Inhibition of insulin secretion in β-cells exposed to arsenic, cadmium and manganese is associated with altered microRNA expression

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Abstract

Diabetes Mellitus (DM) is a global public health epidemic characterized by the inability of the body to properly regulate blood glucose and can manifest in different forms. A variety of risk factors, such as obesity, family history and a high-caloric diet, increase the risk of developing Type II DM. Recently, environmental exposures, including inorganic arsenic (iAs), cadmium (Cd) and manganese (Mn) have been associated with diabetes and altered glucose homeostasis. Previous research from our lab has shown that while iAs, Cd and Mn impair glucose stimulated insulin secretion (GSIS) in rat insulinoma (beta) cells, they may be operating through different mechanisms which remain unknown. MicroRNAs, which are short, non-coding RNA molecules that regulate gene expression post-transcriptionally, have recently emerged as a candidate link between metal exposure and the development of diabetes. The goal of the present study was to evaluate iAs, Cd or Mn exposure on insulin response in rat insulinoma cells and their association with dysregulated miRNA expression. As anticipated, 24-hour exposure to a non-cytotoxic concentration of iAs (1 µM) inhibited GSIS by nearly 50%, while non-cytotoxic concentrations of Cd (5 µM) and Mn (25 µM) inhibited GSIS by more than 50%. These same metal exposures did not have the same effect on the three probed microRNAs (miR-29b, miR-146a, miR-217), which suggests each metal may be operating in a unique way. Small RNA sequencing revealed that these exposures resulted in a unique miRNA profile, with all of the exposures sharing a set of miRNAs found to be highly enriched in pancreatic islets and specifically β-cells. By combining sequencing data with in vitro techniques, we have begun to investigate the relationship between heavy metal exposure, miRNAs, and impaired insulin secretion.
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<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
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<tr>
<td>T2D</td>
<td>Type 2 Diabetes Mellitus</td>
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<tr>
<td>GSIS</td>
<td>Glucose Stimulated Insulin Secretion</td>
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<tr>
<td>SAB</td>
<td>Secretion Assay Buffer</td>
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<tr>
<td>iAs</td>
<td>Inorganic Arsenic</td>
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<tr>
<td>Cd</td>
<td>Cadmium</td>
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<td>Mn</td>
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<td>miRNA</td>
<td>MicroRNA</td>
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<tr>
<td>MCT1</td>
<td>Monocarboxylate Transporter 1</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme Oxygenase 1</td>
</tr>
<tr>
<td>ppb</td>
<td>Parts per billion</td>
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<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
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<tr>
<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>LNA</td>
<td>Locked Nucleic Acid</td>
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Chapter 1: Introduction

1.1 Type 2 Diabetes overview

According to the American Diabetes Association, 29.1 million Americans suffer from diabetes at a cost of $245 billion annually (ADA 2016). This serious, costly, but manageable disease is a public health epidemic. Diabetes manifests in different ways with type 2 diabetes mellitus (T2D) being the most common form of diabetes. This disease centers around insulin, a hormone released by the β-cells within the Islets of Langerhans of the pancreas that signals cells to absorb and/or metabolize glucose. Glucose is used for energy or stored, and if cells cannot properly take up glucose, then energy imbalance and cellular dysfunction will occur. When fat, liver and muscle cells do not respond to insulin properly, a condition called insulin resistance, glucose is unable to enter the cells and builds up in the blood causing individuals to become hyperglycemic (“Standards” 2015). Insulin resistance can develop over time where more and more insulin is required to maintain glucose homeostasis (“Standards” 2015). T2D is a multifactorial disease where both genetic and environmental factors play a role. The presence of certain alleles in multiple genes and a disease-conducive environment have been shown to be instrumental in the development of the disorder (Murea, Ma, & Freedman, 2012). Since T2D is strongly linked to obesity, sedentary lifestyle and a high-caloric diet are also conducive to the development of T2D (Murea, Ma, & Freedman, 2012). A relatively newer area of research is surrounding the role of environmental toxicants and endocrine disruptors, such as inorganic arsenic (iAs), Cadmium and Manganese, in the development of T2D (Navas-Acien et al., 2006).

1.2 Arsenic
Arsenic is a naturally occurring metalloid found in both organic and inorganic forms, with the inorganic form (iAs) being the predominant form of arsenic found in surface and underground water reservoirs (Paul et al., 2007). The most common route of exposure of iAs is through contaminated drinking water, and many countries such as Bangladesh, China, India, Mexico and America have high levels of iAs in their groundwater ("WHO" 2016). The EPA and WHO regulate arsenic in drinking water to 10 µg/L or 10 ppb ("WHO" 2016). It is estimated that more than 50 million individuals in Bangladesh and 13 million residents of the United States are exposed to concentrations of iAs in drinking water at or above 10 ppb (BGS and DPHE, 2001; Focazio et al., 1999). Dietary sources of arsenic exposure can include fish, meat, poultry and dairy products. However, dietary exposure of arsenic from these foods is generally much lower than exposure from contaminated drinking water ("WHO" 2016). Arsenic is currently an established risk factor for cancers, cardiovascular diseases, and neurological disorders (Hou, Wang, & Baccarelli, 2011). The link between arsenic and T2D, however, has been less studied. Exposure to arsenic in contaminated drinking water above 100 ppb is associated with an increased risk of T2D in Taiwan and Bangladesh (Lai et al. 1994; Rahman et al. 1998). An expert review panel from the National Toxicology Program (NTP) in 2011 concluded with support for an association between inorganic arsenic and diabetes in areas where arsenic drinking water levels exceeded 500 µg/L (Maull et al. 2012). One study found that the methylated metabolites of iAs, methylarsonite (MAsIII) and dimethylarsinite (DMAIII), are more potent inhibitors of GSIS in murine pancreatic islets. Furthermore, since arsenic exposure had little to no effects on insulin content, arsenic exposure may be interfering with mechanisms that
are responsible for insulin secretion. Consequently, the authors of this study concluded that insulin-producing β-cells are a target for arsenic exposure (Douillet et al., 2013).

1.3 Cadmium

Cadmium (Cd) is a naturally occurring toxic metal that is mainly found in the Earth’s crust. Since this non-essential metal exhibits a high rate of soil-to-plant transfer, human exposure occurs primarily through food ingestion and tobacco smoke inhalation (Wu et al, 2017). The main targets of cadmium intoxication include the kidneys, liver, bones, respiratory and reproductive systems. Cadmium has been primarily considered a nephrotoxicant, however, recent human and animal studies have suggested an association between exposure and dysregulation of glucose homeostasis (Wu et al, 2017). An epidemiological study found that 31 to 60 year old diabetic males in Pakistan had significantly higher blood and urinary levels of Cd than non-diabetic males (Edwards et al, 2009) and these differences were maintained in both smoker and non-smokers. Another study investigated the accumulation of cadmium in pancreatic β-cells and found that Cd was rapidly taken up in pancreatic tissue. Furthermore, they found that low levels of Cd exposure (5 µM) resulted in an increased rate of GSIS, while high levels of Cd exposure (20 µM) resulted in a significant decrease in insulin release (Nilsson et al., 1986). A review article (Edwards et al., 2009) stated that there is a direct effect of Cd exposure on the pancreas and there is evidence that Cd alters insulin release from pancreatic β-cells. They concluded that the results from both human and animal studies suggest an association between Cd exposure and the development of diabetes and diabetes-related kidney disease (Edwards et al., 2009).

1.4 Manganese
Manganese (Mn) is an essential trace metal that is found in both plant and animal matter. Food is the most common route of Mn exposure in the general population. The Institute of Medicine sets a tolerable upper intake level for Mn at 11 mg/day for adults. Manganese intake from drinking water is significantly lower than food intake at 20 ug/day, assuming a daily water intake of 2 liters. Manganese can exist in 11 different oxidation states, with Mn$^{2+}$, Mn$^{4+}$ and Mn$^{7+}$ being the most important environmental forms. Mn$^{2+}$ is the predominant form found in water at a pH of 4-7 (WHO 2016). Manganese aids the human body in the formation of connective tissue, bones and blood-clotting factors. It is primarily stored in bones, liver, kidney and pancreas (Mason 2018). A cross-sectional study based on the Korean National Health and Examination survey measured blood manganese levels in 3996 participants over the age of 20. They found blood manganese levels were significantly lower in the diabetic group as compared to the non-diabetes group (Koh et al, 2014). The study suggests that blood manganese levels may play a role in glucose homeostasis. Similarly, Kazi et al., 2008 performed a case-control study of 257 type 2 diabetic and 166 non-diabetic controls and found that diabetic patients had significantly lower blood levels of manganese. However, another case-control study of 250 diabetic and non-diabetic individuals revealed that the type 2 diabetic individuals had significantly higher serum levels of manganese than non-diabetic individuals (Ekin et al, 2003). Although these studies show conflicting results, they do suggest an association between serum manganese levels and glucose homeostasis.

1.5 Mechanistic Link - MicroRNAs
MicroRNAs may offer a mechanistic link between increased exposure to inorganic arsenic, cadmium or manganese and the development of diseases such as T2D. MicroRNAs are short, non-coding RNA molecules that regulate gene expression post-transcriptionally (Vrijens et al., 2015). They function as negative regulators through silencing and/or suppressing gene expression (Hou et al., 2011). Research has shown that, “miRNAs are involved in the regulation of gene expression through the targeting of mRNAs during cell proliferation, apoptosis, the control of stem cell self renewal, differentiation, metabolism, development, and tumor metastasis” (Hou et al., 2011). Although the mechanism by which environmental exposures affect miRNA expression is unknown, there are hypothesized models that center around inflammation and oxidative stress, which are implicated in the pathophysiology of various diseases including cancer. Increased oxidative stress and the triggering of inflammatory responses by environmental chemicals may be the cause of alterations in miRNA expression (Hou et al, 2011). Recently, miRNAs have been investigated for their role in the diabetic phenotype. Poy et al., in 2004 was one of the first to establish a role for miRNAs in the regulation of insulin secretion. Since then, dozens of miRNAs have been identified as key players in pathways contributing to the development of type 2 diabetes, which include miR-146a, miR-217 and miR-29b.

1.5.1 miR-146a

MicroRNA-146a has been found to be highly expressed in many cell types under homeostatic conditions (Alipoor et al, 2017). Rong et al., 2013 discovered that circulating miRNA-146a levels were significantly elevated in new T2D patients as compared to healthy controls. Additionally, they found that circulating miRNA-146a
levels were positively correlated with plasma concentrations of protein Heme oxygenase 1 (HO-1). HO-1 exerts an anti-inflammatory and anti-oxidant role on many physiological processes. HO-1 activity has been found to increase more than 100 fold in the presence of cadmium (Trakshel et al., 1986). Arsenic has also been shown to induce the activity and expression of HO-1 (Brown et al, 1997). It is hypothesized that chronic inflammation plays an important role in the development of insulin resistance and complications of diabetes such as neuropathy. MiR-146a is a regulator of the toll like receptor signaling pathway in the innate immune system (Alipoor et al, 2017). Consequently, reduced expression levels of miR-146a could lead to a less effective inhibition of the target genes involved with toll like receptors leading to dysregulation of the inflammatory pathway. Studies have also shown an interaction between miR-146a and interleukin-1 receptor-associated kinase 1/TNF receptor-associated factor 6, which would decrease the inflammatory cytokines production in macrophages (Alipoor et al, 2017).

Additionally, Balasubramanyam et al., 2011 revealed that impairment of miR-146a and its downstream signals leading to inflammation and insulin resistance in type 2 diabetes.

1.5.2 miR-217

Cadmium responsive microRNAs in Daphnia pulex, a widely used toxicological model, revealed a significant network of Cd-responsive microRNAs in relation to insulin metabolism. Reduced insulin levels were found to decrease the activity of transforming growth factor beta 1 (TGFB1), which results in the down-regulation of miR-217 (Chen et al, 2015). Beckman et al., 2011 co-transfected miR-217 with miR-377, which significantly reduced Heme oxygenase-1 protein expression and enzyme activity as compared to the control in HEK 239 cells. As stated previously, HO-1 exerts an anti-
inflammatory and anti-oxidant role on many physiological processes. However, transfection of either miR-217 or miR-377 by themselves did not significantly reduce HO-1 expression level (Beckman et al, 2011). The development of diabetes can lead to liver and kidney impairment. Shao et al., 2016 investigated the correlation between serum miR-217 levels and the severity of diabetic kidney disease. They found that serum miR-217 levels were significantly higher in type 2 diabetic patients as compared to the control suggesting an involvement of miR-217 in pathways relating to the progression of kidney disease.

1.5.3 miR-29b

MiR-29b is widely studied microRNA with an established role in insulin signaling. The miR-29 isoforms are highly expressed in pancreatic islets. Pullen et al., 2011 determined that miR-29b selectively targets both human and mouse Monocarboxylate Transporter 1 (MCT1) 3’ untranslated regions, leading to silencing of the MCT1. MCT1 allows for circulating pyruvate and lactate to enter β-cells, where they can act as substrates for Krebs cycle, providing NADH and FAD for oxidative phosphorylation and ATP synthesis. The increased cytosolic ATP/ADP ratio resulting from this oxidation can trigger insulin release, even in the absence of elevated blood glucose. Therefore, it is likely that maintaining miR-29b expression is required for normal stimulation of insulin secretion by glucose (Pullen et al, 2011). He et al., 2007 studied microRNA expression profiles from normal Wistar and diabetic Goto-Kakizaki rats, and a microarray analysis of skeletal muscles revealed four upregulated and eleven down-regulated microRNAs. Of particular interest is microRNA-29, which was confirmed to have elevated expression levels in the muscle, fat and liver tissues of diabetic rats. Overexpression of microRNA-
29 could repress insulin-stimulated glucose uptake as the muscle, fat and liver tissues are important targets for insulin action (He, Zhu, Gupta, Chang, & Fang, 2007).

1.6 Small RNA sequencing

Previous research has revealed significant expression level changes of miR-29b 146a and 217 with 24 hour iAs III exposure (1 µM) in INS-1 cells (Beck, unpublished 2017) and these same microRNAs were probed through quantitative RT-PCR with Cd and Mn exposure. While a focus on a small subset of miRNAs allows us to associate each miRNA to a specific functionality, there is still a need to assess global changes in the expression of all miRNA genome-wide. High throughput sequencing of small RNAs allows for an unbiased examination of the differential expression levels of all microRNAs in a sample. With small RNA sequencing, we can better understand how iAs, Cd and Mn are affecting microRNAs and identify novel species for future targeted studies.

Chapter 2: Materials and Methods

2.1 Cell Culture and Treatment

Rat insulinoma cells expressing human pre-proinsulin, INS-1 (832/13) cells with passage numbers from 49-60 were cultured at 5% CO₂, 37°C in RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 100 U/ml penicillin, 100 mg/ml streptomycin (all from Gibco, Waltham, MA), and 0.05 mM b-mercaptoethanol (Sigma, St. Louis, MO). INS-1 cells were exposed to iAs III (1 µM), CdCl₂ (5 µM) or MnCl₂ (25 µM) (all from Sigma-Aldrich, St. Louis, MO) for 24 hours. These concentrations significantly inhibited GSIS, while also being non-cytotoxic to INS-1 cells (Dover, unpublished work 2017).

2.2 Glucose Stimulated Insulin Secretion (GSIS)
INS-1 cells were then plated in a 12 well plate at 1,000,000 cells/well 24 hours prior to treatment. The cells were then exposed for 24 hours to iAs\textsuperscript{III} (1 µM), CdCl\textsubscript{2} (5 µM) or MnCl\textsubscript{2} (25 µM). GSIS was measured after exposure in secretion assay buffer (SAB) containing 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, 1.16 mM MgSO\textsubscript{4}, 20 mM HEPES, 2.5 mM CaCl\textsubscript{2}, 0.2% bovine serum albumin, and 25.5 mM NaHCO\textsubscript{3}. Cells were first incubated in 0 mM glucose SAB for 40 minutes, followed by incubation in 2.5 mM glucose SAB for 1 hour and by a final incubation in 16.7 mM glucose SAB for 2 hours. Culture medium was collected after the 2.5 mM glucose SAB and 16.7 mM glucose SAB incubations. The insulin secreted into the medium was measured using a Ultra Sensitive Mouse Insulin ELISA kit (Chrystal Chem). The amount of insulin was normalized for cellular protein content using a bicinchoninic acid assay (BCA).

2.3 RNA Isolation and Quantitative RT-PCR

Total RNA was isolated using the Norgen Total RNA kit (Norgen Biotek, Thorold, Canada) as per the manufacturer's instructions. Nanodrop 2000 was used to quantify RNA. TaqMan microRNA reverse transcription kit (Applied Biosystems) and high capacity RNA to cDNA kit were used as per the manufacturer's instructions to generate complementary DNA for miRNA assays. RT-PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) for miRNA RT-PCR. RT-PCR assays were run on a Bio-Rad CFX96 Touch real time PCR detection system (Bio-Rad). The assays were performed in triplicate using either U6 as an internal control to measure miRNA expression. All TaqMan assays were purchased from Applied Biosystems and include: miR-29b (assay 002082), miR-146a (assay 462788), miR-217 (assay 462171), U6 (assay 001973).
2.4 Small RNA-sequencing

High-quality RNA available from INS-1 832/13 cells exposed to arsenic, cadmium or manganese was used for small RNA sequencing (smRNA-seq) at Genome Sequencing Facility (GSF) in the Greehey Children’s Cancer Research Institute (GCCRI) of UT Health Sciences Center at San Antonio (UTHSCSA). Libraries were created using TriLink’s CleanTag™ Small RNA Library Preparation Kit for Illumina, and 50 bp single-read sequencing was carried out on the Illumina HiSeq platform resulting in an average of over 10 million reads per sample. miRquant 2.0 (Kanke et al, 2016) was used to trim off adapter sequences, align reads to the rat genome (m4), and quantify miRNAs and their isoforms (termed isomiRs). Reads were normalized to reads per millions mapped to miRNAs (RPMMMs). An expression threshold of at least 50 RPMMMs was set to filter out the lowly expressed miRNAs, which resulted in a set of 285 robustly expressed miRNAs.

Chapter 3: Results

3.1 Effects of Metal Exposure on GSIS

A GSIS was performed on INS 832/13 cells after 24 hour exposure to iAs (1 µM), Cd (5 µM) and Mn (25 µM) to assess the effects of these heavy metals on insulin secretion. Insulin response, i.e., insulin excreted from the cells into the medium, was measured in untreated cells after 1 hour incubation in 2.5 mM glucose SAB and after 2 hour incubation in 16.7 mM glucose SAB. As expected, the insulin response of untreated cells increased nearly 10x after incubation in 16.7 mM glucose SAB. Figure 1 also displays insulin response of heavy metal exposure after the 2 hour incubation in 16.7
mM glucose SAB. As expected, exposure to 1 µM iAs resulted in a nearly 50% decline in insulin secretion from 0.3 ng/ug to 0.17 ng/ug. Exposure to 5 µM Cd and 25 µM Cd also resulted in significant declines in insulin secretion, with both metals inhibiting GSIS by over 50%. These results are consistent with past literature and the decline in insulin secretion is statistically significant for all treatment groups.

**Figure 1.** INS 832/13 cells exposed to 24 hour treatment of iAs\textsuperscript{III} (1 µM), CdCl\textsubscript{2} (5 µM) or MnCl\textsubscript{2} (25 µM). Insulin secretion was measured after incubation for 40 minutes in 0 mM glucose SAB, 1 hour in 2.5 mM glucose SAB and 2 hours in 16.7 mM glucose SAB. Values are expressed as mean ± SD for N=6 and (**) indicates statistically significant (p < 0.01) effects of exposure compared to control insulin secretion.

3.2 Effects of metal exposure on miR-29b expression
Real time PCR was performed to determine microRNA-29b expression level changes after exposure to heavy metals. Figure 2 displays the relative quantification values (RQV) of miR-29b after exposure to iAs, Cd and Mn. Exposure to 1 µM iAs increased the relative expression level of 29b 1.28-fold, which was expected, but not statistically significant. Exposure to 5 µM Cd decreased the relative expression level of 29b 0.82-fold, however, this result was not statistically significant. Exposure to 25 µM was found to decrease the relative expression of miR-29b 0.35-fold, which was statistically significant (p < 0.01).

**Figure 2.** INS 832/13 cells exposed to 24 hour treatment of iAs^III^ (1 µM), CdCl\textsubscript{2} (5 µM) or MnCl\textsubscript{2} (25 µM). Real time PCR was performed on these cells with miR-U6 probed as control. Values are expressed as RQV of control ± SD for N=9-12 and (***) indicates
statistically significant \((p < 0.01)\) effects of exposure compared to control microRNA expression level changes.

3.3 Effects of metal exposure on miR-217 expression

Real time PCR was performed to determine microRNA-217 expression level changes after exposure to heavy metals. Figure 3 displays the relative quantification values \((RQV)\) of miR-217 after exposure to iAs, Cd and Mn. Exposure to 1 \(\mu\text{M}\) iAs decreased the relative expression level of miR-217 0.71-fold. This value is statistically significant \((p < 0.01)\). The RQV of miR-217 after exposure to 5 \(\mu\text{M}\) Cd was relatively unchanged. Exposure to 25 \(\mu\text{M}\) was found to decrease the relative expression of miR-217 0.89-fold, but was not statistically significant.
**Figure 3.** INS 832/13 cells exposed to 24 hour treatment of iAs\textsuperscript{III} (1 μM), CdCl\textsubscript{2} (5 μM) or MnCl\textsubscript{2} (25 μM). Real time PCR was performed on these cells with miR-U6 probed as control. Values are expressed as RQV of control ± SD for N=9-12 and (**) indicates statistically significant (p < 0.01) effects of exposure compared to control microRNA expression level changes.

### 3.4 Effects of metal exposure on miR-146a expression

Real time PCR was performed to determine microRNA-146a expression level changes after exposure to heavy metals. Figure 4 displays the relative quantification values (RQV) of miR-146a after exposure to iAs, Cd and Mn. Exposure to 1 μM iAs increased the relative expression level of miR-146a 1.79-fold, which was significant (p < 0.05). The RQV of miR-146a after exposure to 5 μM Cd was relatively unchanged at 0.92-fold. Exposure to 25 μM Mn was found to decrease the relative expression of miR-146a 0.66-fold, which was statistically significant (p < 0.01).
Figure 4. INS 832/13 cells exposed to 24 hour treatment of iAs\textsuperscript{III} (1 µM), CdCl\textsubscript{2} (5 µM) or MnCl\textsubscript{2} (25 µM). Real time PCR was performed on these cells with U6 probed as an internal control. Values are expressed as RQV of control (unexposed cells) ± SD for N=9-12. (*) indicates statistically significant (p<0.05) and (**) indicates statistically significant (p < 0.01) effects of exposure compared to control microRNA expression level changes.

3.5 Sequencing Results

The samples sent for small-RNA sequencing include 3 unexposed controls, 3 with exposure to 1 µM iAs, 3 with exposure to 5 µM Cd and 3 with exposure to 25 µM Mn.
After quality control, a total of 81 million reads mapping to miRNA species were obtained across the twelve samples, with over 1,000 miRNAs or isomiRs identified. Applying an expression threshold of at least 50 RPMMMs resulted in a set of 285 robustly expressed miRNAs, with 10 miRNAs known to be enriched in β-cells responsible for over 50% of the total aligned reads. (Fig. 5) Many of these most highly expressed miRNAs (including miR-375, miR-7-5p, miR-26a-5p and miR-30d-5p) have been previously described in rodent or human islets (de Candia et al., 2017; van de Bunt et al., 2013).
Figure 5. Results of small RNA sequencing on INS-1 832/13 cells after 24 hour treatment of iAs\textsuperscript{III} (1 µM), CdCl\textsubscript{2} (5 µM) or MnCl\textsubscript{2} (25 µM). Expression is normalized as reads per million miRNAs mapped.

Chapter 4: Discussion

This study is the first to characterize the miRNA profile of rat insulinoma cells in the context of heavy metal exposure. Furthermore, by combining sequencing data with in vitro techniques, we have begun to investigate the relationship between heavy metal exposure, miRNAs, and impaired insulin secretion.

Previous in vitro studies conducted in our lab (Douillet et al 2013) show pancreatic islets exposed for 48 hours to low subtoxic concentrations of arsenic and its methylated metabolites display inhibited GSIS without any detrimental effect on insulin transcription. This leads us to believe that arsenic may have targets along the insulin secretory pathway, however, the exact mechanism remains unknown. Research by Dover et al, 2017 (unpublished) assessed the effects of iAs, Cd, and Mn on mitochondrial respiration, and discovered that exposure to iAs significantly impaired the mitochondrial oxygen consumption rate in a manner that other exposures (Cd and Mn) did not. The means by which iAs is exerting an effect on mitochondrial respiration, however, needs to be further investigated.

iAs is of particular interest due to its prevalence in food and drinking water throughout the world. Arsenic levels in some private wells can reach up to 800 ppb, which 80 times the EPA’s regulatory limit of 10 ppb (Sanders et al., 2014). A 1 µM concentration of iAs used in these experiments is equivalent to 75 ppb, a physiologically
relevant concentration many around the world are exposed to. Exposure to 1 µM iAs for
24 hours was enough to reduce GSIS by nearly 50%, however, iAs is just one of many
heavy metals that have been implicated in metabolic diseases.

Cadmium and Manganese are known human developmental toxicants that are
also found in drinking well water (Sanders et al, 2014). 24-hour exposure of INS-1
(832/13) cells to 5 µM Cd resulted in a 53% inhibition of GSIS. Similarly, 24-hour
exposure of INS-1 (832/13) cells to 25 µM resulted in a nearly 70% decline in insulin
secretion. While these non-cytotoxic concentrations of Cd and Mn have been shown to
have no deleterious effect on cell viability or mitochondrial function (Dover, unpublished
2017), there is a lack of data on the effect of these exposures on islet insulin content.
Future studies need to interrogate the effects of Cd and Mn on insulin mRNA levels to
determine if these heavy metals are disrupting the insulin production pathway.

MicroRNAs have recently emerged as a possible mechanistic link between heavy
metal exposure and the diabetic phenotype. Alterations in microRNAs have been shown
to be critical in the pathophysiology of many diseases, including cancer (Calin et al,
2006). While iAs, Cd, and Mn all cause an impairment of GSIS, they may be acting on
different targets, and one way to investigate this is through the examination of their
miRNA profiles.

In this study, miRNAs 29b, 146a and 217 were probed after INS-1 832/13
exposure to iAs, Cd or Mn. Previous research in our lab has shown a statistically
significant increases in the expression of miR-29b and mir-146a with exposure to 1 µM
iAs (Beck, unpublished 2017). Our lab has also shown a statistically significant
decrease in the expression of miR-217 with exposure to 1 µM iAs (Beck, unpublished
This study investigated these same microRNAs in the context of cadmium and manganese exposure. miR-29b is a widely studied microRNA with established roles in insulin signaling (He et al, 2007). Overexpression of miR-29b was found to inhibit insulin-stimulated glucose uptake in a murine insulin-responsive fat cell line. The high levels of miR-29 caused insulin resistance, which mimicked insulin resistance in cells that are incubated with high glucose and high insulin (He et al, 2007). Notably, in our study exposure to 1 µM iAs increased the expression of miR-29b 1.28-fold, which could play a role in the inhibition of GSIS. iAs exposure also resulted in a statistically significant decrease in the RQV of miR-217. High glucose has been shown to increase the expression of miR-217 in rat glomerular mesangial cells (Shao et al, 2016). Therefore, arsenic exposure could be affecting glucose homeostasis during conditions of high glucose, where the expression of miR-217 is dysregulated and unable to perform its normal function. iAs exposure increased the relative expression of miR-146a and manganese exposure decreased the relative expression of miR-146a. MiR-146a is a regulator of the toll like receptor signaling pathway in the innate immune system (Alipoor et al, 2017). Consequently, reduced expression levels of miR-146a could lead to a less effective inhibition of the target genes involved with toll like receptors leading to dysregulation of the inflammatory pathway. Chronic inflammation is hypothesized to play an important role in the development of insulin resistance and complications of diabetes such as neuropathy. Therefore, the dysregulation of the inflammatory pathway by reduced expression levels of miR-146a with manganese exposure could lead to the development of insulin resistance.
Though 24-hour exposure of iAs, Cd or Mn all significantly inhibited insulin response in INS-1 832/13 cells, the metals did not have the same effect on the three probed microRNAs which suggests that they may have different targets in the human body. This was further investigated through high throughput sequencing to view changes in small RNA species transcriptome-wide in response to iAs, Cd, or Mn exposure. Exposure to each metal resulted in a unique miRNA profile, though all exposures shared a set of miRNAs found to be highly enriched in pancreatic islets and specifically β-cells. None of these shared miRNAs were affected by metal exposure, but each metal did have a unique effect on several other miRNAs, which should be investigated further in future studies. While there is no established method of determining the significance of a microRNA fold change (Chugh et al, 2012), functional studies must be performed to evaluate the effect, if any, of the microRNA on the biological phenotype. These studies should target highly upregulated or highly downregulated microRNAs from the small RNA sequencing data. Locked nucleic acids or LNAs can modulate gene expression by binding to specific messenger RNA, pre-mRNA or non-mRNA through Watson and Crick base pairing (Veedu et al, 2010). They can be used in experiments to inhibit microRNA expression. Conversely, microRNA mimics can be used to up-regulate the activity of a specific microRNA. By employing either of these methods, one can observe any phenotypic changes that occur as result of inhibition or overexpression of a specific microRNA.

Additional future studies should also assess the effects of metal mixtures on microRNA expression level changes. Heavy metals, such as arsenic, cadmium and manganese can be found together in drinking water (Buschmann et al, 2008). Studies
investigating metal mixtures would offer insight into whether these metals can have synergistic or antagonistic effects on microRNA expression levels which may further lead to dysregulation of diabetes-related genes.

Regardless of whether or not any gene targets are identified, microRNAs can still be used as biomarkers for correlated disease phenotypes, which could be of high medical and commercial value (Chugh et al, 2012).

In summary, T2D is a global public health epidemic that continues to rise in prevalence. Recently, environmental exposures, including iAs, Cd and Mn, have been linked to the dysregulation of glucose homeostasis and diabetes. In this study, 24 hour exposure to non-cytotoxic concentrations of iAs, Cd or Mn all significantly inhibited insulin secretion in INS-1 (832/13) cells. MicroRNAs represent a possible mechanistic link between these environmental exposures and the development of the diabetic phenotype. These same metal exposures did not have the same effect on the three probed microRNAs (miR-29b, miR-146a, miR-217), which suggests that they may have different targets in the human body. Small RNA sequencing revealed that these exposures resulted in a unique miRNA profile, with all of the exposures sharing a set of miRNAs found to be highly enriched in pancreatic islets and specifically β-cells.
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