1 and NeuroD1 but future work could assess this by performing a chromatin immunoprecipitation assay (ChIP). There is a wide difference in the expression levels shown in figures 3 and 4 that can be attributed to the normalization with 18S rRNA. 18S rRNA expression varied widely between experiments in part because of different genomic DNA standards used in each experiment.

I observed decreased cell death as a result of miR-191 knockdown in both UV and glucolipotoxicity induced apoptosis. This shows that miR-191 promotes apoptosis when

pancreatic beta cells are exposed to stressful environments.The role of miR-191 as a promoter of cell death, though not reported as often as its role in cell survival and proliferation, has been observed before in ovarian cancer cell lines. My results are also consistent with studies that have found cyclin dependant kinase-6 (CDK6) to be a target of miR-191, which causes a decrease in cell proliferation

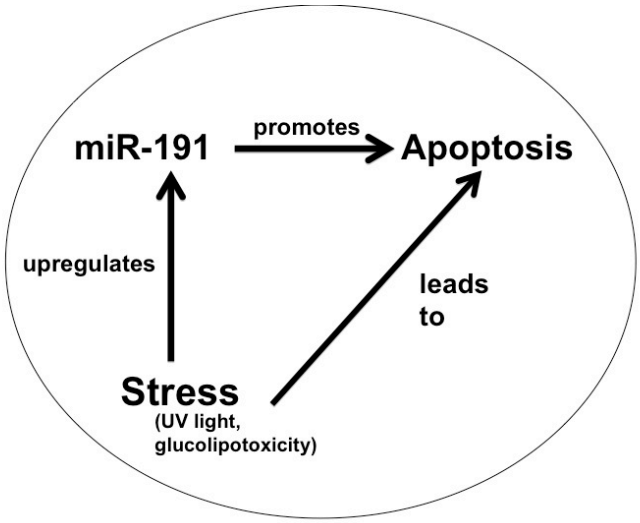

Figure 13 – Model of mir-191 stress response. Visual representation based on data presented. Stress causes cells to undergo apoptosis. miR-191 level increase in response to stress, which promotes this effect.

Knowing how miR-191 may be promoting cell death in pancreatic beta cells will only come to light once target genes are identified. The bioinformatic analysis of miR-191 predicted genes targets suggested that miR-191 has a role in apoptosis but does little to provide viable mechanisms by which miR-191 is promoting cell death. Of the 25 predicted targets of miR-191, 5 of them (CEBPB, DAPK1, MAGEH1, NOTCH2, and RNF216) returned GO terms for apoptosis (Table 1).

It is unlikely that miR-191 is promoting cell death through CEBPB as this gene as been shown to be involved in the ER stress response and promotes apoptosis as well, it has also been shown that deletion of CEBPB increases cell survival. DAPK1, or death-associated protein kinase, is also an unlikely target in miR-191 promoted cell death as it is a known tumor suppressor and promoter of apoptosis. Future studies should focus on identifying targets of miR-191 in order to know how it is working to promote cell death.

During my study I showed that pre-miR-191 levels are increased in the presence of 0.5 mM palmitate. Another study has confirmed the upregulation of miR-191 in samples treated with 25 mM glucose and 1 mM palmitate [62]. It is possible that miR-191 is having an effect somewhere along the line of the ceramide formation response caused by glucolipotoxicity. Palmitate has been shown to be a strong inducer of apoptosis, and has been shown to be directly involved with the de novo synthesis of ceramide that leads to the decrease in active PKB. As discussed previously, ceramide accumulation and decreased levels of PKB lead to decreased level of CREB. A search in miRwalk for miRNAs potentially targeting CREB revealed that there is a predicted miR-191 binding site in the 3' UTR of CREB. It is possible, then, that miR-191 could be contributing to cell death via the ceramide synthesis response caused by glucolipotoxicity.

miR-191 has also been linked to tumor suppressor p53 by a study in HCT-116 cells which contained a null copy of p53. Here it was found that null p53 resulted in decreased levels of miR-191, so it is a possibility that miR-191 works with p53 to promote cell death. p53 is one of the most studied tumor suppressors and has been shown to initiate apoptosis by inducing the transcription of pro apoptotic genes like Bax, Puma,

and Bid; and by repressing the transcription of anti-apoptotic genes like Bcl-2 and survivin. The relationship between p53 and miR-191 has not been studied further but it could be possible for miR-191 to be involved in p53 dependent apoptosis.

Glucolipotoxicity is the hallmark of type 2 diabetes pathophysiology, and there are various pathways involved in glucolipotoxicity where miR-191 could be having an effect. My current study shows that miR-191 has an effect in cell apoptosis but apoptosis does not immediately occur under glucolipotoxic conditions. Pancreatic beta cells undergo a period of compensation where they maintain normal glucose tolerance before experiencing dysfunction. By the time beta cell dysfunction is reached glucose tolerance and insulin secretion are impaired and this is followed by beta cell failure. It is possible, then, that miR-191 could be having an effect earlier before the onset of apoptosis (Figure 14). Studying if miR-191 has an effect on glucose stimulated insulin secretion could assess this.

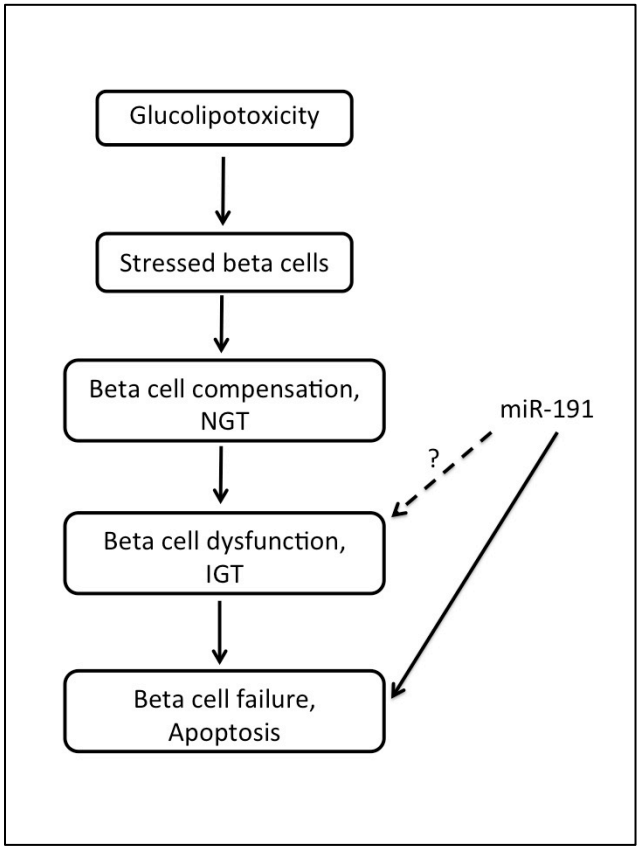

Figure 14 – Type 2 diabetes progression. Glucolipotoxicity causes pancreatic beta cells to compensate for the higher levels of glucose, this leads to beta cell dysfunction and consequently failure. miR-191 is involved in the promotion of apoptosis as a result of glucolipotoxicity but could be involved earlier in type 2 diabetes development.

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