microRNA-375 has opposing effects on cell proliferation in pancreatic α- and β-cells

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by

Pablo Emiliano Diaz

Spring 2017

MicroRNA-375 has opposing effects on cell proliferation in pancreatic α- and β-cells

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ABSTRACT

by

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Master of Science in Biology

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Diabetes is a disease in which patients exhibit deficiencies in their ability to secrete or respond to insulin and is affecting both national and worldwide populations. There are two types of diabetes, type I and type II. In type II diabetes patients, tissues become insulin resistant, requiring pancreatic β-cells to produce more insulin. MicroRNAs (miRNAs) are small 21-23 nucleotide non-coding RNAs that target the 3' untranslated region (UTR) of gene transcripts, inhibiting their expression. One miRNA, miR-375, appears in diabetic's serum more than non-diabetic patients. In diabetic patients with elevated miR-375, β-cell numbers were reduced while pancreatic α-cells increased. In this thesis, I focus on the effects that miR-375 has on β-cell and α-cell proliferation. When inhibiting miR-375 I discovered that α-cells in culture proliferated about 20% less, as one might see in diabetics. Conversely, β-cells in culture proliferated about 20% more, again, as one might see in diabetics. I also focus on target genes of the miR-375: Rasd1 and Pdk1. Rasd1 is a negative regulator of cell proliferation in G coupled protein receptor signaling. Pdk1 is involved in the PI3/Akt pathway for promoting cell proliferation. I predicted that the negative regulator Rasd1 would be less abundant in α-cells compared to β-cells, and in fact this is what I discovered. Though inconclusive, knock down of miR-375 suggests Rasd1 is targeted in α-cells but not in β-cells. The results suggest that miR-375 is important for cell proliferation within α-cells but is inhibitory to β-cell.

CHAPTER I

INTRODUCTION

Diabetes

Diabetes is a disease in which patients exhibit deficiencies in their ability to secrete or respond to insulin, causing hyperglycemia and other systematic impairments within the body. Diabetes has been on the rise at both the national and worldwide levels. On a worldwide scale, 422 million adults have diabetes, per the Centers for Disease Control (CDC, 2014). The prevalence of diabetes worldwide in adults is 8.5% as of 2014 according to the World Health Organization (WHO). In the United States, 9.3% of the total population had diabetes as of 2012 (CDC, 2014). Every year, there are 1.4 million Americans being diagnosed with diabetes and it is known as the 7^{th} leading cause of death.

Insulin is a hormone produced by the pancreatic β-cells that promotes the intake of glucose into cells of the liver, muscle, and adipose tissue. There are two types of diabetes, type I and type II. In type I diabetes, the insulin-producing pancreatic β-cells are destroyed from an autoimmune disorder, causing a patient to be insulin deficient and requiring insulin administration to maintain normoglycemia. β-cell destruction occurs by the body's own T-cells, specifically $CD8^+$ T-cells that recognize a marker specific to β-cells (Graham et al, 2012). Preproinsulin is the most important autoantigen in type I diabetes (Todd, 2010). Type I diabetes is mostly diagnosed within children and comprises 10% of total diabetics. On the other hand, type II diabetes develops over time and is diagnosed in adults rather than children.

In type II diabetes patients, cells of peripheral tissues become insulin resistant, requiring β-cells to produce more insulin. In late term type II diabetes the hyperglycemic conditions the body undergoes can damage the pancreatic β-cells, causing glucotoxicity and apoptosis. Increased metabolism leads to glyceraldehyde autoxidation, producing reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) which then cause cellular damage. This occurs when glucose is metabolized in anaerobic glycolysis to form glyceraldehyde 3-phosphate. Glyceraldehyde 3-phosphate is then oxidized by glyceraldehyde phosphate dehydrogenase (GAPDH), which is an essential step in order for the downstream formation of hydrogen peroxide to occur. H_2O_2 can form highly toxic ROS that cause mutagenic alterations in DNA and apoptosis (Robertson, 2004).

α-cells and β-cells

The pancreatic islets of Langerhans contain the endocrine cells of pancreas. Within the islets are five subsets of hormone secreting cells, each of which secretes a unique hormone (Elayat et al, 1995). The two most abundant are the α-cell, which secretes glucagon, and the β-cell, which secretes insulin (Talchai et al., 2012). The number of each cell type in the pancreatic islets is 20-30% α-cells and 50-70% β-cells (Spijker et al., 2013). Both α- and β-cells are important because they have roles in glucose homeostasis and if one type of cell is impaired, diabetic symptoms potentially arise. Proper control of hormone secretion in these cells depends on many factors, including signaling cross talk between them. This is known as paracrine signaling, the signaling to neighboring cells from the secretion of a hormone. In healthy individuals, paracrine signaling can occur in both directions: β-cell to α-cell and vice versa. When blood glucose is high, glucose enters the β-cell, which then secretes insulin, signaling the

α-cell to stop secreting glucagon, allowing for glucose homeostasis. The receptor responsible for the paracrine signaling of the α-cell is the α-cell specific insulin receptor (αIR). When insulin binds to the receptor, it activates the phosphatidylinositol 3-kinase (PI3K) pathway, which then lowers the sensitivity of potassium dependent ATP channels that are necessary for secretion of glucagon (Kawamori et al, 2009). α-cells secrete acetylcholine as a non-neuronal paracrine signal to regulate β-cell secretion of insulin. Acetylcholine is secreted by α-cells and binds to the muscarinic receptors. When bound, acetylcholine increases cytoplasmic Ca^{2+} concentrations, allowing for the increase of insulin secretion (Rodriguez-Diaz et al, 2011). In late stage type II diabetes, the signaling is not efficient and abnormalities in the paracrine signaling occur. In chronic hyperglycemia, the β-cells are unable to secrete the insulin required to inhibit α-cell glucagon secretion exacerbating the diabetic state (Moon and Won, 2015).

Physiologically, α- and β-cells are quite similar. Both types of cells have transmembrane glucose transporters, although the affinities for glucose in the different cells are different. The α-cell has a high affinity for glucose and transports glucose into the cell even under fasting conditions through the GLUT1 transporter. Even in low glucose, the transporter allows for glucose to enter the cell and begin glycolysis. In the β-cells, the glucose transporter has a low affinity for glucose, which allows the β-cells to respond sensitively to fluctuating glucose levels after consumption of a meal. The glucose transporter for a the β-cell is known as GLUT2 transporter (Quesada et al, 2008). When glucose enters a pancreatic cell, it undergoes glycolysis which produces ATP, which then binds the ATP-dependent potassium leak channels. These channels are necessary to transport potassium outside the cell and when they are bound by ATP the

channels are closed, making an electrical gradient. An electrical gradient occurs when ions cannot leave the cell through its respective channel and therefore cause a change in charge on the cell membrane itself. The accumulation of the charge causes an increase from -70mV, which is the resting potential, to 40mV, the charge necessary for an action potential. This membrane potential opens voltage dependent Ca^{2+} channels that depolarize the membrane and lead to a calcium-dependent exocytosis of hormone-containing secretory granules. While both cells have similar methods of releasing their hormones, the differences lie within the channels and the way they function. When comparing the cells, the glucose transporter is the biggest difference due to their different affinity for glucose. Another difference is the affinities of their ATP dependent potassium channels with the ATP affinity being low within α-cells and high within β-cells. (Quesada et al., 2008). When monitoring the downstream effects of the potassium channels, the depolarization effects are similar in that Ca^{2+} channels are opened to allow secretion of their hormones to occur. This occurs by Ca^{2+} assisted vesicle fusion to a membrane due to the binding of synaptotagmin to Ca^{2+} which is the last trigger to cause the fusion to occur (Pigino et al., 2006).

The role of α-cells in conjunction to β-cells

Although pancreatic β-cells have received the vast majority of attention in the diabetes field, it is believed that α-cells can contribute to the diabetic phenotype as well. This is known as the bihormonal hypothesis. The bihormonal hypothesis states that the development of diabetes is the result of not only insulin resistance but also of excess glucagon secretion (Unger and Orci, 1975). In their classical paper, Unger and Orci (1975) showed the importance of glucagon to diabetes progression and how over-

production of insulin is due to the presence of glucagon causing the hyperglycemic conditions. At first it was believed that insulin was the only hormone responsible for the cause of diabetes because patients that had low insulin secretion could be treated with insulin injections. But now researchers believe that glucagon plays a role in the development of diabetes in conjunction with insulin deficiency. Recently, studies on genetically engineered mice that had both their glucagon receptor gene deleted and their insulin gene deleted showed the mice needed insulin shots and were able to keep glucose levels normal but could not survive for more than 6 days (Neumann et al, 2016). This give rise to the importance the α-cells can be in the studies of diabetes alongside the β-cell and raises the question as to the role that α-cells have in type II diabetes, as shown with the previous studies.

Apart from hormone secretion being affected in diabetics, there have been observations regarding pancreatic β-cell loss and α-cell gain. The ratio of β-cell to α-cell is reported to change in diabetes (Zhao et al., 2010). This was originally attributed to apoptosis of β-cells but recent evidence suggests dedifferentiation of the β-cell into α-cells is the major cause (Talchai et al., 2012). These studies also highlight another factor that affects diabetics and that is loss of cell proliferation of these cells in the pancreas. Many factors play a role in cell proliferation though I will focus on one class of molecules called microRNA (miRNA).

MicroRNAs

MiRNAs are small 21-23 nucleotide non-coding RNAs that target the 3’ untranslated region (UTR) of mRNAs, regulating their expression (Bartel, 2004). Most of the interactions with microRNAs are inhibitory. These miRNAs originate from DNA

after being transcribed by RNA polymerase II to make primary miRNA (pri-miRNA). After being transcribed, pri-miRNA is then cleaved by RNase III enzymes known as Dicer and Drosha. The first step is the cleavage of the pri-miRNA by the Drosha enzyme. The splicing makes a 70 nucleotide product known as precursor miRNA (pre-miRNA), which forms a stem-loop structure and is then transported to the cell cytoplasm by exportin 5. Once the pre-miRNA is in the cytoplasm, the Dicer enzyme cleaves the loop from the pre-miRNA, creating a double stranded 21-23 bp miRNA. The miRNA is then bound by an Argonaute protein to hold the single strand miRNA and begin to be part of the RNA induced silencing complex (RISC). The RISC is composed of an Argonaute protein which binds the miRNA and other proteins that are involved in binding to target mRNA. It is important to note that binding of the miRNA is semi complimentary and thus forms a bulge from the binding within the RISC. This binding also causes the RISC to inhibit translation initiation (Djuranovic et al, 2012) MiRNAs have many targets with a wide range of biological impacts such as proliferation, metabolism, cell differentiation, and apoptosis (Alvarez-Garcia and Miska 2005). MiRNAs are known to have important roles in development of various tissues and organs and because of this, have attracted attention in the diabetes field as potential gene therapies.

miR-375 importance and role in Diabetes

In my study, I focus on a specific miRNA, miRNA-375. MiRNA-375 is present and has an important role in the pancreatic islet development. miR-375 was first identified in pancreatic α- and β-cells, and in the β-cells it was shown to inhibit glucose-stimulated insulin secretion (GSIS) because it represses myotrophin, a protein responsible for mobilizing actin filaments around insulin secretory granules (Poy et al., 2004). MiR-

375 targets insulin secretion by physiological means as well. When monitoring Na^+ channels, miR-375 has been shown to regulate the membrane voltage that plays a crucial role for insulin secretion. When miR-375 is down regulated, membrane voltage increased, showing the role miR-375 is that of regulating the steps necessary for insulin secretion. MiR-375 also regulates insulin secretion by reducing Ca^{2+} dependent exocytosis. These results show miR-375 affects insulin secretion by multiple methods within the β-cells (Salunkhe et al, 2015).

MiR-375 is also very important in the early stage development of β-cells in the pancreas. There are transcription factors such as Pdx1 and NeuroD1 that bind on to the miR-375 promoter, activating its expression (Keller et al, 2007). Pdx1 and NeuroD1 are known for development and cell growth of β-cells. In knock-out miR-375 mice (KO375), the β-cell percentage dropped while α-cell percentage increased within the pancreas (Poy et al., 2009). These results highlight the importance that miR-375 has in the development of the pancreas since miR-375 is needed for the β-cell proliferation at early developmental stages of the pancreas.

In contrast to its role in promoting the β-cell identity in development, miR-375 may have a suppressive effect on β-cell growth in the adult and in disease. For example, El Ouaamari et al (2008) discovered that miR-375 suppressed cell proliferation in a β-cell line through the protein phosphoinositide dependent kinase 1 (Pdk1). It is unknown what, if any, effect miR-375 has on α-cell proliferation. Consistently, in diabetics miR-375 overexpression is correlated with reduced β-cell counts and increased α-cell counts (Zhao et al, 2010). Elevated levels of serum miR-375 are being evaluated as biomarkers for

identifying type II diabetes (Higuchi et al, 2015). **In my thesis, I will test the hypothesis that miR-375 promotes the proliferation of α-cells.**

In order to test whether miR-375 regulates cell proliferation, I focused on known miR-375 target genes which are known regulators of cell proliferation. Dexamethasone-induced Ras-related protein (Rasd1) and Pdk1 are genes targeted by miR-375. These target genes play a role in cell proliferation either a negative role such as Rasd1 (Graham et al., 2001), or a positive role such as Pdk1 (El Ouaamari et al., 2008). Rasd1 has been shown to be a negative regulator of cell proliferation since it is involved in the inhibition of the α subunit of the heterotrimeric G protein in G coupled protein receptor (GPCR) signaling. Rasd1 is a small G protein which binds to the Gα subunit of the heterotrimeric G protein, thereby suppressing the downstream mitogen-activated protein (MAP) kinase signaling cascade which normally leads to cell proliferation (Fig. 1). This study of the Rasd1 negative regulation was done in COS-7 cells, a kidney cell line (Graham et al, 2002). Pdk1 plays a role in promoting cell proliferation in the PI3K/Akt pathway. Pdk1 binds to activated phophatydylinosotol 3,4,5 triphosphate (PIP_3) and recruit protein kinase B (Akt) which inhibits the p27, a cyclin dependent kinase 1 (Cdk1) inhibitor and ultimately allow the cyclin to bind with the Cdk1 to activate the cells to proliferate by initiating S phase (Nakamura et al, 2008). These studies suggest the signaling mechanism of the Pdk1 to be crucial for the cyclin interaction with the cdk1 protein for cell proliferation (Fig. 2). Rasd1 and Pdk1 were also chosen to be targets due to their complimentary binding with the miR-375 (Peterson et al, 2014).

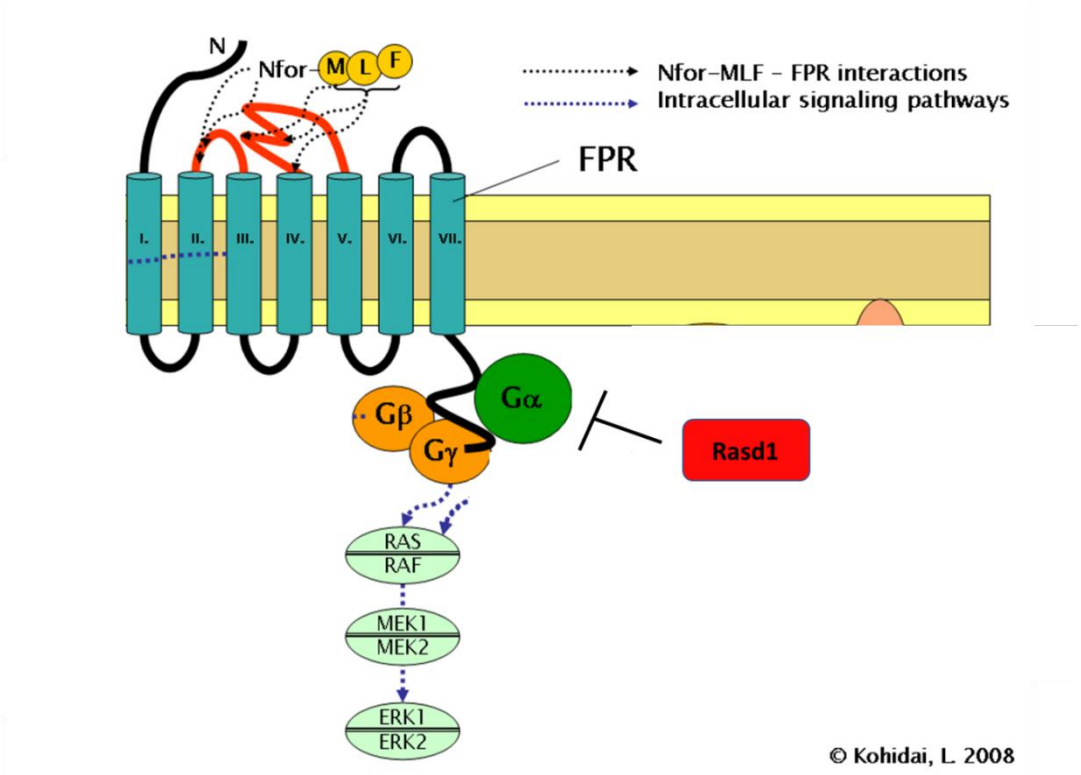

Figure 1. Suggested model for Rasd1 in cellular proliferation. Rasd1 binds to the heterotrimeric G protein α subunit to inhibit the separation of the β/γ subunits and begin the signaling cascade or RAS/RAF, MEK1/MEK2 and ERK1/ERK2 activation. This suggested model shows the importance of Rasd1 in being a negative regulator in cell proliferation (Kohidai 2008).

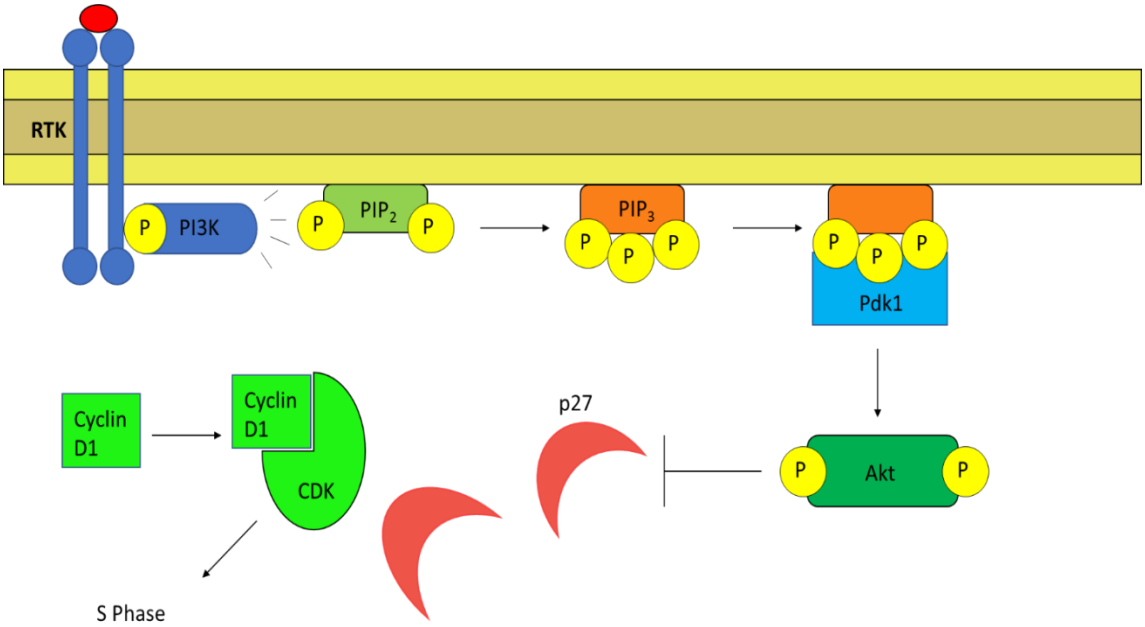

Figure 2. Suggested pathway that Pdk1 plays in the PI3K/Akt signaling mechanism. Pdk1 interacts with Akt, causing a downstream effect o inhibit proteins targeting cyclin, which is important for the transition of cell cycle stages. MiR-375 is suggested to target Pdk1, inhibiting the interaction of Pdk1 and Akt to not occur and halting cell proliferation.

It has been noted that most of the work on miR-375 has been on β-cells and not a lot of information is known in the α-cells. MiR-375 has been shown to effect hormone secretion and cell proliferation within β-cells but no work has been done in α-cells. This raises the question of what the effects of miR-375 has on α-cells regarding cell proliferation. I hypothesize that the miR-375 will have opposing effects within the two cells in their cell proliferation and their expression patterns of genes responsible for proliferation. In my studies, I perform metabolic activity assays to monitor the metabolic activity of the two cell lines when transfected with miR-375 hairpin inhibitors. It is expected that miR-375 will inhibit β-cell proliferation but enhance α-cell proliferation. Another focus is to monitor the expression of various known target genes of miR-375. Monitoring these target genes with transfection of miR-375 inhibitor will enable us to analyze the role of the miRNA in both cell lines and support our hypothesis.

CHAPTER II

MATERIALS AND METHODS

Cell Culture

Mouse αTC1-6 glucagonoma and Min6 insulinoma cell lines were used in an MTS assay and reverse transcriptase PCR. Human Embryonic Kidney 293 (HEK 293) cell line was used for the Taqman assay. αTC1-6 cells were cultured using Dulbecco's Modified Eagle Media (DMEM), supplemented with 17.9 mM $NaHCO_3$, 15 mM Hepes, 10% fetal bovine serum (FBS), 1x non-essential amino acids, 1x penicillin/streptomycin/glutamine and incubated at 5% CO_2 in 37ºC. Min6 cells were cultured using DMEM which was supplemented with 15% FBS, 50μM β-mercaptoethanol, 1mM sodium pyruvate, 1x penicillin/streptomycin/glutamine and incubated 5% CO_2 in 37ºC. HEK 293 cells were cultured in DMEM, 10% FBS, 1x penicillin/streptomycin/glutamine and incubated at 5% CO_2 in 37ºC. Cells were fed every three days and split the day after.

MicroRNA inhibitor Assay

Serum free media was used for transferring the transfection reagent with the inhibitors. Lipofectamine 2000 reagent was mixed with the serum free media and incubated at room temperature for 5 mins. Inhibitor and control inhibitor was then mixed with the reagents and incubated for 15min at room temperature. After incubation, the mix was then introduced to the wells. When performing a 12-well plate transfection, 200uL of serum free DMEM was prepared with 20nM inhibitor and 2μL of Lipofectamine 2000. Within

the 96-well plate, cells were transfected with 40nM of inhibitor and 0.25μL of Lipofectamine 2000.

MTS Assay

Both cell lines were seeded in a 96-well culture plate at 50,000 cells per plate for both cell lines. The cells were incubated in 37^{o}C for 24 hours and then transfected with 40nM of miR-375 hairpin inhibitor or control inhibitor by using Lipofectamine 2000 reagent. Cells were incubated for 24 hours and then treated with Hank's balanced salt solution (HBSS) to normalize cells. After a 30min incubation, the HBSS was removed and replaced with HBSS with various glucose concentrations: 0mM, 0.156mM, 0.312mM, 0.625mM. 20μL MTS reagent was then added to samples and then incubated at 37^{o}C for 1-4 hours. After incubation, the 96-well plate was read at a wavelength of 490nm.

Reverse Transcriptase

RNA was extracted from cell lines after inhibitor assays in 12-well plates. Media from each well was removed and replaced with 500μL of Trizol reagent. Cells were incubated and placed on a plate shaker for 10 minutes at room temperature. After shaking, the Trizol reagent containing lysed cells were transferred to 1.5mL tubes and mixed with 200μL of chloroform. After spinning down at 10,000 RPM for 15min in 4^{o}C, the organic and aqueous layers were separated and 250μL of 100% isopropyl alcohol was added to the aqueous. After incubation at room temperature for 10min, another spin of 10,000 RPM for 10min at 4^{o}C was performed and the supernatant is dispensed. RNA pellets were washed in cold 75% ethanol.

cDNA Synthesis

Recovered RNA was treated with DNase I in 37^{o}C water bath for 30min and then deactivated in 70^{o}C for 15min. After RNA measurements on the spectrophotometer at wavelengths 260nm and 280nm were measured, 500ng of RNA were reverse transcribed using 250 ng of random primers. Reverse transcription was performed using Moloney Murine Leukemia Virus reverse transcriptase and 10 μM deoxynucleoside triphosphates. Samples underwent incubation of 37^{o}C for 60 min followed by a heat inactivation in 70^{o}C for 15 min. DEPC treated water was used to make 1:5 dilutions of samples and then run in polymerase chain reaction (PCR).

Quantitative PCR

Synthesized cDNA was analyzed by quantitative real time PCR (qRT-PCR) by using an Eppendorf Mastercycler. The fluorescent dye SYBR green (Thermo-Fisher HiGreen reagent) was used to quantify the genes of interest. PCR was run under an initial denaturation step of 95^{o}C for 10min, followed by 95^{o}C for 15sec and 68^{o}C for 45sec for forty cycles. Mouse genomic DNA diluted 1:5 in 5 serial dilutions were used as the standards for each primer set used.

Primer Design

Table 1. Primer sequences for target genes and quantitative PCR reactions. The primers used were designed using Primer3. Gene transcripts for In-Silico PCR with the primers were tested using UC Santa Cruz Genome Browser.

mRasd1_RT 115_F	GGGCCGTTTCGAGGATGCTT
mRasd1_RT 115_R	CGGATGATTGCCGGATGTGTC
m/rPdk1_RT 77_F	AGCTGAAGCCCGACCAAGCA
m/rPdk1_RT 77_R	GGCGTTTCTTGCGAGGCTGT
mActB_RT 96 F	TCGTACCACAGGCATTGTGATGG
mActB_RT 96 R	AGACGCAGGATGGCGTGAGG

Taqman Assay

100 ng of RNA is used for the first strand synthesis. The RNA is treated with 5x 1st strand buffer, 10μM deoxynucleoside triphosphates, and Moloney Murine Leukemia Virus reverse transcriptase. The samples were then run in 16^{o}C for 30min, 42^{o}C for 30min and 85^{o}C for 5mins. The cDNA was then diluted at a ratio of 1:9 to be prepared with 20x Taqman Probe, 10x Buffer, 25 mM MgCl, 10 mM deoxynucleoside triphosphates and 5U/ul of Taq DNA pol. In the qPCR, the samples were run at 95^{o}C for 30 sec, 95^{o}C for 15sec and 60^{o}C for 1min for 40 cycles.

CHAPTER III

RESULTS

Characterization of cell lines

Primers specific for insulin were used to verify the MIN6 cells to be of β-cell origin. MIN6 cells showed a higher expression of insulin than αTC1-6 and Human embryonic kidney 293 cells (HEK 293) (Fig. 3A). In a similar fashion, primers specific for glucagon were used to determine that our cell lines are of α-cell origin. The αTC1-6 cell line shows a high expression of glucagon when compared to the MIN6 and the HEK 293 cells (Fig. 3B). Using the Taqman qPCR assay, mature miR-375 is present within α-cell line αTC1-6 and β-cell line MIN6, as shown in previous studies (Poy et al., 2004).

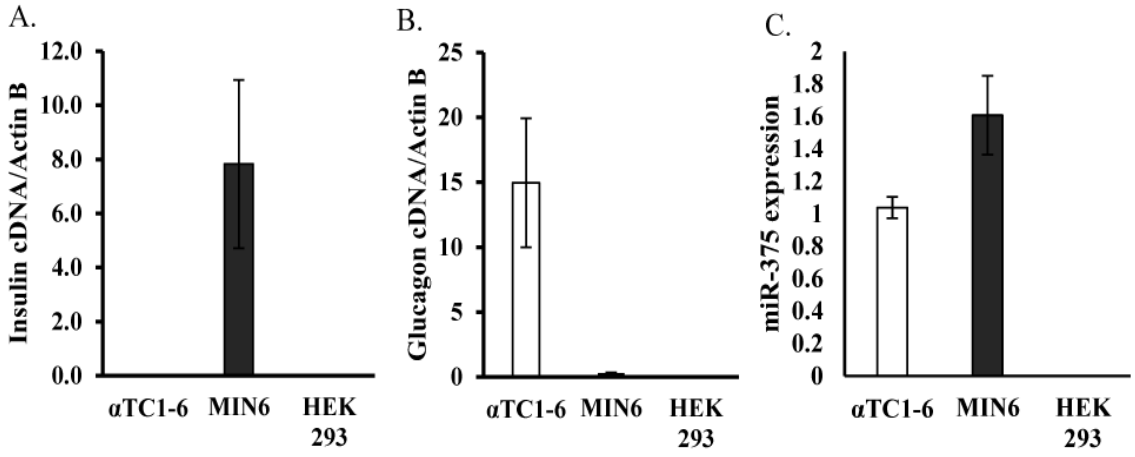

Figure 3. miR-375 is present within both α-cell and β-cell lines. RNA was extracted from mouse glucagonoma αTC1-6 cells, mouse insulinoma MIN6 cells, and from human embryonic kidney (HEK) 293 cells as a negative control. Reverse Transcriptase (RT) PCR was performed to generate cDNA and levels of transcripts were measured using qPCR. **(A)** Insulin and **(B)** glucagon transcripts were amplified and normalized to actin B (ActB) transcripts which served as a housekeeping control gene. **(C)** miR-375 was analyzed by Taqman assay and qPCR. Insulin transcript is detected in MIN6 cells while glucagon transcript is detected in αTC1-6 cells. miR-375 is detected in both αTC1-6 and MIN6 cells. N=4 for **A** and **B** but N=3 for **C**. Error bars represent +/- 1 standard deviation. P value for **A** was 0.007 when β-cell line is compared to both α- and HEK cells. P value for **B** was 0.004 when α-cell line is compared to both β-cells and HEK cells. P value for **C** was 0.04 when comparing α-cell and β-cell lines. P-values were calculated using a student's T-test.

The detection pattern shows higher levels within the MIN6 cells compared to αTC1-6 cell

line, though the significance for this is still not fully understood. Within the HEK 293 cells, there was no detection of the mature miR-375 sequence (Fig. 3C). These initial findings are used to confirm that the cells are pancreatic cell lines and because of their unique expression for the transcripts necessary for hormone production. These cells being pancreatic cell lines are important to monitor gene expression and interaction with miR-375. The levels of miR-375 monitored in the Taqman assay give an understanding of how prevalent it is within the two cell lines in comparison to a non-pancreatic cell line. High miR-375 levels will make it a target for follow up experiments, making these cell lines ideal for knockout experiments to monitor the effects of miR-375 for other cellular processes such as cell proliferation and gene target transcripts.

MiR-375 has opposing roles on cell proliferation in α- and β-cells

Previous studies have shown that miR-375 has an important role in maintaining pancreatic homeostasis within mice. In a study by Poy et al (2009), mice that are lacking miR-375, known as knock out 375 (375KO), resulted in an increase of a total cell count for α-cells while consequently lowering β-cell mass. These past findings have increased interest to test whether miR-375 has opposing roles in cell proliferation in the cell lines. We transfected a miR-375 hairpin inhibitor using Lipofectamine, a lipid molecule that fuses with nucleic acids to form a lipid-RNA complex to facilitate endocytosis of the lipid and introduce the hairpin inhibitor to bind to the miR-375. A control inhibitor derived from cel-miR-67 does not have any sequence identity within human, rat and mouse cells and serves to show the transfection protocol does not affect the expression pattern. Previous work in our laboratory has shown that the inhibitor could block miR-375 activity by 5-fold in a fluorescent reporter assay (M. French, unpublished data). Cell

proliferation was assessed with a colorimetric MTS assay. The cell growth was compared to a normalized ratio of cell growth between the inhibitor treatment to the control which was treated as the standard of our experiments. As expected from our hypothesis, the α-cells showed a decreased cell proliferation when treated with the miR-375 inhibitor, opposite of the β-cells showing an increase after the inhibitor treatment (Fig. 4). These results support the hypothesis on the importance of miR-375 on cell growth is different within the cell lines. MiR-375 is important in understanding cell proliferation in each cell

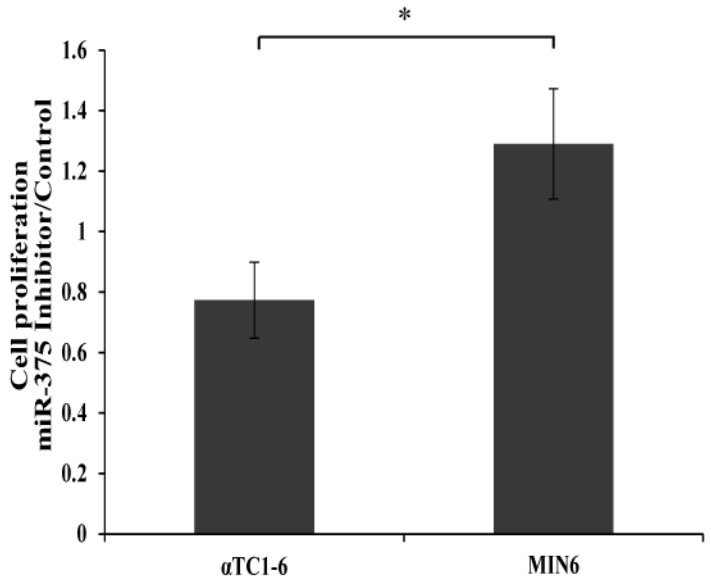

Figure 4. miR-375 has opposing effects in cellular proliferation within α-cell and β-cell lines. An MTS proliferation assay was performed in a 96-well plate to monitor cellular proliferation with cells transfected with the miR-375 inhibitor or the control inhibitor. Cells were transfected with 40nM of inhibitor or control inhibitor. After 24-hour incubation period, the cells were collected and an MTS assay was performed. Error bars represent +/- 1 standard deviation. P value between miR-375 inhibitor and control in α cell line was 0.018 and 0.02 within the β-cell line. The P value between the miR-375 inhibition treatments were 0.015. P values were determined using a student's T-test. N=3.

line, but the mechanisms of this activity are not fully understood.

Target gene expression between different cell lines

Studies have shown there are genes targeted by the miR-375 and are categorized by their initial role in cell proliferation and cell growth. There are genes that have a negative effect in cell proliferation such as RasD1 (Poy et al., 2009) and genes having a positive role in cell growth such as Pdk1 (El Ouaamari et al., 2008). These previous findings have been confirmed in β-cells but not in α-cells. To monitor the role of the target gene, the transcripts are monitored using qPCR to understand the level of expression of the genes within each cell line (Fig. 5). We found that Rasd1 was expressed

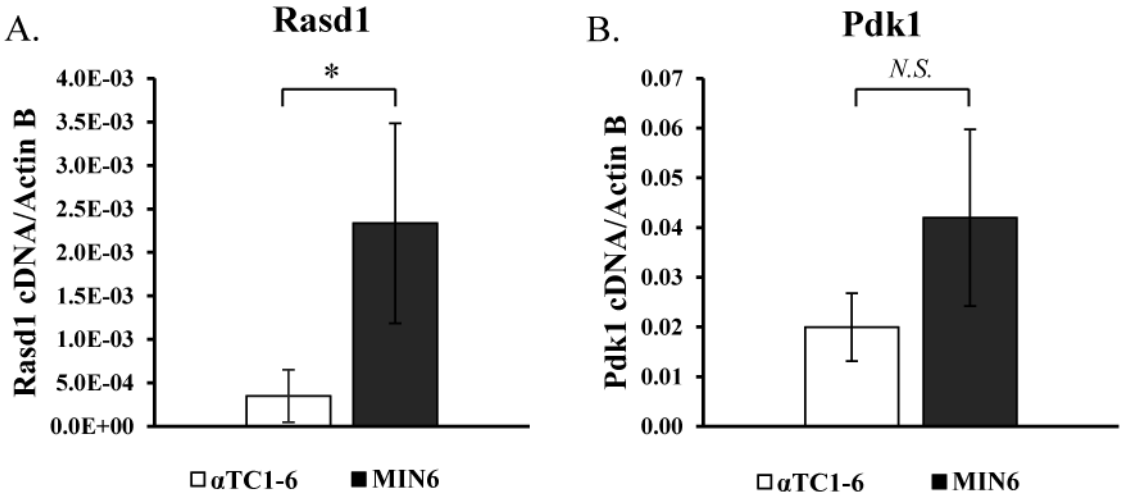

Figure 5. Target gene expression within α- and β-cell lines. RNA was extracted from mouse glucagonoma aTC1-6 cells, mouse insulinoma MIN6 cells, and from human embryonic kidney (HEK) 293 cells as a negative control. Reverse Transcriptase (RT) PCR was performed to generate cDNA and levels of transcripts were measured using qPCR. Rasd-1 transcript was expressed at a higher amount within the MIN6 cell line. Pdk1 transcript expression was the same within both cell lines. N=3. * P value= 0.035 for Rasd1 between both cell lines. P value= 0.12 for PDK1 between both cell lines, N.S. = not significant. P-values were calculated using a student's T-test.

approximately 5-fold less in aTC1 cells as compared with MIN6 cells (Fig. 5A). Pdk1, on the other hand, showed approximately equal expression between the two cell lines (Fig. 5B).

To follow up with the target gene transcripts, we transfected miR-375 inhibitor and control inhibitor to monitor their expressions. The results showed no significant changes for αTC1-6 cells and MIN6 cells when observing the gene transcripts. Rasd1 levels were shown to not have a significant increase in expression in either cell line treated with the inhibitor (Fig. 6). However, there is a trend toward increased Rasd1 expression in the αTC1-6 cell line. The expression patterns are compared to the standard, which is the normalized value of the inhibitor expression to the control expression.

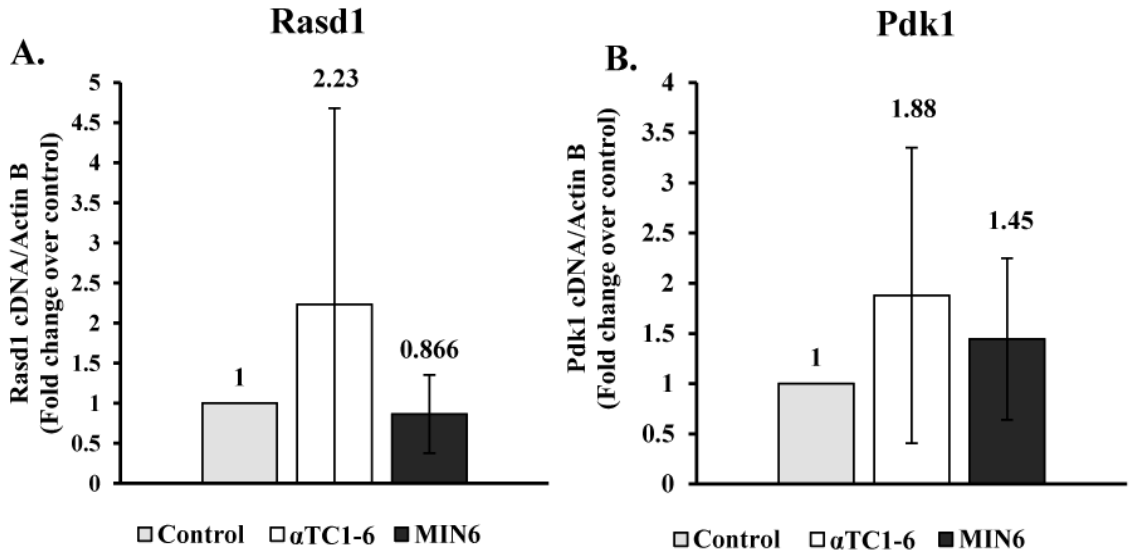

Figure 6. Fold expression change of Rasd1 is dependent on miR-375 but not for Pdk1. The cell lines were transfected with 20nM miR-375 hairpin inhibitor. After a 48-hour incubation, cells were retrieved and treated with RT-PCR for cDNA. qPCR analysis was used to measure the expression of target gene transcripts Rasd1 and Pdk1. Fold expression was measured to the effects of miR-375 inhibition. Pdk1 fold change for the αTC1-6 cell line is the only significant change within the treatments given to the cell lines. P-value for αTC1-6 for Pdk1 transcript is 0.04. N=5 for all runs. P-values were calculated using a student's T-test.

CHAPTER IV

DISCUSSION

The role of miR-375 in pancreatic development and its potential role in diabetes pathology has made it a subject of intense research. For example, when the miR-375 gene is knocked out, the ratio of β- to α-cells decreases (Poy et al., 2009). A similar problem is seen in diabetic patients, except that gain of miR-375 is associated with a decrease in the β- to α-cell ratio. Thus, miR-375 may become a biomarker to monitor diabetes in patients. Elevated miR-375 may contribute to decreased insulin secretion in diabetics (Poy et al., 2004) and decreased β-cell numbers (El Ouaamari et al., 2008). My research confirms that miR-375 decreases proliferation in a β-cell line and extends this to show that miR-375 increases proliferation in an α-cell line.

We report that miR-375 has opposite effects on cell proliferation in the αTC1-6 and MIN6 cells (Fig. 4). We can conclude this as a proliferation assessment of the cells because we allowed a full day for the cells to undergo the MTS assay, allowing a full cycle of cell division. The results indicate miR-375 is important for α-cell proliferation and reduced in the β-cell line, supporting the hypothesis that miR-375 has different effects in α- and β-cell proliferation.

Because miR-375 has different roles in the cell proliferation in these two pancreatic cell lines, it raises the question of how this occurs and through which mechanisms. To explore this, we monitored known target genes of miR-375in each cell line in the hopes of suggesting a mechanism in which miR-375 inhibits or promotes cell proliferation. Targets were selected based on known miR-375 target genes by Poy et al

(2009). The target genes chosen were Rasd1, Cav1, Id3, Aifm1, Eefe1, C1q and Pdk1. These targets were selected because they were monitored to be upregulated in KO375 mice (Poy et al, 2009). We predicted that growth inhibitory target genes Rasd1, Cav1, Id3, Aifm1, Eefe1, and C1q would be expressed higher in MIN6 cells. We reasoned that miR-375 stimulates α-cell proliferation through inhibition of these genes. To determine if this was the case, the target transcripts were amplified and monitored for expression within each cell line. The expression patterns show a significant difference within the Rasd1 target transcript with more of the gene being expressed in the β-cell as predicted (Fig. 5A). We also predicted that the cell proliferation gene Pdk1 would be higher in α-cells. We reasoned that miR-375 blocks β-cell proliferation through inhibition of this gene. However, Pdk1 expression was uniform between the two cell lines, in contrast to our hypothesis (Fig. 5B) We now believe that Pdk1 is targeted in both cell lines to an equal extent. Perhaps the effects of other genes override Pdk1's influence on cell proliferation in β-cells. Next we asked whether miR-375 inhibition would affect Rasd1 and Pdk1 expression. Because we saw low expression of Rasd1 in α-cells, we expected to see the greatest increase in Rasd1 expression in α-cells when miR-375 was blocked. However, when monitoring Rasd1, there was no significant change between the α- and β-cells lines (Fig. 6A). We did notice a trend that supported our prediction, as the fold change in the αTC1-6 is doubled when comparing the miR-375 inhibitor with the control inhibitor. Because we saw equal expression of Pdk1 in the α- and β-cells, we predicted that Pdk1 would be equally elevated in the cells. Again, we saw no significant increase in Pdk1 transcript when the cells were transfected with the miR-375 inhibitor (Fig. 6B). We did notice a trend that supported our prediction, as the Pdk1 transcript was expressed at a

higher rate than the control. One caveat to our findings is that miR-375 is known to suppress the translation of protein through inhibition of translation initiation (Djuranovic et al, 2012) which means that we need to study protein expression as well. There is a rationale for monitoring mRNA levels, however, as miRNA inhibition leads to deadenylation and subsequent degradation of the transcript (Djuranovic et al., 2012).

In summary, based on the data in Fig. 5, we propose that miR-375 targets Rasd1 to a greater extent in α-cells but targets Pdk1 in both α- and β-cells (Fig. 7). It's important to note islet proliferation originates only from pre-existing pancreatic cells. This is because the pancreatic cells are terminally differentiated and cannot proliferate rapidly. Pancreatic β-cells have been able to proliferate after loss of β-cells in the islet as well as reduced insulin secretion (El-Gohary et al., 2014). Although cell proliferation is

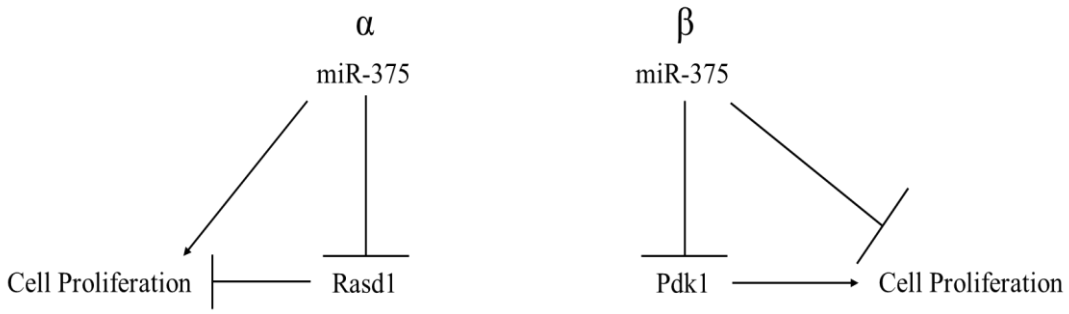

Figure 7. Schematic of the suggested role miR-375 plays in cell proliferation within α-cells and β-cells. The role of miR-375 is different when comparing the two pancreatic cells. Rasd1 is a negative regulator of cell proliferation but in the α-cells, is targeted by the miR-375, increasing cell proliferation. In β-cells, Pdk1 is a positive regulator of cell proliferation and is targeted by miR-375, decreasing cell proliferation as previously tested by Poy et al, 2009.

not rapid within the developed pancreas, our mechanism to explain the action of miR-375 in the cells can help understand the proliferation of the islet when investigating α- and β-cells.

If this work is to be translated to human disease, we need to determine whether miR-375 regulates cell proliferation in human cells. While we have not yet obtained human pancreatic cells, we can at least predict whether miR-375 could target Rasd1 and Pdk1 in human cells using sequence comparison. Using the database provided by miRNA.org, statistical analysis was used to calculate the binding capability and the repression by the miR-375 for each target figure within the mouse and human genomes (Figs. 8 and 9). The binding capabilities of the miR-375 was determined using a mirSVR score. This score predicts whether the miRNA will target the transcript based of the seed

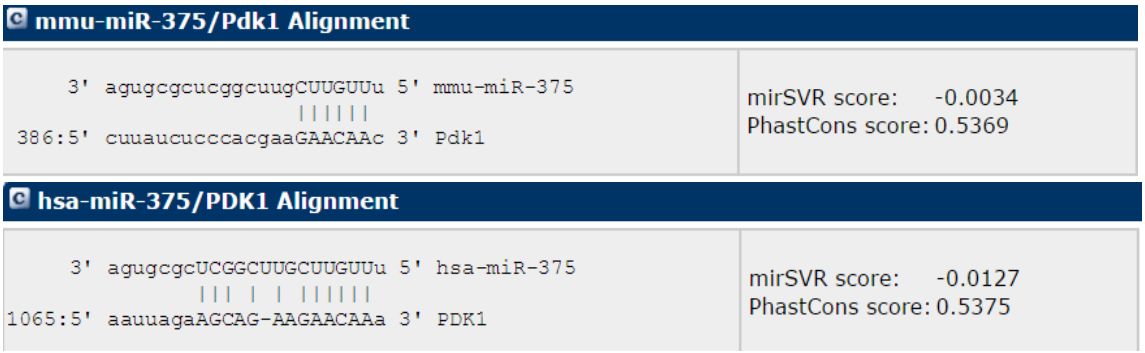

Fig. 8. miR-375 alignment database predictions with target gene Pdk1. The database provided shows the semi-complimentary binding of the miR-375 in both human and mouse genomes. miRSVR scores and PhastCons scores are given with the binding. mirSVR score predicts whether miR-375 targets the transcript. A score of less than -0.1 is suggested to be a high confidence interaction. PhastCons score represents the percentage of the sequence is conserved within all mammals.

binding site for the miR-375. A miRSVR score $\leq$ -0.1 was suggested to be a high confidence target (Peterson et al, 2014). Based on this interpretation, Pdk1 is not predicted to be a target in human cells, while Rasd1 is predicted to be a target. This makes the case stronger for following up on the role of Rasd1 in α-cell proliferation.

Fig. 9. miR-375 alignment database predictions with target gene Rasd1. The database provided shows the semi-complimentary binding of the miR-375 in both human and mouse genomes. miRSVR scores and PhastCons scores are given with the binding. mirSVR score represents how much repression is occurring within the interaction of miR-375 and the transcript. A score of less than -0.1 is suggested to be a high confidence interaction. PhastCons score represents the percentage of the sequence is conserved within all mammals.

MiR-375 is an important miRNA for development of the pancreas in early stages but has an opposite role in late stage of development. The miR-375 has been targeted in past studies in β-cells in regard to target genes, insulin secretion and diabetic markers within type II diabetics. This highlights the importance of miR-375 in this study on cell proliferation and we propose suggested mechanisms based off their target genes. These studies have been on RNA transcripts but not in protein level, making that an ideal follow up experiment in future studies to monitor the interaction of miR-375 at a post translational study. The hypothesis of miR-375 has opposite effects in cell proliferation in α- and β-cells is supported in these studies. These results suggest miR-375 is a potential target for cell proliferation and could be tested using other known target genes to see the importance of miR-375 by other signaling mechanisms.

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