Investigation of Promoter Histone Deacetylation as a Mechanism for Cyclic Adenosine Monophosphate-Stimulated Repression of microRNA-375

A Thesis
Presented
to the Faculty of
California State University, Chico

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
in
Biological Sciences

by
Alexander R. Stanton
Summer 2015
Investigation of Promoter Histone Deacetylation as a Mechanism for Cyclic Adenosine Monophosphate-Stimulated Repression of microRNA-375

A Thesis
by
Alexander R. Stanton
Summer 2015

APPROVED BY THE INTRIM DEAN OF GRADUATE STUDIES:

______________________________
Sharon Barrios, Ph.D.

APPROVED BY THE GRADUATE ADVISORY COMMITTEE:

______________________________
David M. Keller, Ph.D., Chair

______________________________
Jeff Bell, Ph.D.

______________________________
Jonathan R. Day, Ph.D.
ACKNOWLEDGEMENTS

I would like to thank my advisor David Keller whose consistent advice and direction was not without the freedom to explore independence, fostering my development as a researcher. Additional thanks to my thesis committee, Jeff Bell and Jonathan Day, for asking the hard hitting questions needed to keep me on my toes.

Likewise, thanks to my fellow graduate students, undergraduate coworkers, and teaching associates who endured work and academics alongside me. Your support and companionship – sharing in the joys and woes over the last three years – has enhanced the process tremendously.

And finally, thank you to all my friends and family for encouraging and understanding the time investment and obsession which graduate school requires.
TABLE OF CONTENTS

PAGE

Acknowledgements ........................................................................................................... i

Table of Contents ............................................................................................................. ii

List of Tables .................................................................................................................... iv

List of Figures ................................................................................................................... v

Abstract ............................................................................................................................ vi

CHAPTER

I. Introduction ......................................................................................................................

Type 2 Diabetes .................................................................................................................. 1

Pancreatic β-Cells ............................................................................................................. 2

microRNA ......................................................................................................................... 3

microRNA-375 ................................................................................................................. 5

Epigenetics and Histone Acetylation ............................................................................... 6

II. Materials and Methods ............................................................................................... 9

Bioinformatic Analysis ..................................................................................................... 9

Cell Culture ..................................................................................................................... 9

Cloning ............................................................................................................................ 9
Luciferase Reporter Assay................................................................. 10
RNA Extraction.................................................................................. 10
cDNA Synthesis.................................................................................. 11
Chromatin Immunoprecipitation.......................................................... 11
Micrococcal Nuclease Protection Assay................................................. 12
Quantitative Polymerase Chain Reaction............................................. 12

III. Results.......................................................................................... 13

Characterization of the Putative microRNA-375 Promoter................... 13
Importance of the Upstream Promoter Region of miR-375................. 15
Repression by cAMP-Stimulation......................................................... 16
Effect of HDAC Inhibition on miR-375 Repression.............................. 19
Decreased Transcription Initiation by cAMP-Stimulation.................... 21
Promoter Histone Deacetylation by cAMP-Stimulation....................... 24
Overall Chromatin Structure.............................................................. 25

IV. Discussion....................................................................................... 29

V. References....................................................................................... 33
# LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Cloning Primers</td>
<td>15</td>
</tr>
<tr>
<td>2. microRNA-375 Gene and Promoter Primers</td>
<td>21</td>
</tr>
<tr>
<td>3. Control Primers</td>
<td>22</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Model of cAMP-induced HDAC-mediated Repression of microRNA-375</td>
<td>13</td>
</tr>
<tr>
<td>2. Schematic of the miR-375 Cloning Sites</td>
<td>14</td>
</tr>
<tr>
<td>3. Schematic of the miR-375 Upstream Promoter in Relation to Tested Regions</td>
<td>14</td>
</tr>
<tr>
<td>4. Transcriptional Activity Conferred by miR-375 Upstream Promoter Regions</td>
<td>16</td>
</tr>
<tr>
<td>5. Repression of miR-375 in Response to cAMP</td>
<td>18</td>
</tr>
<tr>
<td>6. HDAC-Inhibition on Expression of pre-miR-375 During cAMP-Signaling</td>
<td>20</td>
</tr>
<tr>
<td>7. Transcriptional Initiation at the miR-375 Promoter After cAMP Induction</td>
<td>23</td>
</tr>
<tr>
<td>8. Acetylation of Histone H3 Lysine 9 and 14 (Ac-H3K9/14) Reduction at the Distal Upstream miR-375 Promoter Regions</td>
<td>25</td>
</tr>
<tr>
<td>9. Chromatin Structure Analyzed by Susceptibility to micrococcal nuclease (MNase) digestion</td>
<td>27</td>
</tr>
<tr>
<td>10. Overall Model of HDAC 1/2 in Relation to miR-375 Regulation</td>
<td>31</td>
</tr>
</tbody>
</table>
ABSTRACT
by
Alexander R. Stanton
Master of Science in Biology
California State University, Chico
Summer 2015

Type 2 Diabetes (T2D) is a disease which affects a large percentage of the United States and the risk of developing T2D is increasing according to recent CDC estimates. T2D can be fatal, and although patients are living longer with it, the costs of caring for this disease are burdensome on both the patients and the health care system. Better understanding of the aberrations in cell signaling during T2D could lead to insights that may restore proper signaling. Investigations of genetic dysregulation in these patients has implicated the overexpression of the small regulatory RNA microRNA(miR)-375. These miRNA are post-transcriptional regulators that function through translational silencing, and two of the genes which miR-375 silences are involved in the pancreatic β-cell viability and insulin exocytosis (phosphoinositide-dependent kinase-1 and myotrophin respectively). The factors which govern the activation and repression of miR-375 are not well known, but it is downregulated by the cyclic adenosine monophosphate (cAMP)/ protein kinase A (PKA) pathway through an unknown mechanism. Since cAMP activates PKA, which then activates histone deacetylase (HDAC) 1 and 2 through phosphorylation, deacetylation is a possible mechanism for repression during this pathway. This study has identified two regions of the miR-375 upstream promoter which are deacetylated at histone H3 lysine 9/14 corresponding with decreased transcriptional initiation by cAMP induction. Observed cAMP-induced deacetylation does not cause overall changes in the chromatin structure of the miR-375 upstream promoter as analyzed by a micrococcal nuclease
protection assay. The necessity of HDAC in this process is still unclear due to the broad effects of HDAC inhibition by HDAC inhibitor trichostatin A. While some regions of the promoter are deacetylated during cAMP signaling, the moderate changes it confers suggest that it may only be partially responsible for the repression of miR-375.
Type 2 Diabetes

In the United States, 8.3% (25.8 million people) of the population has either Type 1 or Type 2 Diabetes Mellitus (T1DM and T2DM) as of 2011 (CDC 2011). Of these, Type 2 Diabetes is the most prominent (ADA 2014). During 2012, the health care cost of diabetes was $245 billion dollars, costing diabetic patients 2.3 times more on average than they would otherwise pay (ADA 2013). Therefore, conducting research into understanding and treating this disease is a pressing issue in the United States.

T2DM is classified as inadequate sensitivity to insulin accompanied by inability to produce adequate insulin (Kudva et al. 1997, DeFronzo et al. 1992, Stumvoll et al. 2005). The pathogenesis of the disease begins with insensitivity to insulin, which is initially compensated for by hypersecretion of insulin by the β-cells (Prentki et al. 2006). Initially the β-cells are able to compensate for the insensitivity to insulin, but eventually due to myriad reasons the β cells are unable to keep up the hypersecretion of insulin and hyperglycemia develops - at which point the person becomes type 2 diabetic (Prentki et al. 2006).

The dysfunction of β-cells to adequately produce insulin is not completely elucidated but can sometimes be associated with a deficit in the density of β-cells (Butler et al. 2003). There is evidence that β-cell apoptosis is occurring, but new evidence also points to β-cell dedifferentiation into alpha-cells (Prentki et al. 2006, Talchhai et al. 2012). Just as with other forms of diabetes, this inability to process or produce insulin adequately results in hyperglycemia while somatic cells are unable to utilize adequate glucose (Stumvoll et al. 2005). Chronic exposure to these issues leads to even more devastating medical problems such as nephropathy (Mordchai et al. 1998, Herman et al. 1998).
Pancreatic β-Cells

β-cells of the pancreas play an important role in the regulation of blood glucose concentrations. The primary function of these cells is to release the hormone insulin, which induces the uptake of glucose from the blood by other cell types. Insulin production is regulated by physiological changes such as elevated blood or intestinal glucose concentrations and signaling by incretin hormones, both of which are the result of consuming a meal (McIntyre et al. 1965, Holst et al. 2004). Presence of high glucose and incretin secretion then stimulates signaling which acts to reduce blood glucose concentrations, thereby maintaining homeostasis.

After meal consumption, the rise in intestinal and blood glucose concentration triggers the production and exocytosis of insulin by the pancreatic β-cells. Insulinotropic effects of β-cells are initiated by the internalization and subsequent metabolism of glucose (Henquin et al. 2000). β-cells can internalize glucose in the absence of insulin due to their expression of the insulin-independent glucose transporter type 2 (GLUT-1 in humans and GLUT-2 in rats) over the insulin-dependent glucose transporter type 4 (GLUT-4) found in other cell types (De Vos et al. 1995, James et al. 1988). Glycolysis causes an elevation in ATP concentration which depolarizes the β-cells by closing ATP-sensitive K+ channels, triggering voltage-gated calcium channels to transport Ca^{2+} into the cell (Henquin et al. 2000). Influx of Ca^{2+} ions stimulates exocytosis, and thereby secretion of insulin (Henquin et al. 2000).

The other means by which β-cells are induced to secrete insulin is through incretin hormone signaling. Incretin hormones are molecules which decrease blood glucose concentrations, primarily as an insulin secretagogue, of which only two have been detailed: glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP) (Holst et al. 2004). Both of these proteins are produced by cells in the small intestine, L-cells produce GLP-1 and K-cells produce GIP, in response to nutrient presence (Mortensen et al. 2003, Orskov et al. 1992, Ebert et al. 1980). After their secretion, they bind to their respective G-protein coupled receptors on pancreatic β-cells and stimulate the cAMP signaling cascade through activation of adenylyl cyclase (Ramos et al. 2008).

Production of cAMP triggered through the binding of incretin hormones is propagated by cAMP-activation of protein kinase A (PKA) and exchange protein directly activated by cAMP (Epac). The catalytic subunits of both PKA and Epac are inhibited by their own regulatory
subunits, which are released after binding cAMP, thus making PKA and Epac catalytically active (Cheng et al. 2008). It has been observed that the activation of these two enzymes function to exocytose insulin granules through independent pathways (Seino et al. 2005). Epac signaling regulates the release of small vesicles and mobilizes exocytotic granules to the membrane, whereas PKA signaling stimulates the release of large dense-core vesicles and in conjunction with Epac stimulates granule fusion and upregulates number exocytotic sites (Cheng et al. 2008, Seino et al. 2005).

Exocytosed insulin is secreted into the blood where it stimulates signaling pathways related to the utilization of the increased blood glucose. Sensing of serum insulin causes skeletal muscle and adipose cells to uptake glucose for metabolism or storage (White and Kahn 1994). Additionally, serum insulin also inhibits the process of gluconeogenesis from glycogen by hepatic cells (White and Kahn 1994). Therefore, the proper functioning of insulin signaling should lower blood glucose levels by utilizing absorbed glucose and preventing the production of new glucose.

**microRNA**

MicroRNAs (miRNAs) are a family of short noncoding RNAs that are around 22 nucleotides in length and function to regulate the expression of genes post-transcriptionally. These miRNAs have varied origins; they can be derived from their own genes or from intronic sequences of other genes, and they can have varied mechanisms of action, with respect to repressing gene expression, depending on their associated proteins (Siomi et al. 2009). The biogenesis of these molecules all follow the same process of two cleavage steps and the eventual loading into protein complex.

Whether the miRNA is coded for by its own gene or is intronic, it is transcribed by RNA Polymerase II (Lee et al. 2004). This initial stage is a long stem-looped RNA, complete with cap structure and polyadenylated tail of normal mRNA, called the primary (pri)-miRNA (Cai et al. 2004). While still in the nucleus the pri-miRNA undergoes its first cleavage. The RNase-III type enzyme Drosha in complex with the protein DiGeorge syndrome critical region gene (DGCR8) binds to the pri-miRNA via the DGCR8 dsRNA binding domain, then Drosha cleaves the pri-
miRNA into a shorter stem-loop of about 65-70 nucleotides called the precursor (pre)-miRNA (Han et al. 2004, Siomi et al. 2009).

At this point in the miRNA biogenesis, all nuclear processing has occurred and the pre-miRNA hairpin is exported out of the nucleus into the cytoplasm by exportin-5 using RanGTP as a cofactor (Yi et al. 2003, Bohnsack et al. 2004). All subsequent steps in the creation of functional mature miRNA are carried out in the cytoplasm. The second and final cleavage step is performed by another RNase-III type enzyme called Dicer which removes the loop from the pre-miRNA stem-loop leaving an ~22 nucleotide duplex (Hutvagner et al. 2001, Ketting et al. 2001, Knight et al. 2001). This duplex is called the miRNA-5p/miRNA-3p duplex because of the 5’-end and 3’-end of the stem structure, either of which may become the mature miRNA (http://www.mirbase.org).

The remaining processing no longer requires pruning of the RNA sequence, but does require strand selection and incorporation into a protein complex. Depending upon the species in which the miRNA is found, the strand selection and regulatory functionality is performed by different Dicer-associated proteins such as R2D2, FMR-1, RDE-4, and argonaute proteins (Tabara et al. 2002, Liu et al. 2003, Ishizuka et al. 2002, Caudy et al. 2002, Jin et al. 2004, Hammond et al. 2001). Strand selection is thought to occur by an inherent difference in thermodynamic stability between the two strands of RNA, where the Dicer-associated protein preferably binds to the more stable strand, which then becomes the mature miRNA (Schwarz et al. 2003, Khvorova et al. 2003, Tomari et al. 2004). While this mechanism has only been described for small interfering RNA, due to similarities in their biogenesis, this selection mechanism is thought to be largely the same for miRNA.

In humans the processing of pre-miRNA and the strand sorting are actually performed by two proteins in a trimeric complex called the RNA induced silencing complex (RISC). This complex is formed by Dicer, HIV transactivating response RNA-binding protein (TRBP), and argonaute-2 (ago2) (Gregory et al. 2005). Dicer cleaves the pre-miRNA into the miRNA-5p/miRNA-3p duplex, TRBP is responsible for the strand selection, and ago2 is the catalytic center which is responsible for gene silencing (Siomi et al. 2009, Gregory et al. 2005). Incorporation of the miRNA strand into the RISC allows for the sequence targeting of mRNA transcripts. Binding of the miRNA to the target mRNA occurs typically through binding of nucleotides 2-8 of the miRNA to the 3’-UTR of the target (Doench et al. 2004). Once bound, the
argonaute protein destabilizes the mRNA which acts through transcriptional silencing or through triggering mRNA degradation (Filipowicz et al. 2008).

The expanding role of miRNA in normal cellular regulation is becoming clear. Investigations into cell-type specific miRNAs are also demonstrating their necessity in maintaining cell-specific functions (Sood et al. 2006). It is also becoming better understood how RNA interference from dysregulated miRNA are driving the progression of diseases like diabetes, cancer, and even HIV-1 infection (Zampetaki et al. 2010, Zhao et al. 2010, Wang et al. 2014, Lu et al. 2005, Yueng et al. 2005). Understanding how miRNAs become dysregulated in different diseases will lead to development of much more robust models of these diseases.

**microRNA-375**

One miRNA which is involved in the proper functioning of β-cells is miRNA-375 (miR-375). Expression of miR-375 is limited to the islet cells of the pancreas and the pituitary gland in the brain (Poy et al. 2004, Poy et al. 2009, Kapsimali et al. 2007). This selective expression is limited to cell types linked by secretion of hormones, a functional similarity between these two organs. Although, abnormal expression of miR-375 in other cell types does occur in cancers such as breast, cervical, and prostate cancer (Ye et al. 2014, Shen et al. 2013, Bryant et al. 2012).

There is evidence that miR-375 is incorrectly regulated during type 2 diabetes mellitus (T2DM). Two studies have assessed the differences in expression of miR-375 in the pancreatic β-cells between patients with T2DM and non-diabetic patients. One small study analyzing autopsied human pancreatic islet cells found a significant increase in miR-375 expression in 40 diabetic patients compared to 15 non-diabetic patients (Zhao et al. 2010). Later, a larger study confirmed these findings, discovering a nearly two-fold increase in miR-375 expression in 54 T2DM patients compared to 53 control patients with normal glucose tolerance, where no overexpression was seen in 44 impaired glucose tolerance patients (Wang et al. 2014). These two studies indicate that there may be a link between excessive regulation by miR-375 and the phenotypes of T2DM.

One of the confirmed targets that miR-375 silences is myotrophin (MTPN). Mtpn has been studied for its involvement in the exocytosis of neurotransmitters via vesicular transport in neurons, another expressor of miR-375 (Yamakuni et al. 2002). Overexpression of miR-375
decreases the expression of Mtpn in a similar manner compared to its inhibition by siRNA (Poy et al. 2004). In addition, this effect correlated with a Ca\(^{2+}\)-independent inhibition of insulin exocytosis as well - suggesting a similar mechanism for Mtpn in \(\beta\)-cells and a role for miR-375 overexpression to play a role in hyperglycemia (Poy et al. 2004).

Another confirmed target is phosphoinositide-dependent kinase-1 (PDK1). The site predicted for miR-375 binding in the 3’ UTR of PDK1 has been highly conserved, and indeed miR-375 expression mediates post-transcriptional repression experimentally (El Ouaamari et al. 2009). This same study overexpressed miR-375 and observed a decrease in Pdk1 protein expression levels without noticing a decrease in the gene’s mRNA, and additionally inclusion of the PDK1 3’-UTR in a luciferase reporter gene was sufficient to knock down luciferase expression relative to a mutated control 3’-UTR (El Ouaamari et al. 2009). The Pdk1 protein is involved in maintaining the viability of pancreatic \(\beta\)-cells through downstream propagation of various signaling pathways, including insulin response (Hashimoto et al. 2006, Taniguchi et al. 2006). Pdk1 knockout in pancreatic \(\beta\)-cells results in many diabetes-like conditions including decreased \(\beta\)-cell size and density, which corresponded to hyperglycemia (Hashimoto et al. 2006). Therefore incorrect regulation of miR-375 during T2DM may play an important role in the progression of the disease.

More detailed understanding of how miR-375 is regulated may lead to insights in understanding its misregulation and current therapies, or provide ideas for future therapies. Some studies have gone into understanding the factors which drive its expression, but not many have focused on understanding how that expression is dampened (Avnit-Sagi et al. 2009, Avnit-Sagi et al. 2012, Keller et al. 2007). Previous work has indicated that stimulation of the cAMP/PKA pathway is sufficient to repress the expression of miR-375 (Keller et al. 2012). This mechanism fits in with the current understanding of insulin secretion induced by GLP-1 signaling (Drucker 2006). How this pathway mediates the reduced production of miR-375 is not well understood.

Epigenetics and Histone Acetylation

Epigenetic regulation is a form of transcriptional control enacted by changes in the superstructure of chromatin rather than strictly through the sequence of the genetic code. dsDNA is wound around an octameric protein complex of histone subunits, generally composed of two
of each histone H2A, H2B, H3, and H4. The histone complex with associated DNA is termed a nucleosome and encompasses around 147 base pairs of DNA wrapped approximately 1.67 times around the histone octamer (Luger et al. 1997). How the DNA is wrapped around the histone depends on two different different epigenetic mechanisms. One, cytosines in CpG positions can be methylated into 5-methylcytosine (Jones et al. 2001). Although 5-methylcytosine is functionally identical as a nucleotide to normal cytosine, concentrated methyl groups presented in some CpG islands can recruit transcription factors to the sequence (Jones et al. 2001). Second, the histone subunits can be post-translationally modified within N-terminal tails that extend from the core (Peterson et al. 2004). The modifications that can be added to residues in these N-termini are acetylation, methylation, ubiquitination, phosphorylation, sumoylation, ribosylation, and citrullination (Peterson et al. 2004). These modifications can change the structure of the histone subunits, giving them different interactions with the associated DNA.

The focus of this study is on acetylation at histone subunit N-terminal lysine residues. These reactions are catalyzed by two classifications of enzymes. Acetylation by the transfer of an acetyl group from acetyl-coenzyme A to a lysine of a histone is carried out by histone acetyltransferase (HAT) enzymes. Whereas the removal of acetyl groups from histones to rejoin with coenzyme A (deacetylation) is carried out by histone deacetylase (HDAC) enzymes.

Changes in acetylation status of histones affect the transcriptional activity of genes associated with the nucleosomes of the altered histones. Acetylation of histone lysines removes the positive charge conferred by the unmodified lysine residue, thereby repelling the negatively charged DNA in proximity to the histone tail (Brower-Toland et al. 2005). Likewise, deacetylation restores the positive charge and increases the histone tail’s affinity for DNA, more tightly coiling the DNA in the nucleosome. Changes in the histone core’s ability to bind DNA affect the ability of transcription factors and machinery to access regulatory regions of genes (Tse et al. 1998, Clapier et al. 2002, Hassan et al 2001). This effect is exemplified by the use of HDAC inhibitory molecules, such as trichostatin A (TSA), which increases the acetylation of particular genes which correlates with their reduced expression (Hassig et al. 1998).

One particular class of HDAC enzymes, Class I HDACs, have properties that make them good candidates for the repression of miR-375 by the stimulation of the cAMP/PKA pathway. This classification includes four proteins denoted as HDAC1, 2, 3, and 8 which primarily consist of one enzymatically active domain (Mihaylova et al. 2013). However, HDAC1 and 2 have
domains on the more N-terminal end of the polypeptide from the deacetylase domain which contain sites for posttranslational modification (Cai et al. 2001). In these N-termini are two serines (Ser421 and Ser423) which are modified by phosphorylation to activate them for inclusion into histone deacetylation complexes such as NuRD, Sin3, and CoREST (Pflum et al. 2001). It is in these complexes that HDAC1 and 2 are transported to the nucleus and perform their function to deacetylated histones (Pflum et al. 2001). There are two kinases which phosphorylate these serines, casein kinase II and PKA (Cai et al. 2001). Additionally, various Class I HDACs have been found to have a role in metabolic regulation, such as the role of HDAC1 and 2 in adipogenesis (Haberland et al. 2009). Therefore it is possible that activation of the cAMP pathway by GLP-1 sensing may lead to the activation of HDAC 1 and 2, thereby deacetylating miR-375-associated histones and reducing transcription of the miRNA under normal circumstances.
CHAPTER II

MATERIALS AND METHODS

Bioinformatic Analysis and Primer Locations

Analysis of the gene and promoter region for pri-miR-375 was done using the University of California Santa Cruz (UCSC) Genome Browser using the 2004 *Rattus norvegicus* assembly. The genomic location of the pri-miRNA-375 gene is on chromosome 9: 74,233,499-74,233,573. Primers to detect the production of pre-miR-375 were designed within this region. The remaining primers for the putative promoter region were designed within 2,533 bp upstream of the gene, between chromosome 9: 74,233,573-74,236,106.

Cell Culture

All experiments were conducted using the INS-1 832/13 rat insulinoma cell line. Cells were cultured using RPMI 1640 (Corning) supplemented with 25mM Hepes, 2mM L-glutamine, 10% fetal bovine serum (FBS) (Hyclone), 1mM sodium pyruvate (Hyclone), 1x Penicillin/Streptomycin/glutamate (Hyclone), 50µM β-mercaptoethanol (Fischer) and incubated in 5% CO\textsubscript{2} at 37°C. The day before treatment with forskolin (FSK) (MP Biomedicals, LLC) and/or 3-isobutyl-1-methylxanthine (IBMX) (Acros Organics), cells were starved overnight with 1.5% FBS RPMI 1640 supplemented as normal except for serum. Media was replaced every two days, and cells were passaged in ratios between 1:2 to 1:5 once cells reached 80%-90% confluence.

Cloning

Amplification of different promoter regions upstream of the miR-375 gene were carried out by polymerase chain reaction using a BioRad iCycler 96 Well Reaction Module. The
program used was initial denaturation for 95°C for 1 minute. Then 95°C for 15 seconds, variable
temperature annealing for 30 seconds, and 68°C for 45 seconds repeated for thirty cycles, where
the annealing temperature differed depending on the combination of primers used. These
fragments were cloned into the pGL3 luciferase reporter gene vector (Promega) using competent
*E. coli* cells. Cloning was screened using polymerase chain reaction. Plasmids were isolated
using PerfectPrep Endofree Maxi Kit (5-PRIME). Sequencing of the inserted DNA fragment was
used to ensure fidelity of the cloned region of putative promoter.

Luciferase Reporter Assay

INS-1 832/13 cells were cultured in a 96-well plate, seeded at 2,500 cells per well. After
one day, 50ng of cloned miR-375 upstream promoter constructs in pGL3 vector (Promega) or
pGL3-control vector (Promega) were transfected into cells along with 50ng of TK-*Renilla*
luciferase plasmid (Promega) as a standard. Transfections were done using TransIT-LT1
transfection reagent (Mirus). Transfected cells were grown for two days to allow for expression
of transfected plasmids and assayed using Dual-Glo Luciferase Assay System (Promega).
Luminescence from firefly luciferase was standardized against luminescence from *Renilla*
luciferase. Luminescence readings were performed using a BioTek Synergy HT plate reader.

RNA Extraction

INS-1 832/13 cells were seeded in 3cm culture plates at 500,000 cells per plate. The cells
were cultured for two days before replacing media, then starved on the third day overnight (some
with TSA (Cell Signaling Technology) treatment) before FSK and/or IBMX treatment. To
harvest RNA, media was replaced with 500uL RiboZol RNA Extraction Reagent (Amresco) and
incubated with shaking for 10 minutes. After adding 100uL of 100% chloroform, the organic and
aqueous phases were separated by 15 minute centrifugation at 10,000 rpm at 4°C. 250uL of
100% isopropyl alcohol was added to the aqueous layer to separate nucleic acids by 10 minute
centrifugation at 10,000 rpm at 4°C. RNA was purified from DNA by incubation with DNase I
(Promega) treatment at 37°C for 30 minutes, and DNase I inactivation at 70°C for 15 minutes.
cDNA Synthesis

After extraction, 500ug of RNA was reverse transcribed into cDNA. 250ng of random primers (Promega) were annealed at 70°C for 5 minutes. Synthesis of cDNA was performed using 200 units of Moloney Murine Leukemia Virus reverse transcriptase (Promega) and 10µM deoxynucleotide triphosphates incubated at 37°C for 60 minutes, then heat inactivated at 70°C for 15 minutes. Samples were diluted 1:5 in diethylpyrocarbonate (DEPC)-treated water before analysis.

Chromatin Immunoprecipitation

DNA and associated proteins were crosslinked with 1% paraformaldehyde for 15 minutes, then quenched with 125mM glycine for 5 minutes. Collected cells were ruptured using 600uL of lysis buffer (0.1% sodium dodecyl sulfate, 0.5% triton x-100, 20mM Tris-HCl pH 8.1, 150mM NaCl in nanopure water) per 10cm plate of cells. Chromatin was fragmented by six separate 10 second sonications. The chromatin was separated by 10 minute centrifugation at 14,000 rpm in 4°C. Before immunoprecipitation, chromatin was pre-blocked with 40µL of protein G-conjugated agarose beads (Millipore).

100ug of protein, as measured by Bradford assay, was used for each immunoprecipitation along with 1ug of antibody (anti-RNA Polymerase II-phosphorylated Ser 5 antibody from AbCam, anti-acetylayed histone H3 lysine 9/14 antibody from Santa Cruz Biotech). For use as a standard, 20ug of unprecipitated chromatin was used. Immunoprecipitation occurred during rotation at 4°C overnight, and was then allowed to bind to protein G-conjugated agarose beads during another two hours of rotation at 4°C. Pelleted beads were washed twice with lysis buffer, once with LiCl buffer (10mM Tris-HCl pH 8.0, 1mM EDTA, 0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate in nanopure water), and twice with TE buffer (10mM Tris-HCl pH 8.0, 1mM EDTA in nanopure water). Eluted twice using 200uL of 1% sodium dodecyl sulfate and 0.1M NaHCO3 in DEPC-treated water by 15 minute rotation at room temperature. Proteins were
denatured with 0.33M NaCl and 17 hour incubation at 65°C. DNA was then isolated by phenol-chloroform extraction and resuspended in 500uL of 10mM Tris-HCl pH 8.0.

**Micrococcal Nuclease Protection Assay**

Chromatin was extracted using the same methods as the chromatin immunoprecipitation. Fragmentation of chromatin was performed by three separate 10 second sonications. The chromatin containing solution was dialyzed (Cel Seamless Celulose 12,000 to 16,000 MWCO) overnight into micrococcal nuclease (MNase) reaction buffer (50mM Tris-HCl pH 8.0, 5mM CaCl$_2$ in nanopure water). Digestion of chromatin used five units of MNase (in 10mM Tris-HCl pH 7.5, 50mM NaCl, 1mM EDTA, 50% Glycerol in DEPC-treated water) (New England Biolabs) incubated at 37°C for 30 minutes. MNase was inactivated with 0.33M NaCl and protein was denatured and DNA isolated using the same methods as the chromatin immunoprecipitation. DNA was resuspended in 80uL of 10mM Tris-HCl pH 8.0.

**Quantitative Polymerase Chain Reaction**

Analysis of cDNA by quantitative polymerase chain reaction was performed using an Eppendorf RealPlex$^2$ Mastercycler. All quantitative polymerase chain reaction analysis used 5-PRIME SYBR Green reagents, except for TSA-treatment cDNA experiments which used Thermo-Fisher HiGreen (heat activated polymerase containing SYBR Green based) reagents. The initial 95°C denaturation step lasted two minutes using 5-PRIME reagents, and 10 minutes using Thermo-Fisher reagents - to heat activate the polymerase. The program then repeated 95°C for 15 seconds and 68°C for 45 seconds for 40 cycles. Genomic DNA and four 1:5 serial dilutions of it were analyzed for each primer for each experiment to generate standard curves.
CHAPTER III

RESULTS

Figure 1 – Model of cAMP-stimulated HDAC-mediated repression of miR-375. Figure adapted from the Muscular Distrophy Association. This model demonstrates the proposed mechanism of inhibition following incretin stimulation of cAMP signaling. Incretin hormones (represented by the more ubiquitous GLP-1) stimulate the production of cAMP by binding to their G-protein coupled receptor. A series of conformational changes in associated proteins activates adenyl cyclase to produce cAMP from ATP. Then cAMP binds to PKA, removing its repressor domains, allowing it to activate HDAC1 and/or 2 for complex formation through phosphorylation of C-terminal serines. Then HDAC1 and/or 2 are able to deacetylate promoter histones of miR-375, repressing its transcriptional activity.

Characterization of the Putative microRNA-375 Promoter

Previous studies elucidating regions of the promoter associated with repression have been conducted in mice models as well as mouse or hamster-derived cell lines (Avnit-Sagi et al. 2009). Given that my model system is using INS-1 832/13 rat insulinoma cell lines, first
developing a comparison and understanding of the rat microRNA-375 gene and upstream promoter is key. This undertaking seeks to identify corresponding conserved sequences defined in previous studies, as well as expand the number of conserved sites analyzed.

Within a 2,533 bp stretch of DNA upstream of the gene for miR-375 there are a variety of sequences with high mammalian conservation. The four regions of conservation clustered closest to the gene constitute the four regions defined in Avnit-Sagi et al. 2009. For consistency with the previous literature, these were named numerically 1-4, increasing as you move toward the gene (Fig. 1). Primers used to produce combinations of these regions are annotated against a map of conservation in Figure 1. These primers were used to clone promoter constructs with different composition for use in luciferase reporter assays.

Another set of primers were used during qPCR analysis of the upstream promoter. To keep them distinct from the primers used for cloning, these qPCR primers are named alphabetically A-F, beginning closest to the TATA box and moving away from the gene (Fig. 3). Primers were designed to represent regions of high conservation, a property indicative of an important functional sequence.

Only one gene is as proximal to these sites of conservation as miR-375: coiled-coil domain containing 108 (CCDC108). This gene has been discovered in the human genome and no evidence shows its expression in rats or β-cells.

Figure 2 – Schematic of the miR-375 cloning sites. Schematic outlining the highly conserved regions used in the Avnit-Sagi et al. 2009 paper, labeled 1-4. Each arrow displays a primer used in cloning parts of the promoter for a luciferase reporter assay. Forward arrows are forward primers and reverse arrows are reverse primers. In order from left to right, the primers represent the ‘increased upstream region,’ ‘region 1,’ and ‘region 2’ (Table 1). Created using UCSC Genome Browser.

Figure 3 - Schematic of the miR-375 upstream promoter in relation to tested regions. Primers for the potential promoter region of miR-375 are labeled A-F in descending alphabetical
order starting from the TATA box and extending away from the gene. Primer products for regions C and B share some sequence identity. Sequence conservation is shown as a potential indicator of function. Created using UCSC Genome Browser.

**Importance of the Upstream Promoter Region of miR-375**

**Table 1 - Cloning primers.** These primers were designed to amplify specific individual or combinations of conserved regions of the microRNA-375 5’-UTR. Forward primers contain extensions to add an NheI recognition site and reverse primer contains an extension to add a HindIII recognition site.

<table>
<thead>
<tr>
<th>PRIMER REGION</th>
<th>DIRECTION</th>
<th>PRIMER SEQUENCE</th>
<th>ADDED 5’-RESTRICTION SITE</th>
</tr>
</thead>
<tbody>
<tr>
<td>microRNA-375 Promoter Increased Upstream Region</td>
<td>Forward</td>
<td>5’-atgctagcgttcagagggcatagct-3’</td>
<td>NheI</td>
</tr>
<tr>
<td>microRNA-375 Promoter Conserved Region 1</td>
<td>Forward</td>
<td>5’-atgctagccagaggtggaaa-3’</td>
<td>NheI</td>
</tr>
<tr>
<td>microRNA-375 Promoter Conserved Region 2</td>
<td>Reverse</td>
<td>5’-ataagctttatatagccaaatcgtct-3’</td>
<td>HindIII</td>
</tr>
</tbody>
</table>

In order to investigate the involvement of more upstream promoter regions than in previous literature (Avnit-Sagi et al. 2009), a luciferase reporter assay was used. Using the primers listed in Table 1, regions from upstream of the gene were isolated for use in a luciferase reporter assay. Isolated regions cloned into the pGL3 firefly luciferase expression vector were the entire upstream promoter sequence and regions 1/2 (Fig. 3). The results were standardized to the expression of co-transfected renilla luciferase.

Results from this experiment show that sequences farther upstream are necessary for full transcriptional activity. The construct with just regions 1/2 had significantly different transcriptionally active than the control vector (p=0.01), however the increased upstream
promoter region construct was more transcriptionally active with about 26 times more standardized luminescence (p=0.01) (Fig. 4). This result indicates that there is more to understand about the regulation of miR-375 in relation to the upstream promoter. It appears that there are regions necessary for transcription there which might be involved in repression during cAMP signaling.

**Figure 4 – Transcriptional activity conferred by miR-375 upstream promoter regions.** INS-1 832/13 cells were transfected with different components of the miR-375 upstream promoter in a luciferase expressing plasmid along with a Renilla expressing plasmid. After 48 hours, cells were lysed and luminescence from Firefly/Renilla luciferase was recorded. The transcriptional activity of the 1050 bp upstream region of miR-375 (“increased upstream”) compared to the activity in just regions 1 and 2 (Reg1/2) (shown in Avnit-Sagi et al. 2009 to be necessary for transcription). Expression of firefly luciferase expressed by the attached promoter regions was standardized against expression of Renilla luciferase. pGL3-basic was used as a negative control. ** represents p=0.01, n=3 wells in one experiment.

Repression by cAMP-Stimulation

To understand how to optimally treat cells, I conducted preliminary experiments to understand how pre-miR-375 levels decrease in response to artificial elevation of cAMP concentration. This aim was achieved through two different approaches, a time course and dose response. Since the INS-1 832/13 cells used in these experiments are insensitive to incretin hormones, intracellular concentration of cAMP is elevated using an adenylyl cyclase activator, forskolin (FSK). Where a strengthened and sustained response is desired, cells were treated with a phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX), to prevent turnover of cAMP. For the time course, RNA was extracted from cells treated for 5, 10, and 15 minutes with
1µM FSK/100µM IBMX and compared to a mock 0 minute sample treated for 15 minutes with the vehicle control, dimethyl sulfoxide (DMSO) (Sigma-Aldrich). For the dose response, RNA was extracted after 15 minutes of treatment with 0.125µM, 0.500µM, and 2.000µM FSK again compared to a 15 minute DMSO treatment. Extracted RNA was then assayed using quantitative reverse transcriptase PCR (qRT-PCR) for abundance of pre-miR-375, as a measure of the production of mature miR-375. Abundance of pre-miR-132, upregulated due to cAMP signaling, was assayed as an indicator of adenylyl cyclase activation by FSK. All results were standardized to abundance of U6 snRNA abundance.

During the time course, the strong combined dosage of FSK and IBMX was sufficient to deplete the production of pre-miR-375 below 50% of the untreated samples after only 5 minutes (Fig. 4). Depleted abundance of pre-miR-375 was maintained over the course of the treatments indicating that 5 minutes of treatment is sufficient to observe the extent of repressive effects from 1µM FSK/100µM IBMX treatment. Abundance of pre-miR-132, a gene upregulated by cAMP/PKA signaling, was assessed as an indication of successful induction of the proper signaling pathway. The abundance of pre-miR-132 was elevated to around 10-fold higher over the course of treatment (Fig. 4).

Treating cells with lower doses of FSK without addition of IBMX was also able to induce cAMP-stimulated repression of miR-375. Each increasing dose repressed the production of miR-375 progressively more with the first statistically significant decrease occurring at 0.500µM FSK (Fig. 5). Whereas 1µM FSK/100µM IBMX lowered pre-miR-375 abundance below 50% within 5 minutes of treatment, only the 2.000µM FSK treatment induced a similarly strong response, reducing pre-miR-375 to 44.3% of untreated abundance (Fig. 5).
Figure 5 – Repression of pre-miR-375 in response to cAMP. A) Time course of pre-miR-375 depletion by 1μM forskolin (FSK)/100μM 3-isobutyl-1-methylxanthine (IBMX) treatment via qRT-PCR of INS-1 rat insulinoma cell extracts. An increase in cAMP-activated miRNA-132 confirms stimulation of cAMP production. For all time points n=3, except miR-375 at 15 min where n=1. B) Dose response of pre-miR-375 depletion by lower concentrations of FSK treatment. The increase in pre-miR-132 confirms cAMP stimulation. For pre-miR-375 n=6, 4, 4, and 6 respectively. For pre-miR-132 n=4, 2, 2, and 4 respectively.
Effect of HDAC Inhibition on miR-375 Repression

As an initial test of HDAC involvement in cAMP-stimulated repression of miR-375 I performed an assay measuring pre-miR-375 expression during cAMP signaling with and without HDAC activity. Utilizing the information gained from the previous experiments, the same assay was performed using 1µM FSK/100µM IBMX for 15 minutes to ensure significant repression of miR-375 during the course of the experiment. One pair of DMSO and FSK/IBMX-treated cells were pre-treated for 17 hours with 400nM trichostatin A (TSA), an inhibitor of Class I and II HDACs. The other pair was pre-treated with ethanol (EtOH) as a vehicle control.

Only one experiment was able to be conducted during the time-frame, so results are difficult to draw information from. However, at this point in data collection it appears that the HDAC inhibition from TSA may be having broader effects than anticipated. Repression of pre-miR-375 by cAMP induction is observed between both the EtOH- and TSA-treated samples (Fig. 6). Although the amount of repression between the TSA/DMSO and TSA/FSK+IBMX samples is less than between their EtOH-treated counterparts, the amount of pre-miR-375 transcribed is less with TSA than with EtOH (Fig. 6). Likewise, the TSA/FSK+IBMX sample transcribed less pre-miR-375 than the EtOH/FSK+IBMX sample (Fig. 6). This effect suggests that TSA is causing a synergistic effect which is adding another variable to the experiment. Inhibition of all class I and II HDACs increases the acetylation of more than just miR-375, and one of those genes could potentially be an inhibitor of miR-375 – and since the chromatin for miR-375 is open due to acetylation, that repressor would have access. While this is happening it is difficult to obtain a clear picture of the necessity of HDAC in the cAMP-induced repression of miR-375. Expression of miR-132 increases over the 15 minute treatment with TSA, but not with the ethanol control samples. With only one experiment conducted, it is hard to know the cause of this and it should be repeated.
Figure 6 – HDAC-Inhibition on expression of pre-miR-375 during cAMP-signaling. INS-1 832/13 cells were starved (1.5% serum) and treated with 400nM trichostatin A (TSA) for 17 hours. Then cAMP production was induced with 1μM forskolin (FSK)/100μM 3-isobutyl-1-methylxanthine (IBMX) treatment for 15 minutes. RNA was extracted and cDNA was created using reverse transcriptase (RT)-PCR. Samples of cDNA were assayed for amount of miR-375 using primers for pre-miR-375, and primers for cAMP-activated pre-miR-132 were used to confirm cAMP induction. All data is standardized to the expression of the housekeeping gene 18s rRNA. TSA treatment was compared against its vehicle control, ethanol (EtOH), and FSK/IBMX was compared against its vehicle control dimethyl sulfoxide (DMSO). For all samples, n=1.
Decreased Transcription Initiation by cAMP-Stimulation

Table 2 - microRNA-375 gene and promoter primers. These primers were used to amplify particular regions of the upstream promoter during analysis of transcriptional activity and promoter histone acetylation states. Primers are labeled corresponding to the region displayed in figure 2 which they amplify.

<table>
<thead>
<tr>
<th>TARGET GENE/REGION</th>
<th>DIRECTION</th>
<th>PRIMER SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>microRNA-375 Gene</td>
<td>Forward</td>
<td>5′-cctcgacaaaccggacct-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-gcctcacgagccgaac-3</td>
</tr>
<tr>
<td>microRNA-375 Promoter A</td>
<td>Forward</td>
<td>5′-gccaattcagtctctgccccta-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-cccgagaggtgtgtgtgtg-3′</td>
</tr>
<tr>
<td>microRNA-375 Promoter B</td>
<td>Forward</td>
<td>5′-tggtgttttgccagaggcgtcc-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-cagagggcttgccagccggag-3′</td>
</tr>
<tr>
<td>microRNA-375 Promoter C</td>
<td>Forward</td>
<td>5′-agcgctcttggaacaacaccag-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-ctgtctctgtctctctctgagc-3′</td>
</tr>
<tr>
<td>microRNA-375 Promoter D</td>
<td>Forward</td>
<td>5′-ccaccacccacagctccgttt-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-agcacctccagggccacic-3′</td>
</tr>
<tr>
<td>microRNA-375 Promoter E</td>
<td>Forward</td>
<td>5′-gctgaetgtgtgaggcaatga-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-actggtagagccagccgtggag-3′</td>
</tr>
<tr>
<td>microRNA-375 Promoter F</td>
<td>Forward</td>
<td>5′-tcctctctgcctccagcttt-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5′-cttcaccatcccttgccctgct-3′</td>
</tr>
</tbody>
</table>
Table 3 - Control primers. These primers were used to amplify sequences used as controls. U6 snRNA and 18s rRNA are housekeeping genes used to standardize transcriptional activity. Pre-miR-132 is a miR that is upregulated due to cAMP signaling used as a positive indicator of forskolin effectivity. MyoD1 is a muscle differentiation factor not expressed in INS-1 832/13 cells, therefore used as a negative control for active chromatin. cFos is a transcription factor which is highly expressed in INS-1 832/13 cells and is used a positive control for active chromatin.

<table>
<thead>
<tr>
<th>TARGET GENE/REGION</th>
<th>DIRECTION</th>
<th>PRIMER SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>U6 snRNA</td>
<td>Forward</td>
<td>5’-ttcggcagcataactaaaattgga-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-aggggccatgtatcttctctgt-3’</td>
</tr>
<tr>
<td>18s rRNA</td>
<td>Forward</td>
<td>5’-cgacagggacaggtgacaga-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-accaccacggagaaaga-3’</td>
</tr>
<tr>
<td>pre-microRNA-132</td>
<td>Forward</td>
<td>5’-tccggttcccacgtaacaatcg-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-ccggtctcagggcaac-3’</td>
</tr>
<tr>
<td>MyoD1 Promoter</td>
<td>Forward</td>
<td>5’-gacgcgactgtttttccaccac-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-ttccctctgtctgtctgtc-3’</td>
</tr>
<tr>
<td>cFos Promoter</td>
<td>Forward</td>
<td>5’-cttccagttcctgtttccgctca-3’</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’-cggtctctatccagttctcagttgc-3’</td>
</tr>
</tbody>
</table>

Observing a decrease in the production of pre-miR-375 could represent either a decrease in the gene’s transcription or an increase in the degradation of the gene. To clarify which type of process is causing this decreased production, I assayed for the presence of transcriptional machinery positioned upstream of the gene. Cells were treated with 1µM FSK/100µM IBMX for 5 minutes to induce elevated levels of cAMP enough to decrease pre-miR-375 to below half of baseline levels. After treatment, cells were chromatin immunoprecipitated using antibodies against RNA polymerase II with phosphorylated serine 5 (RNAPol2-Ser5p), a modification indicative of transcriptional initiation.

With the exception of region C and A, there was a modest, but statistically significant (p=0.01) decrease in RNAPol2-Ser5p associated with the upstream promoter DNA during repression (Fig. 7). Considering that region A is close in proximity to the TATA box, this region showed considerably higher amounts of associated RNAPol2-Ser5p and therefore may saturate
the amount of antibody used for the assay. If the assay were saturated then a decreased amount of RNApol2-Ser5p may not drop below the abundance necessary to saturate the amount of antibody - explaining the decreased presence of RNApol2-Ser5p in adjacent regions of DNA, but not at the site of binding. No change in transcriptional activity was observed for the negative control gene, myogenic differentiation-1 (MyoD1), which is a muscle-specific gene not expressed in pancreatic β-cells and whose transcription is not affected by cAMP signaling pathways (Fig. 7).

Given the decrease in promoter-associated RNApol2-Ser5p when cAMP levels are artificially elevated, it suggests transcriptional repression is responsible for repression of miR-375. This may not account for the entirety of cAMP-stimulated repression of miR-375, there could be repression by degradation as well. However, the data suggests that at least a component of cAMP-stimulated repression of miR-375 is due to decreased transcriptional activity.

**Figure 7 – Transcriptional initiation at the miR-375 promoter after cAMP induction.** INS-1 832/13 cells were starved overnight and then treated with 1μM forskolin (FSK)/100μM IBMX for 15 minutes. Chromatin was extracted and immunoprecipitated with anti-RNA polymerase 2-phosphorylated serine 5 (RNApol2-Ser5p) antibody. Samples were also chromatin immunoprecipitated with rabbit IgG to correct for non-specific binding. After denaturation of associated proteins, DNA was isolated and assayed using quantitative PCR using primers which amplify different regions of the miR-375 upstream promoter. This is a measure of how much initiation-associated form of RNApol2 is active at the promoter after cAMP induction. MyoD1 is a muscle differentiation factor not transcribed in INS-1 832/13 cells. **represents p=0.01, n=4.
Promoter Histone Deacetylation by cAMP-Stimulation

Samples assayed for transcriptional activity at the miR-375 promoter were concurrently assayed for differences in histone acetylation at the same locations. To begin this investigation, antibodies against histone H3 acetylated at lysine 9 and 14 (H3K9/14) were used to precipitate DNA associated with histones modified in this manner. Given that during the same treatment and time-course there is an observable change in transcription, a concomitant decrease in acetylation should be seen if histone deacetylase activity is involved in conferring that repression.

Two regions of the miR-375 upstream promoter show decreased acetylation during the cAMP-stimulated repression of miR-375. Regions A, B, C, and D have statistically identical abundance of the H3K9/14 modification, whereas there is a significant decrease in this histone modification (p=0.01 and p=0.05 respectively) in regions E and F (Fig. 8). These regions are the two sites most distal to the gene, region E is 1,524 and F is 2,533 bp upstream (Fig. 2). Observing this difference suggests that deacetylation is playing a role in decreasing the transcription of miR-375 by decreasing chromatin accessibility at a distance from where RNA Polymerase II binds.

Although the deacetylation at regions E and F are statistically significant, there are a couple factors to take into account regarding their interpretation. First, the magnitude of the deacetylation is moderate and therefore may only confer a moderate change in the chromatin. Second, the H3K9/14 modification only represents a portion of the possible acetylation sites that could be affected by HDAC activity.
**Figure 8 - Acetylation of histone H3 lysine 9 and 14 (Ac-H3K9/14) reduction at the distal upstream miR-375 promoter regions.** INS-1 832/13 cells were starved overnight and then treated with 1μM forskolin (FSK)/100μM IBMX for 15 minutes. Chromatin was extracted and immunoprecipitated with anti-acetylated histone H3 lysine 9 and 14 (Ac-H3K9/14) antibody. Samples were also chromatin immunoprecipitated with rabbit IgG to correct for non-specific binding. After denaturation of associated proteins, DNA was isolated and assayed using quantitative PCR using primers which amplify different regions of the miR-375 upstream promoter. This is a measure of how much of the promoter-associated histone 3 is acetylated at lysines 9 and 14 after cAMP induction. MyoD1 is not transcriptionally active (see Fig. 5) and it’s promoter should have a largely deacetylated histone composition.

* represents p=0.05, **represents p=0.01, n=4.

---

**Overall Chromatin Structure**

After observing a decrease in transcriptional initiation at the miR-375 upstream promoter that coincided with deacetylation of H3K9/14, I conducted an assay to evaluate changes in the binding affinity for histones. A micrococcal nuclease (MNase) protection assay was used to determine histone binding by degrading chromatin DNA unbound to histones with nucleases, leaving histone-bound DNA protected. INS-1 832/13 cells were treated under the same conditions as previous assays, with 1μM FSK/100μM IBMX for 15 minutes against a control treated with DMSO for 15 minutes. Chromatin was then extracted from the treated cells and then
digested with MNase. DNA protected from MNase by being tightly bound to histones should be less easily degraded than DNA less tightly bound to histones. Using qPCR I analyzed the protection of DNA in different regions of the miR-375 upstream promoter denoted in Figure 3. These regions were evaluated against a negative control (MyoD1) and a positive control (cFos) for degradation of DNA. MyoD1, as a muscle differentiation factor, has tight chromatin structure preventing access by transcription factors and is not degraded by MNase. cFos, as a transcription factor abundant in pancreatic β-cells, has very open chromatin structure and is highly degraded.

The positive degradation control, cFos, demonstrated complete digestion of DNA as expected from a highly expressed protein with open chromatin. FSK/IBMX or DMSO treatment did not change the amount of digestion between samples, both treatments were over 98% digested (Fig. 9). The negative degradation control, MyoD1, was not degraded - although there was a strange phenomenon where the assay registered more DNA after nuclease treatment (Fig. 9). This occurrence may be due to concentration-based effects. If the MyoD1 promoter DNA is not digested, but a considerable amount of other DNA is, then a higher proportion of the DNA is the correct template for the primers being used.

Neither of the two regions which were deacetylated during the previous assays displayed any significant changes in chromatin structure. Regardless of treatment with FSK/IBMX there was no difference in the protection from MNase at region E or F, and both regions were degraded below 50% of the amount in the undigested sample (Fig. 9). Additionally, the two highly conserved regions directly preceding the site of RNA Polymerase II binding are equally unchanged. It does not appear that the deacetylation of miR-375 upstream promoter histones observed at H3K9/14 have an effect on chromatin structure. However, it may be possible that the changes in DNA-binding affinity are too subtle to be detected during the MNase protection assay.
Figure 9 – Chromatin structure analyzed by susceptibility to micrococcal nuclease (MNase) digestion. INS-1 832/13 cells were starved overnight and then treated with 1μM forskolin (FSK)/100 μM IBMX, or its vehicle control dimethyl sulfoxide (DMSO), for 15 minutes to induce cAMP production. At this treatment and time point miR-375 production has already been repressed, so all chromatin changes should have occurred. Chromatin was extracted digested with either 0 gel units (0 gu) or 5 gel units (5 gu) of MNase. The MNase was deactivated and denatured. Then remaining DNA was assayed using quantitative PCR using primers for upstream promoter regions of miR-375 to compare pre- and post-digestion abundance of promoter regions. All samples are set relative to the amount of DNA present in their corresponding undigested sample. A) MyoD1 promoter primers were used as a negative control for digestion. It is not expressed in this cell type, so it should have closed chromatin and not be susceptible to nuclease
digestion. B) cFos promoter primers were used as a positive control for digestion. It is highly expressed in this cell type, so it should have very open chromatin and be very susceptible to nuclease digestion. C) The two regions of the miR-375 upstream promoter which were deacetylated during cAMP induction were assayed. Since these regions’ histones were less acetylated after cAMP induction, they should be more protected after FSK/IBMX treatment. For all samples, n=3.
CHAPTER IV

DISCUSSION

The intent of this study was to investigate whether HDAC was involved in repressing miR-375 in response to incretin hormone binding. I have provided evidence that during stimulation of the cAMP/PKA pathway there appears to be deacetylation of distally associated histones in the miR-375 upstream promoter. These changes in acetylation coincide with decreasing presence of RNA Polymerase II as well as repressed miR-375 production. A weakness of this study is that it was not able to cover a wider breadth of acetylated lysine changes that could result from deacetylase activity. Acetylation states of H3K9/14 was chosen for their general role in transcription and in other models of miR-375 regulation. In general, acetylation of H3K14 is associated with transcriptional activation, therefore it would likely be deacetylated during transcriptional repression (Strahl et al. 2000). Additionally, a study on the downregulation of miR-375 in trastuzumab-resistant breast cancer induced miR-375 expression using TSA and noted an increase in H3K9/14 (Ye et al. 2014). However, more lysines in the N-terminal tails of histone H3, as well as the remaining histone subunits, should be assayed for a more thorough understanding of the changes that HDAC imparts to miR-375 promoter-histone interactions.

An unexpected result was the lack of changes in the overall promoter-histone interactions during cAMP-stimulated repression of miR-375. Since the method of transcriptional modulation from (de)acetylation stems from changing the histones’ affinity for DNA, the observed histone deacetylation should confer increased DNA binding affinity. However, no significant increase in nuclease protection was seen during the MNase protection assay. This lack of change could be the result of very slight changes to the promoter-histone associations which the assay is not sensitive enough to detect. Or, it could be true result caused by compensatory changes in other epigenetic modifications in the miR-375 promoter histones.

Testing for the necessity of HDAC activity in the repression of miR-375 during cAMP signaling could be altered to possibly produce more useful data. Since TSA is a broad spectrum inhibitor of all Class I and II HDACs there is assuredly inhibition of deacetylation in a number of
other genes. Use of TSA seems to be working to open chromatin as expected as seen in the data for pre-miR-132 in figure 6. Where FSK/IBMX treatment already induces pre-miR-132 production, treatment with TSA induces even greater transcriptional activity (Fig. 6). These genes’ chromatin would then open up, allowing for increased production of what could be an inhibitor of miR-375 transcription. An attempt to alleviate this potential conflict is to treat cells simultaneously with cycloheximide, an inhibitor of protein synthesis. If there is a transcription factor being artificially upregulated by TSA, this will prevent that effect. Since that assay is measuring the production of an RNA, cycloheximide will not affect the creation of pre-miR-375. This should help eliminate variables and present a better picture of the necessity for HDAC in this model.

Although deacetylation was selected due to its relation to the cAMP/PKA pathway through activation of HDAC I and II, there is a caveat to be recognized and considered for future study. Pursuing changes in acetylation states and deriving conclusions from that data is much like inspecting a couple candidates from a list of crime suspects and expecting to determine a meaningful verdict. Acetylation is just one epigenetic modification, albeit a well understood and explored one. There are other epigenetic modifications to consider such as methylation, ubiquitination, and sumoylation. Only after understanding how all of these modifications are altered in the miR-375 promoter chromatin can the entirety of its epigenetic regulation be elucidated.
Figure 10 – Overall model of HDAC 1/2 in relation to miR-375 regulation. This is what is currently known after this study on how miR-375 is regulated by cAMP stimulation. Incretin hormones (GLP-1 or GIP) bind to G-protein coupled receptors which activate the production of cAMP through adenylyl cyclase. Elevated cyclic adenosine monophosphate (cAMP) concentration activates more protein kinase A (PKA). PKA phosphorylates HDAC1 and 2, allowing them to form into deacetylation complexes with other proteins. These complexes then deacetylate histones, causing the DNA that they’re associated with to be more transcriptionally active. In the miR-375 upstream promoter this effect is seen in the more distal conserved regions (Fig. 8), and it corresponds to the repression of miR-375 by transcriptional inhibition (Fig. 5, Fig. 7). However, this isa moderate change and doesn’t seem to affect the chromatin structure significantly (Fig. 9) and HDAC inhibition by trichostatin A (TSA) represses miR-375 further, potentially through increased expression of a miR-375 inhibitor (Fig. 6).
REFERENCES
REFERENCES


Keller, D., Clark, E., Goodman, R. (2012). Regulation of microRNA-375 by cAMP in Pancreatic B-
Cells. *Molecular Endocrinology*, 26(6), 0000-0000.


35, 701-711.


