INORGANIC ARSENIC AS AN ENDOCRINE DISRUPTOR: MODULATION OF THE GLUCOCORTICOID RECEPTOR PATHWAY AND IMPLICATIONS FOR PLACENTAL PHYSIOLOGY

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ABSTRACT

Cassandra Jean Meakin: Inorganic Arsenic as an Endocrine Disruptor: Modulation of the Glucocorticoid Receptor Pathway and Implications for Placental Physiology
(Under the direction of Rebecca Fry)

Prenatal exposure to inorganic arsenic (iAs) has been associated with adverse developmental and reproductive outcomes, and may be tied to altered glucocorticoid receptor (GR) in the placenta. In this study, we investigated whether iAs exposure modulates GR signaling in placental cells. Trophoblast JEG-3 cells were exposed to environmentally-relevant doses of iAs and mRNA expression and DNA methylation were quantified. Results demonstrated that iAs exposure alters the expression of 12 GR-genes that play a role in fetal and placental development. Furthermore, supporting a role for the epigenome in control of this expression, the mRNA alterations were associated with changes in DNA methylation patterning in placental cells. The identified target genes have been implicated in associations with prenatal arsenic exposure, placental physiology, and fetal development. This study provides evidence of iAs as an endocrine disruptor and insight into the mechanisms by which prenatal iAs exposure induces adverse birth outcomes.

To my family, friends, and my lab members, who have loved and supported me throughout this process. You all inspire me to be the best scientist and version of myself that I can be each and every day.

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LIST OF ABBREVIATIONS

ACTB	Beta-actin
AFF1	AF4/FMR2 family, member 1
AQP1	Aquaporin 1
ARID5B	AT Rich Interactive Domain 5B
CpG	Cytosine proximal to Guanine
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
H6PD	Hexose-6-phosphate dehydrogenase
iAs	Inorganic Arsenic
MT2A	Metallothionein 2A
NR3C1	Nuclear Receptor Subfamily Group 3C Member (Glucocorticoid Receptor)
PDGFRB	Platelet-derived growth factor receptor, beta
PER2	Period Homolog 2
SBSB1	SplA/ryanodine receptor domain and SOCS box containing 1
SGK1	Serum/GC-regulated kinase 1
SLC22A5	Solute carrier family 22 (organic cation/carnitine transporter), member 5
TFO	Transcription Factor Occupancy

CHAPTER 1: INTRODUCTION

Inorganic arsenic (iAs) is an ubiquitous contaminant representing a worldwide health concern as more than 100 million individuals are exposed to potentially harmful levels of arsenic in drinking water that exceeds the World Health Organization's (WHO) limit of 10 μg/L (Naujokas et al., 2013). In the United States alone, arsenic has been found to be present in drinking water at levels that exceed 800 μg/L (Sanders et al., 2012). These exposures are a significant public health concern as they have been associated with skin lesions, diabetes mellitus, cancer, and high blood pressure in adult populations (Naujokas et al., 2013; Yunus, Sohel, Hore, & Rahman, 2011).

Early life exposures to inorganic arsenic are associated with adverse reproductive and developmental outcomes such as preterm birth, low birth weight, and susceptibility to infection, among others (Naujokas et al., 2013; Bailey et al., 2016). Additionally, prenatal exposure to inorganic arsenic exposure may cause spontaneous abortion, stillbirth, and infant mortality (Rahman et al., 2007; Rahman et al., 2010). Previously, the molecular mechanisms that underlie these observed health outcomes associated with arsenic exposure have been thought to be related to arsenic's ability to induce epigenetic modifications, which have been shown to mediate disease development and susceptibility (Laine & Fry, 2016; Smeester & Fry, 2018; Bailey & Fry, 2014; Kile et al., 2014; Green et al., 2016; Appleton, Jackson, Karagas, & Marsit, 2017).

Of relevance to arsenic-induced developmental outcomes, inorganic arsenic has been shown to cross the placenta, which subsequently leads to fetal exposure and modulation of

the fetal epigenome (Concha, Vogler, Lezcano, Nermell, & Vahter, 1998). Furthermore, placental arsenic concentrations have been shown to correlate with fetal and maternal blood concentrations, which provides a basis for the utilization of placental tissue as a primary organ of interest to quantify fetal exposures during development (Punshon et al., 2015). Additionally, previous studies have demonstrated arsenic's ability to accumulate in placental tissue once arsenic crosses the placental barrier (Punshon et al., 2015). The placenta acts as an endocrine organ during fetal development and mediates fetal exposures to exogenous compounds (Evain-Brion & Malassine, 2003; Burton, Fowden, & Thornburg, 2016). Other functions of the placenta include regulating fetal nutrition, controlling the production of fetal and maternal cortisol, producing additional hormones key for fetal development such as growth factors and cytokines, and ultimately controls the fetal environment (Novakovic & Saffery, 2012; Godfrey, 2002; Kertes et al., 2016; De Bonis et al., 2012). Additionally, the placenta also acts as driver of early and later life outcomes and functions as a biosensor for environmental exposures and impacts on later life health (Maccani & Marsit, 2009). Taken together, there is substantial evidence for the use of placental tissues to quantify epigenetic marks that may result from environmental iAs exposures *in utero* that may mediate early and later in life health outcomes.

Previously arsenic has been shown to act as a potential endocrine disruptor (Sun, Sun, Xiang, Luo, & Hong, 2016). For example, in non-placental hepatic cells, arsenic has been shown to display a non-monotonic dose relationship where lower concentrations (0.1-1 μM) induce activity but higher concentrations (2-3 μM) repressing activity (Bodwell, Kingsley, & Hamilton, 2004). The specific molecular mechanisms by which arsenic alters activity of the glucocorticoid receptor (GR) pathway and acts as an endocrine disruptor remain relatively

understudied. However, it is well established that epigenetic modifications, such as DNA methylation, may underlie the arsenic-associated modulation of biological pathways associated with adverse health outcomes and placental gene expression (Laine & Fry, 2016; Green et al., 2016; Sun et al., 2016; Rojas et al., 2015).

The observed activation of the GR-pathway could potentially be linked to DNA methylation patterning through the transcription factor occupancy (TFO) theory (Martin & Fry, 2016). This theory posits that DNA methyltransferase cannot gain access to DNA when transcription factors have bound to and occupied response elements of selected genes, thereby influencing CpG methylation patterning and subsequent gene expression (Martin & Fry, 2016; Zhu, Wang, & Qian, 2016). This is significant as disruptions in placental pathophysiology *in utero* have been linked to functional outcomes such as cardiovascular disease and hypertension later in life (Barker, Bull, Osmond, & Simmonds, 1990). Additionally, epigenetic marks in the placenta have been associated with cognitive functioning in newborns (Appleton et al., 2017; Paquette et al., 2015; Paquette et al., 2014; Conradt, Lester, Appleton, Armstrong, & Marsit, 2013; Monk et al., 2016). Specifically, DNA methylation of the glucocorticoid receptor, *NR3C1*, has been shown to impact fetal behavior and neurocognition (Paquette et al., 2015; Conradt et al., 2013; Bromer, Marsit, Armstrong, Padbury, & Lester, 2013). Taken together, this data demonstrates the ability of in utero arsenic exposure to disrupt endocrine associated pathways, such as the GR-pathway, which may have functional outcomes later in life.

With respect to biological pathways associated with fetal exposures and adverse health outcomes, the GR-pathway has been shown to mediate the effects of stress during pregnancy on the developing fetus (Cottrell & Seckl, 2009). The GR serves as the master

regulator of glucocorticoid action during fetal development and throughout the life course of an individual (Zannas & Chrousos, 2017; Emin Turkay Korgun, 2012). When glucocorticoids, such as cortisol and corticosterone, bind to the GR, the GR dimerizes and enters the nucleus and subsequently binds to DNA response elements. Downstream genes of glucocorticoid response elements (GRE) have been shown to affect placental and fetal growth, development, and function (Emin Turkay Korgun, 2012; Moisiadis & Matthews, 2014). While excess exposures to glucocorticoids have been associated with adverse fetal outcomes, glucocorticoids are necessary for proper fetal and placental development (Moisiadis & Matthews, 2014; Ozmen, Unek, & Korgun, 2017; Bivol, Owen, & Rose'Meyer, 2016; Fowden & Forhead, 2015). Specific developments driven by glucocorticoid programming include the heart, the brain, the lungs, the kidneys, and maturation of the fetal HPA axis (Moisiadis & Matthews, 2014). Due to the integral nature of glucocorticoid exposure during fetal development, aberrations in the GR-signaling pathway have been associated with fetal abnormalities and potentially lifelong adverse health effects (Ozmen et al., 2017; Bivol et al., 2016). Furthermore, the gene that encodes for the GR (*NR3C1*) is highly expressed in the placenta and modulation of the GR-pathway in the placenta and other tissues have been associated with aforementioned neurobehavioral outcomes, cardiovascular disease, cancer, and inflammatory disease (Paquette et al., 2015; Monk et al., 2016; Emin Turkay Korgun, 2012; Argentieri, Nagarajan, Seddighzadeh, Baccarelli, & Shields, 2017).

Literature that details arsenic's role in modifying the placental glucocorticoid pathway are limited. Given the evidence that arsenic modulates fetal disease development and may act as an endocrine disruptor, we determined the effects of arsenic exposure on placental GR-gene expression. We hypothesized that increased iAs exposure would result in a significant

alteration of mRNA gene expression levels that would reflect previous studies where lose doses of arsenic exposure induce GR-gene expression and higher doses of arsenic repress GR-gene expression. Additionally, we hypothesized these changes in expression are significantly associated with DNA methylation patterning in placental cells, leading to functional outcomes associated with disease. In this novel study, we are among the first to examine GR-pathway response to arsenic exposure in the placenta. Placental JEG-3 cells were treated with levels of inorganic arsenic at environmentally-relevant, non-cytotoxic doses at concentrations of 0.5, 1, and 3 μM iAs. Isolated DNA and RNA were used to quantify DNA methylation and GR-pathway gene expression.

CHAPTER 2: METHODS

Cell Culture and iAs Treatments

The JEG-3 choriocarcinoma cell line was purchased from the American Type Culture Collection (Manassas, VA). JEG-3 cells were grown in Dulbecco's modified Eagle's Minimum Essential Medium, supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1mM sodium pyruvate at 37 °C in 50% CO_2. Cells were plated at 5 x 10^6 cells per 25cm^3 flask and incubated under standard conditions until achieving 80-90% confluence. In order to investigate the effects of iAs *in vitro*, JEG-3 cells were cultured in a 6-well culture plate for 24 at 0.5 x 10^6 cells per well. iAs was dissolved in deionized water and vortexed to create final concentrations of 0.5 μM, 1 μM, and 3 μM iAs. Cells were exposed to these concentrations of iAs for 24 h, at which point cells were harvested for DNA and RNA isolation. Vehicle controls were treated with Dulbecco's modified Eagle's Minimum Essential Medium, supplemented with 10% FBS, 1% penicillin/streptomycin, and 1mM sodium pyruvate for 24 h.

mRNA Expression Assessment by Quantitative Real-Time Polymerase Chain Reaction (RT-PCR)

Treated and untreated JEG-3 cells were harvested using ice cold PBS and Buffer RLT. Harvested cells were then placed in a QIAcube (Qiagen, Valencia CA) for RNA and DNA extraction according to the manufacturer's protocol. Subsequent extracted RNA were quantified through the use of a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA). RNA integrity was analyzed with the use of the QIAxcel Screengel (Qiagen,

Valencia CA) according the manufacturer's protocol. Extracted RNA was converted to cDNA and subsequent mRNA expression was analyzed with the RT² Profiler™ PCR Array for Human Glucocorticoid Signaling, which quantifies 84 genes that are downstream targets of the GR-pathway, according to the manufacturer's protocol (Qiagen, Valencia CA)). Results of RT-PCR cycle times were normalized against the housekeeping gene Beta-actin (*ACTB),* and fold changes in expression were calculated based on the ΔΔCT method (Livak & Schmittgen, 2001).

DNA Methylation Analysis

Extracted DNA were quantified through the use of a Nanodrop 1000 spectrophotometer (Thermo Scientific). To analyze placental CpG methylation, extracted DNA sequences were sent to Wayne State (Detroit, MI) and bisulfate-converted using the EZ DNA methylation kit (Zymo Research, Irvine, CA) and then subsequently hybridized on the Illumina HumanMethylation850 Bead Chip array (Illumina, Inc., San Diego, CA), which assesses the DNA methylation levels of 853,307 individual probes at single nucleotide resolution. Methylation levels were calculated and expressed as β values (β= intensity of the methylated allele (M)/ [intensity of the unmethylated allele (U) + intensity of the methylated allele (M) + 100. Data were normalized for both arrays using the *minfi* package in R (Aryee et al., 2014). Specifically, image files were used to produce background-corrected and quantile normalized β-values.

Statistical Analysis

Each experiment was performed in biological triplicate. Statistically significant findings are reported as the mean value ± the standard error of the mean (SEM). DNA methylation and mRNA expression changes between exposed and unexposed cells were

analyzed using a one-way analysis of variance (ANOVA) in Partek® Genomics Suite™ Softwate (St. Lewis, MO). In order to investigate direct comparisons, a post-hoc Tukey test was utilized. Statistical significance was set at a Bonferroni corrected $p<0.05$ (McDonald, 2014). For the purposes of identifying biological functions and regulators associated with an identified gene set, Ingenuity Pathway Analysis (IPA) was used. Significant pathways associated with the gene set were identified using a right-tailed Fisher's Exact test ($p<0.05$).

CHAPTER 3: RESULTS

In order to gain insight as to whether iAs exposure modulates expression of genes involved in the GR-signaling pathway, isolated mRNA was extracted from exposed JEG-3 cells and analyzed with a Qiagen GR-signaling array, which measures mRNA expression of 84 GR target genes (Qiagen, Valencia CA). Results demonstrated iAs exposure to induce a significant alteration (p<0.05) in mRNA expression amongst 12 GR-signaling genes represented in **FIGURE 1**.

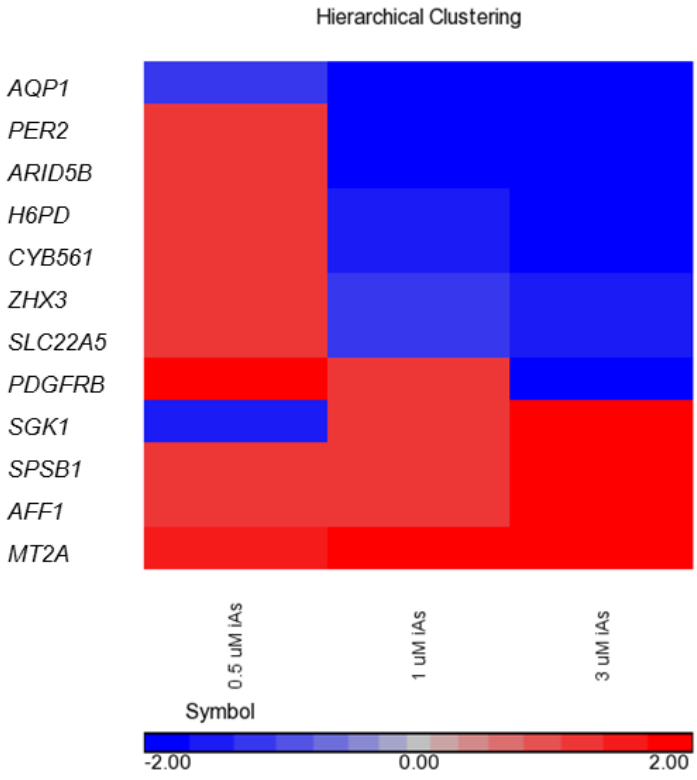

Figure 1. mRNA expression heatmap displaying the 12 genes that demonstrated significant (p<0.05) altered mRNA expressing with iAs treatment.
A total of 12 genes displayed a significant alteration (p<0.05) in mRNA expression with inorganic arsenic treatment relative to controls. Red coloring denotes an increase in expression while blue coloring indicates a decrease in expression.

Specifically, three genes displayed an increase in expression as iAs exposure increased (0.5-3 μM), which included Metallothionein 2A *(MT2A),* AF4/FMR2 family, member 1 (*AFF1),* SplA/ryanodine receptor domain and SOCS box containing 1 (*SPSB1).* Conversely, Aquaporin 1 (*AQP1)* displayed a decrease in expression across all treatment groups relative to controls. Serum/GC-regulated kinase 1 (*SGK1)* demonstrated a dose-response relationship where decreases in mRNA expression were observed at 0.5 μM iAs and increases in mRNA expression at increasing treatments of 1-3 μM iAs. An additional seven genes displayed a unique trend where low doses (0.5-1 μM iAs) resulted in an increase in mRNA expression, but the (1-3 μM iAs) doses caused a reduction in mRNA expression. These genes were Period Homolog 2 (*PER2*), AT Rich Interactive Domain 5B (*ARID5B*), Hexose-6-phosphate dehydrogenase (*H6PD*), Cytochrome b-561 (*CYB561*), Zinc Fingers and Homeoboxes (ZHX3), Solute carrier family 22 (organic cation/carnitine transporter), member 5 (*SLC22A5),* and Platelet-derived growth factor receptor, beta *(PDGFRB).*

SUPPLEMENTAL TABLE 1 displays all genes on the GR-signaling array and their associated p-values, fold change values, and standard error values.

To further investigate how iAs exposure modulates the GR-signaling pathway in placental cells, genome-wide DNA methylation was quantified using the 850k Illumina array (Wayne State, Detroit, MI). Significance was set at a Bonferroni-corrected *p*-value of $p<0.05$ (McDonald, 2014). Additionally, β differences were calculated as β (treated)-β (untreated) to determine how CpG methylation changed with treatment groups. When DNA methylation data was integrated with the expression data of the 12 significant ($p<0.05$) identified genes from the GR-signaling array (Qiagen, Valencia, California), 65 probes representing 12 GR-signaling genes were found to have significantly altered levels of DNA methylation and

mRNA expression in treatment groups compared to controls. Of these genes, probes on all 12 identified genes displayed changes representative of the transcription factor occupancy (TFO) theory in at least one treatment group. The term TFO patterning means the identified probes demonstrated either hypermethylation in association with decreased mRNA expression or hypomethylation in association with increased mRNA expression.

AFF1, *SBSB1*, *AQP1*, *ARID5B*, *SLC22A5*, and *H6PD* had at least one probe site that displayed TFO patterning across all treatment groups and represented in **FIGURE 2**.

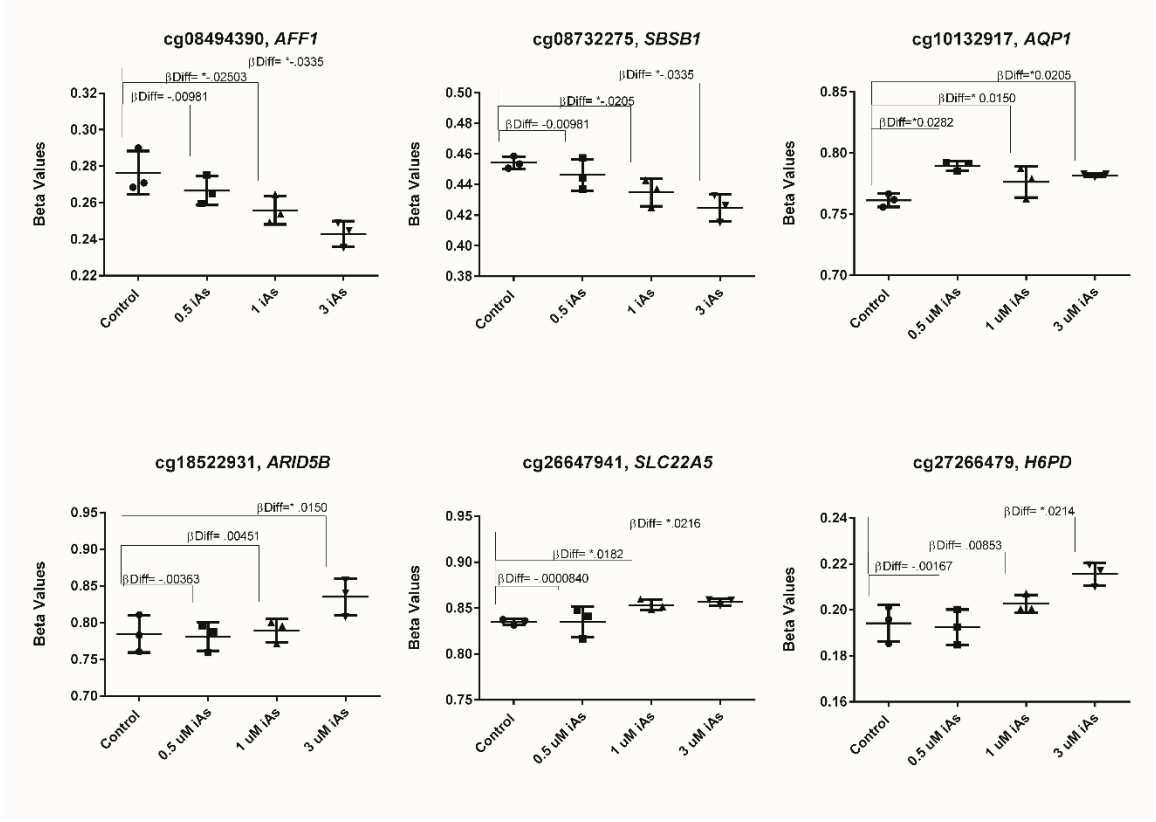

Figure 2. The six genes that displayed TFO patterning across all treatment groups. The six identified genes that displayed TFO patterning across all treatment groups indicating decreases in mRNA expression are associated with increases in methylation and increases in mRNA expression are associated with decreases in methylation. Significant alterations in beta differences are indicated with an asterisk.

Specifically, *AFF1* and *SBSB1*, displayed hypomethylation and an increase in gene expression compared to controls. Conversely, *AQP1* displayed hypomethylation compared to

controls as treatment increased and had a subsequent relative decrease in mRNA expression. The other three genes, *ARID5B, SLC22A5,* and *H6PD* displayed a unique pattern where the 0.5 μM iAs treatment resulted in a decrease in methylation and an increase in mRNA expression, while 1 and 3 μM iAs treatment groups displayed an increase in methylation and a decrease in mRNA expression relative to controls. The remaining six genes, *PER2, CYB561, ZHX3, PDGFRB, SGK1,* and *MT2A* displayed TFO patterning across some but not all treatments. In summary, half of the 12 identified genes had probes that displayed TFO patterning across all treatment groups signifying a relationship between placental DNA methylation alterations and mRNA expression in GR-signaling genes. Beta differences as well as beta values for all 12 identified genes that displayed significant ($p<0.05$) alterations in methylation and mRNA expression can be found in **SUPPLEMENTAL TABLE 2**.

To identify canonical pathways, upstream targets, and functional outcomes associated with the 12 significant genes that displayed overlap between methylation altered GR-target gene expression identified network analysis was performed. For this analysis, IPA constructs networks based on relationships with the literature (Kramer, Green, Pollard, & Tugendreich, 2014). Significant pathways associated with the gene set were identified using a right-tailed Fisher's Exact test.

The 12 significant genes that displayed overlap between significant alterations in DNA methylation and mRNA expression can be found in **SUPPLEMENTAL TABLE 2**. Interestingly, one of the top canonical pathway associated with the identified gene set was the circadian rhythm signaling pathway ($p = 1.84 \cdot 10^{-2}$). Diseases and functions associated were quantity of cells ($p = 1.93 \cdot 10^{-4}$) and size of body ($p = 3.06 \cdot 10^{-4}$), abnormal adrenal gland function ($p = 4.30 \cdot 10^{-4}$), and diabetic complications ($p = 5.85 \cdot$

10^{-4}), among others. Previous literature has supported the theory that environmentally-responsive transcription factors influence CpG methylation patterns (Martin & Fry, 2016). Given this, to further investigate for transcription factor binding sites within the genes that displayed altered CpG methylation transcriptional regulators of GR-signaling genes were subsequently identified. Analysis revealed that the promoter region of the genes that displayed altered CpG methylation were enriched for binding sites of Tumor Necrosis Factor (TNF) ($p = 1.79 \cdot 10^{-4}$). **(TABLE 1)**.

Table 1. Identified functional outcomes, diseases, canonical pathways, and transcription factor regulators of the 12 identified GR-signaling genes that displayed significant (p<0.05) alterations in mRNA expression and DNA methylation.

Functional outcomes/TF regulators	p-value, genes associated
Tumor Necrosis Factor (TNF)	($p = 1.79 \cdot 10^{-4}$), *AQP1, ARID5B, MT2A, PER2, SGK1, SPSB1*
Circadian Rhythm Signaling	($p = 1.84 \cdot 10^{-2}$), *PER2*
Quantity of cells	($p = 1.93 \cdot 10^{-4}$), *AFF1, AQP1, ARID5B, MT2A, PDGFRB, PER2, SGK1*
Size of Body	($p = 3.06 \cdot 10^{-4}$), *AFF1, AQP1, ARID5B, H6PD, PER2*
Diabetic Complications	($p = 5.85 \cdot 10^{-4}$), *AQP1, MT2A, PDFGRB*
Abnormal Adrenal Function	($p = 4.30 \cdot 10^{-4}$), *ARID5B, H6PD*

CHAPTER 4: DISCUSSION

Millions of people around the world are exposed to inorganic arsenic (iAs) through contaminated drinking water (Naujokas et al., 2013). These exposures have been associated with adverse health outcomes in adult populations as well as adverse pregnancy and neonatal outcomes (Naujokas et al., 2013; Rahman et al., 2007). Furthermore, iAs exposure has been to act as an endocrine disruptor and has been shown to modulate the GR-signaling pathway, which is integral for adult stress response and fetal development (Zannas & Chrousos, 2017; Emin Turkay Korgun, 2012). In this study, we investigated how iAs modulates GR-target gene expression in placental cells and whether iAs exposure influences methylation patterns, which are associated with disease. Our results confirm our hypothesis that mRNA expression levels of downstream targets of the GR-signaling pathway were significantly altered in response to iAs exposure. Additionally, iAs exposure was shown to significantly alter CpG methylation patterning of GR-signaling genes in placental cells. Finally, the differentially modified genes observed in this study have been shown to be associated with placental physiology and fetal development (Guo et al., 2016; Salker et al., 2011; Tekin, Kayaalti, Aliyev, & Soylemezoglu, 2012; Grube et al., 2005; Chhabra et al., 2012). These results suggest that prenatal iAs exposure may disrupt the GR-signaling pathway in placental cells and that this disruption may have functional implications for DNA methylation patterning and health outcomes.

In support of arsenic's role as an endocrine disruptor, we investigated the role that iAs exposure plays in modulating expression of a major endocrine pathway, the GR- signaling

pathway through the use of a Qiagen Human GR-signaling array. Results demonstrated a dose-response relationship amongst 12 genes that are targets of the GR-signaling pathway. These genes included Aquaporin 1 (*AQP1),* Metallothionein 2A *(MT2A),* AF4/FMR2 family, member 1 (*AFF1),* and Serum/GC-regulated kinase 1 (*SGK1),* among others. The observed dose-response of these genes is significant as many of these identified genes play a role in placental physiology and fetal growth and development. Specifically, *AQP1,* a maternally-expressed gene, has been shown to be involved in successful placental growth and development (Guo et al., 2016). Previous studies have revealed the dysregulation of *SGK1* to result in errors in embryonic implantation and cell survival (Salker et al., 2011). *PDGFRB* has been shown to regulate placental hematopoietic stem cell generation in trophoblast cells and also has been shown to prevent premature hematopoietic stem cell differentiation, which is important for proper fetal development (Chhabra et al., 2012). Additionally, in the placenta *MT2A* and *SLC22* have been postulated to regulate the transfer of micronutrients to the fetus as well as the transfer of L-carnitine uptake, which is critical for fetal development (Tekin et al., 2012; Grube et al., 2005).

Previous literature has demonstrated iAs's ability to selectively alter glucocorticoid-target gene expression at low, environmentally relevant doses (Bodwell et al., 2004; Kaltreider, Davis, Lariviere, & Hamilton, 2001). Specifically, higher doses of inorganic arsenic (1-3 μM) induced an inhibition of gene expression while lower doses (0.5-1 μM) show an induction of expression (Kaltreider et al., 2001). In this study, our data substantiated these findings as we observed low doses (0.5-1 μM) of iAs to induce mRNA expression of GR-target genes in placental cells, while higher doses (1-3 μM) repressed expression of GR-target genes. It must be noted that the extent to which iAs modulates the GR-signaling

pathway is largely dependent on the quantity of the GR present in the tissue of interest, indicating iAs effects on the GR-signaling pathway may differ by tissue type (Bodwell et al., 2004). Of relevance to placental function, previous studies have shown the GR gene, *NR3C1*, to be highly expressed in the placenta, which could explain differences in GR-target gene induction demonstrated in placental cells by this study (Conradt et al., 2013).

To investigate whether iAs-induced alterations of GR-target gene expression is tied to altered CpG methylation patterns in placental cells, genome-wide methylation was evaluated with an 850 K Illumina Array. Previous studies have shown that DNA methylation is able to influence mRNA gene expression of many genes in placental tissue and may serve as a mediating mechanism for iAs-induced health outcomes (Laine & Fry, 2016; Kile et al., 2014; Green et al., 2016; Sun et al., 2016). Our results displayed a total of 65 CpG sites representing 12 GR-target genes displayed both significantly altered CpG methylation and mRNA expression levels in relation to iAs exposure. Past literature has demonstrated inorganic arsenic to preferentially bind to the GR and alter the glucocorticoid's ability to act a transcription factor in liver cells (Kaltreider et al., 2001). These findings suggest that when inorganic arsenic binds to the GR, the GR is able to bind to GREs and induce transcription at low doses but inhibit transcription at higher doses (Kaltreider et al., 2001).

Transcription factor binding has been postulated to drive the transcription factor occupancy (TFO) theory. As previously mentioned, the TFO theory postulates that environmentally-responsive transcription factors influence methylation patterns by blocking the enzymes that methylate DNA from gaining access to the DNA sequence (Martin & Fry, 2016). Interestingly, all 12 genes displayed patterning associated with the TFO theory in at least one treatment group, with half of these genes (n=6) displayed TFO patterning across all

treatments and the remaining six genes displayed TFO patterning across some but not all treatments. Interestingly, in three of the six genes that displayed TFO patterning across all treatments, *H6PD, ARID5B* and *SLC22A5*, increases in mRNA expression were associated with decreases in methylation at 0.5 μM iAs treatment and at 1 and 3 μM iAs decreases in mRNA expression were associated with hypermethylation relative to controls. Taken together, our findings in conjunction with existing literature suggest that inorganic arsenic is able to bind to the GR and induce expression at low doses while inhibiting expression at higher doses (Bodwell et al., 2004; Kaltreider et al., 2001). In addition to this, we have demonstrated that there is some interplay between the GR binding to GREs and DNA methylation patterning. This suggests that these observed arsenic-induced alterations in mRNA expression are also associated with DNA methylation patterning in placental cells, **FIGURE 3**.

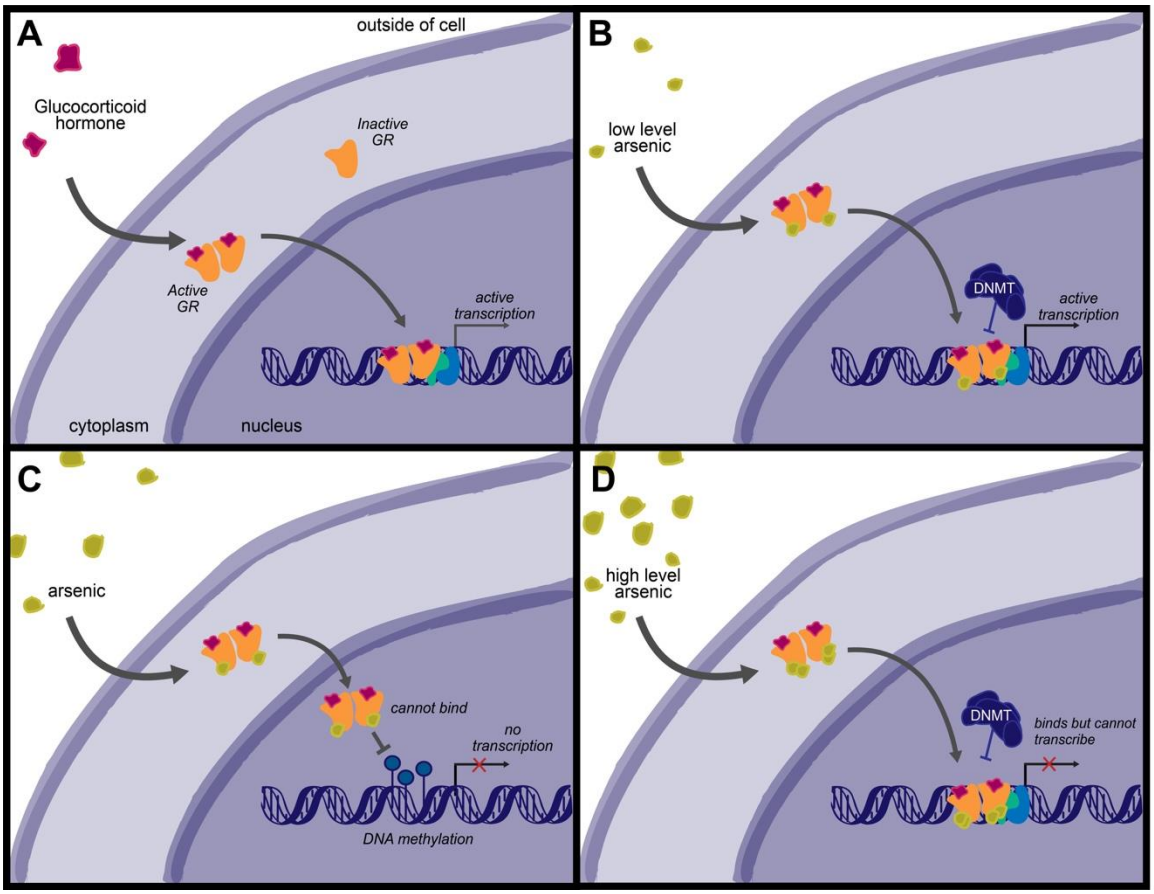

Figure 3. Graphic illustrating how iAs may modulate the GR signaling in a biological system at high and low doses of iAs.
A. Glucocorticoid receptor (GR) binding to glucocorticoid response elements (GREs) under normal physiologic conditions, in human cells, and inducing dimerization, translocation, and induction of mRNA expression. B. At low levels, inorganic arsenic (iAs) may bind to GR and induce mRNA expression while denying DNMT access to the DNA sequence, resulting in an increase in mRNA expression and a decrease in CpG methylation. C. iAs exposure may result in DNA methylation, preventing the GR with iAs bound from binding to GREs, resulting in a decrease in mRNA expression. D. iAs also may bind to GR and repress mRNA expression, resulting in a decrease in mRNA expression and a decrease in CpG methylation.

In our study, the observed CpG methylation patterning could be due to the fact the iAs selectively induces the expression of transcription factors along the GR-signaling pathway in placental cells in a dose-response manner. This altered binding of the GR as a transcription factor prevents DNA methylation enzymes (DNMT) from accessing GREs on the DNA, which ultimately reduces methylation levels and selectively increases mRNA expression of

GR-target genes. Previous studies have postulated that higher doses of arsenic, which bind to the GR, induce allosteric changes in the DNA binding domain, ultimately altering target mRNA gene expression and subsequent methylation patterns, which we have demonstrated in this study (Bodwell et al., 2004). Altered methylation patterns in the placenta are significant as these epigenetic marks have been associated with gene expression alterations and adverse fetal health outcomes such as spontaneous abortion, infant mortality, altered cognitive functioning, higher rates of infant infection, and preterm birth (Naujokas et al., 2013; Rahman et al., 2007; Appleton et al., 2017; Punshon et al., 2015). It must be noted that differences in methylation patterning and gene expression not associated with the TFO can potentially be explained through the impact of site-specific CpG methylation. Previous studies have suggested that the location of the methylation mark may serve as a differential driver of gene expression. Specifically, methylation in the promoter region of the genome has been shown to reduce mRNA expression while methylation in the gene body has been associated with an induction in mRNA expression (Rojas et al., 2015).

In support of the TFO theory, we investigated the major upstream regulators of the 12 identified GR-target genes that displayed both altered mRNA expression and CpG methylation patterns in response to iAs exposure. Size of body and quantity of cells were identified as major functional outcomes associated with the identified 12 genes. This is interesting as previous studies have demonstrated prenatal iAs exposure to be associated with, low infant birthweight, low gestational age, newborn length, and small head circumference (Laine et al., 2015; Gilbert-Diamond, Emond, Baker, Korrick, & Karagas, 2016). Additionally, iAs has been associated with diabetes development, which has also identified as a functional outcome through IPA analysis (Farzan et al., 2016). The circadian

rhythm signaling pathway ($p = 1.84 \cdot 10^{-2}$) was identified as a top canonical pathway associated with the 12 GR-target genes. This is interesting as previous studies have postulated that the placenta may possess circadian properties, which affect fetal and placental physiology and development (Waddell, Wharfe, Crew, & Mark, 2012). Dysregulation of circadian genes have been implicated in the development of cancer and obesity (Froy, 2010). Furthermore, these results are substantiated by previous studies, which have shown glucocorticoids to play a role in circadian rhythm signaling and the regulation of pro-inflammatory cytokines, such as TNF (Waddell et al., 2012; Mark, Wharfe, Lewis, & Waddell, 2011). In the current study, we identified binding sites of TNF ($p = 1.79 \cdot 10^{-4}$) as the as the primary transcriptional regulator of the identified gene set. TNF has been shown to regulate observed proteomic alterations in response to prenatal iAs exposure in previous studies (Laine & Fry, 2016; Bailey et al., 2014). TNF serves as a modulator of cellular signaling pathways that regulate cell growth and proliferation as well as inflammatory pathways (Holtmann & Neurath, 2004).

In summary, we investigated whether inorganic arsenic disrupts the expression levels of genes that act within the GR-pathway in JEG-3 placental cells. Our results have shown that GR-target genes are modulated by iAs exposure at increasing concentrations in placental cells. Furthermore, these expression alterations are at least partially related to DNA methylation patterning in placental cells, which has been implicated in functional adverse health outcomes associated with prenatal iAs exposure, placental physiology, and fetal development. These alterations in CpG methylation patterning provide support for the TFO theory as decreases in CpG methylation have been associated with increases in mRNA expression. The identified genes that display TFO patterning have been associated with

circadian signaling, growth and development, and endocrine function. Taken together, our data substantiates findings from previous studies, which have shown iAs to bind to the GR and selectively alter GR-target genes. Our future work will aim to elucidate how inorganic arsenic alters the DNA Binding Domain of the GR in placental cells and to investigate how these exposures modulate GR-target gene expression in human placental tissues.

APPENDIX 1: TABLE S1

Column ID	p-value (Treatment)	p-value (0.5 vs. Control)	p-value (1 vs. Control)	p-value (3 vs. Control)	Average Fold Change 0.5 As	SE of 0.5 FC	Average fold change 1 As	SE 1 AS FC	Average fold change 3 As	SE 3 FC
ADARB1	0.82	0.48	0.97	0.93	1.23	0.27	-1.04	0.05	-1.06	0.15
AFF1	0.02	0.93	0.13	0.01	1.13	0.33	1.26	0.17	3.09	0.34
AK2	0.78	0.46	0.95	0.56	1.17	0.23	-1.06	0.07	-1.02	0.03
AMPD3	0.93	0.91	0.59	0.67	1.25	0.61	-1.15	0.19	-1.02	0.14
ANGPTL4	0.25	0.39	0.53	0.26	1.31	0.15	-1.18	0.14	-1.25	0.05
ANXA4	0.54	0.92	0.42	0.23	1.11	0.35	-1.35	0.16	-1.33	0.11
AQP1	0.00	0.97	0.24	0.00	-1.05	0.17	-2.14	0.14	-10.04	0.02
ARID5B	0.03	0.42	0.10	0.04	1.05	0.13	-1.81	0.07	-2.30	0.03
ASPH	0.12	0.99	0.32	0.04	1.04	0.22	-1.25	0.10	-2.34	0.08
ATF4	0.38	0.55	0.43	0.40	1.08	0.10	-1.09	0.10	1.16	0.05
BCL6	0.86	0.71	0.44	0.57	1.20	0.32	-1.26	0.18	-1.08	0.07
BMPER	0.46	0.96	0.19	0.94	N/A	N/A	-1.69	0.27	N/A	N/A
CALCR	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
CEBPA	0.89	0.74	0.91	0.49	1.35	0.31	1.10	0.07	1.08	0.03
CEBPB	0.36	0.34	0.93	0.17	1.40	0.46	-1.08	0.09	1.24	0.03
COL4A2	0.73	0.83	0.52	0.50	1.24	0.16	-1.06	0.06	-1.08	0.07
CREB1	0.83	0.41	0.52	0.55	1.29	0.20	1.06	0.07	1.01	0.08
CREB3	0.33	0.94	0.47	0.11	1.08	0.12	1.05	0.06	1.33	0.07
CREB3L4	0.30	0.45	1.00	0.24	1.11	0.20	1.00	0.03	-1.51	0.05
CTGF	0.81	0.99	0.56	0.48	1.02	0.11	1.01	0.16	-1.14	0.09
CYB561	0.05	0.91	0.06	0.03	1.09	0.13	-1.43	0.05	-1.79	0.00
DDIT4	0.39	0.67	0.43	0.36	1.75	0.75	1.04	0.63	1.96	0.08
DIRAS2	0.09	0.14	0.04	0.45	N/A	N/A	N/A	N/A	N/A	N/A

Column ID	p-value (Treatment)	p-value (0.5 vs. Control)	p-value (1 vs. Control)	p-value (3 vs. Control)	Average Fold Change 0.5 As	SE of 0.5 FC	Average fold change 1 As	SE 1 AS FC	Average fold change 3 As	SE 3 FC
DUSP1	0.19	0.40	0.49	0.04	1.17	0.18	1.02	0.08	1.93	0.11
EDN1	0.85	0.42	0.54	0.70	4.96	3.88	1.98	0.30	1.48	0.38
EHD3	0.17	0.52	0.06	0.10	1.11	0.47	-1.97	0.11	-1.62	0.08
ERRFI1	0.88	0.86	0.98	0.51	1.23	0.35	-1.02	0.07	1.17	0.13
FKBP5	0.36	0.19	0.89	0.20	1.82	0.35	1.27	0.43	N/A	#VALUE!
FOSL2	0.09	0.82	0.20	0.04	1.26	0.44	-1.63	0.08	-2.61	0.01
GDPD1	0.19	0.76	0.16	0.16	1.38	0.54	-1.87	0.13	-1.96	0.08
GHRHR	0.35	0.19	0.72	0.57	N/A	N/A	-1.64	0.14	-1.07	0.31
GLUL	0.08	0.85	0.08	0.06	1.16	0.22	-1.25	0.03	-1.38	0.02
GOT1	0.77	0.88	0.52	0.54	1.16	0.17	-1.03	0.06	1.00	0.04
H6PD	0.03	0.33	0.22	0.03	1.19	0.13	-1.41	0.04	-2.45	0.02
HAS2	0.96	0.75	0.92	0.98	N/A	N/A	N/A	N/A	N/A	N/A
HNRPLL	0.83	0.42	0.50	0.51	1.21	0.17	1.06	0.06	1.04	0.05
IL10	0.90	0.98	0.95	0.58	1.77	1.30	1.22	0.56	1.10	0.76
IL1RN	0.32	0.32	0.42	0.83	1.24	0.20	#REF!	0.13	35.30	0.70
IL6	0.08	0.55	0.06	0.02	2.12	0.95	3.55	0.64	4.66	0.32
IL6R	0.80	0.99	0.87	0.44	N/A	N/A	N/A	N/A	3.14	1.21
KLF13	0.06	0.26	0.28	0.09	1.26	0.20	-1.21	0.04	1.14	0.05
KLF9	0.50	0.44	0.81	0.46	1.27	0.26	-1.21	0.15	-1.17	0.06
LOX	0.93	0.64	0.91	0.93	1.27	0.50	-1.04	0.27	1.06	0.38
MERTK C	0.05	0.27	0.91	0.06	1.22	0.10	1.08	0.11	-1.35	0.01
MT1E	0.62	0.40	0.74	0.91	N/A	N/A	N/A	N/A	N/A	N/A
MT2A	0.01	0.95	0.54	0.00	1.47	0.32	11.37	7.68	80.07	24.91
NFKBIA	0.17	0.52	0.08	0.06	1.13	0.22	-1.30	0.02	-1.43	0.01

Column ID	p-value (Treatment)	p-value (0.5 vs. Control)	p-value (1 vs. Control)	p-value (3 vs. Control)	Average Fold Change 0.5 As	SE of 0.5 FC	Average fold change 1 As	SE 1 AS FC	Average fold change 3 As	SE 3 FC
NR3C1	0.09	0.99	0.10	0.05	1.07	0.29	-1.96	0.04	-2.39	0.03
PDCD7	0.39	0.89	0.18	1.00	1.13	0.16	-1.08	0.04	-1.00	0.07
PDGFRB	0.01	0.03	0.42	0.10	1.85	0.53	1.06	0.06	-1.86	0.07
PDP1	0.15	0.27	0.10	0.04	1.26	0.17	1.14	0.05	1.62	0.10
PER1	0.61	0.32	0.88	0.98	1.70	0.67	-1.13	0.18	-1.02	0.08
PER2	0.00	0.86	0.02	0.00	1.17	0.19	-2.14	0.04	-5.19	0.02
PIK3R1	0.15	0.48	0.07	0.06	1.08	0.28	-1.40	0.06	-1.56	0.03
PLD1 Ph	0.25	0.33	0.97	0.27	1.32	0.28	-1.04	0.05	-1.46	0.04
PLEKHF1	0.85	0.90	0.80	0.56	1.17	0.24	-1.19	0.21	1.05	0.08
POU2F1	0.09	0.68	0.12	0.08	1.38	0.30	-1.21	0.08	-1.46	0.07
POU2F2	0.79	0.56	0.70	0.86	1.98	0.84	1.17	0.52	1.35	0.44
RASA3	0.54	0.19	0.56	0.81	2.07	0.96	1.50	0.73	1.48	0.72
RGS2	0.28	0.49	0.96	0.11	1.07	0.18	-1.05	0.03	1.44	0.41
RHOB	0.06	0.84	0.06	0.04	1.16	0.11	-1.36	0.03	-1.54	0.04
RHOJ	0.90	0.85	0.78	0.67	2.58	2.01	2.33	1.40	1.72	1.33
SESN1	0.10	0.84	0.10	0.07	1.18	0.22	-1.13	0.08	-1.33	0.07
SGK1	0.03	0.06	0.53	0.14	-1.46	0.18	1.05	0.23	1.91	0.20
SLC10A6	0.52	0.52	0.16	0.49	1.71	0.79	2.52	0.78	1.74	0.81
SLC19A2	0.29	0.39	0.98	0.11	1.21	0.24	1.03	0.07	1.41	0.09
SLC22A5	0.04	0.27	0.31	0.05	1.27	0.09	-1.15	0.12	-1.51	0.03
SNTA1	0.44	0.70	0.50	0.13	1.26	0.41	-1.09	0.08	-1.45	0.02
SPHK1	0.98	0.70	0.80	0.75	2.31	1.76	1.69	1.05	1.23	0.30
SPSB1	0.04	0.32	0.10	0.01	1.21	0.17	1.17	0.06	2.58	0.12
STAT5A	0.60	0.67	0.80	0.42	2.31	1.53	3.75	3.26	-2.68	0.16
STAT5B	0.40	0.22	0.28	0.95	1.30	0.18	1.17	0.04	-1.04	0.06

Column ID	p-value (Treatment)	p-value (0.5 vs. Control)	p-value (1 vs. Control)	p-value (3 vs. Control)	Average Fold Change 0.5 As	SE of 0.5 FC	Average fold change 1 As	SE 1 AS FC	Average fold change 3 As	SE 3 FC
TBL1XR1	0.08	0.85	0.08	0.06	1.16	0.20	-1.21	0.04	-1.34	0.03
TNF	0.10	0.04	0.66	?	N/A	0.46	-1.23	0.11	N/A	0.17
TNFAIP3	0.83	0.72	0.61	0.92	1.48	1.92	-1.02	0.59	1.13	0.21
TSC22D3	0.39	0.72	0.61	0.21	2.53	0.35	-1.07	0.18	-2.47	0.15
USP2	0.31	0.38	0.44	0.08	1.33	0.17	1.22	0.11	1.85	0.06
USP54	0.17	0.20	0.29	0.41	1.42	0.34	1.21	0.06	-1.02	N/A
VDR	0.54	0.29	0.67	?	-1.53	0.31	-2.82	0.12	N/A	N/A
VLDLR	0.59	0.62	0.58	0.46	1.11	0.25	1.02	0.21	N/A	0.09
XDH	0.05	0.06	0.76	0.26	1.56	0.36	-1.13	0.03	-1.36	0.03
ZFP36	0.21	0.89	0.43	0.17	1.17	0.08	-1.09	0.10	1.08	0.07
ZHX3	0.05	0.09	0.79	0.16	1.18	0.20	-1.18	0.10	-1.52	0.04
NF281	0.08	0.85	0.08	0.06	1.23	0.00	1.11	0.00	1.03	0.00

APPENDIX 2: TABLE S2

ID	pval	qval	Location	UCSC_RefGene_Name	Avg Cont Beta	Avg 0.5 Beta	Beta Diff 0.5 v Cont	Avg 1 Beta	Beta Diff 1 v Cont	Avg 3 Beta	Beta Diff 3 v Cont	UCSC_RefGene_Group	pval (Tx)	pval (0.5 vs. Cont)	pval (1 vs. Cont)	pval (3 vs. Cont)	Avg Fold Change 0.5 As	Avg Fold Change 1 As	Avg Fold Change 3 As
cg00154587	0.01	0.41	Body	AFF1	0.90	0.91	0.01	0.90	0.00	0.92	0.02	Body	0.02	0.93	0.13	0.01	1.13	1.26	3.09
cg03714884	0.05	0.50	Body	AFF1	0.93	0.95	0.02	0.94	0.01	0.95	0.02	Body	0.02	0.93	0.13	0.01	1.13	1.26	3.09
cg08494390	0.01	0.40	Body;Body	AFF1	0.28	0.27	-0.01	0.26	-0.02	0.24	-0.03	Body;Body	0.02	0.93	0.13	0.01	1.13	1.26	3.09
cg17279557	0.02	0.44	Body;TSS1500	AFF1	0.04	0.04	0.00	0.04	0.00	0.04	0.00	Body;TSS1500	0.02	0.93	0.13	0.01	1.13	1.26	3.09
cg19170233	0.05	0.50	TSS1500;Body	AFF1	0.70	0.67	-0.02	0.72	0.02	0.67	-0.02	TSS1500;Body	0.02	0.93	0.13	0.01	1.13	1.26	3.09
cg19320820	0.02	0.43	Body;Body	AFF1	0.43	0.41	-0.02	0.37	-0.06	0.37	-0.06	Body;Body	0.02	0.93	0.13	0.01	1.13	1.26	3.09
cg21571135	0.02	0.43	Body;Body	AFF1	0.42	0.40	-0.01	0.34	-0.08	0.31	-0.11	Body;Body	0.02	0.93	0.13	0.01	1.13	1.26	3.09
cg24551420	0.02	0.44	TSS200	AFF1	0.06	0.06	0.00	0.06	0.00	0.06	0.01	TSS200	0.02	0.93	0.13	0.01	1.13	1.26	3.09
cg10132917	0.01	0.40	1stExon	AQP1	0.76	0.79	0.03	0.78	0.01	0.78	0.02	1stExon	0.00	0.97	0.24	0.00	-1.05	-2.14	-10.04
cg18080604	0.04	0.49	1stExon;5'UTR	AQP1	0.75	0.79	0.04	0.77	0.02	0.75	0.00	1stExon;5'UTR	0.00	0.97	0.24	0.00	-1.05	-2.14	-10.04
cg18307978	0.05	0.50	Body	AQP1	0.70	0.72	0.02	0.71	0.01	0.73	0.03	Body	0.00	0.97	0.24	0.00	-1.05	-2.14	-10.04
cg21289714	0.01	0.42	TSS1500	AQP1	0.74	0.76	0.02	0.75	0.01	0.77	0.03	TSS1500	0.00	0.97	0.24	0.00	-1.05	-2.14	-10.04
cg03028088	0.01	0.39	Body;Body	ARID5B	0.67	0.68	0.01	0.69	0.01	0.73	0.06	Body;Body	0.03	0.42	0.10	0.04	1.05	-1.81	-2.30
cg06854180	0.00	0.32	Body	ARID5B	0.89	0.90	0.01	0.86	-0.03	0.89	0.00	Body	0.03	0.42	0.10	0.04	1.05	-1.81	-2.30
cg08241406	0.05	0.50	TSS200;Body	ARID5B	0.58	0.58	0.01	0.59	0.02	0.60	0.02	TSS200;Body	0.03	0.42	0.10	0.04	1.05	-1.81	-2.30
cg17633222	0.01	0.42	TSS1500;Body	ARID5B	0.91	0.91	0.01	0.91	0.01	0.92	0.02	TSS1500;Body	0.03	0.42	0.10	0.04	1.05	-1.81	-2.30
cg18522931	0.05	0.50	Body	ARID5B	0.78	0.78	0.00	0.79	0.00	0.84	0.05	Body	0.03	0.42	0.10	0.04	1.05	-1.81	-2.30
cg18980410	0.04	0.49	Body	ARID5B	0.76	0.75	-0.01	0.73	-0.03	0.77	0.01	Body	0.03	0.42	0.10	0.04	1.05	-1.81	-2.30

ID	pval	qval	Location	UCSC_Ref Gene_Name	Avg Cont Beta	Avg 0.5 Beta	Beta Diff 0.5 v Cont	Avg 1 Beta	Beta Diff 1 v Cont	Avg 3 Beta	Beta Diff 3 v Cont	UCSC_Ref Gene_Group	pval (Tx)	pval (0.5 vs. Cont)	pval (1 vs. Cont)	pval (3 vs. Cont)	Avg Fold Change 0.5 As	Avg Fold Change 1 As	Avg Fold Change 3 As
cg22368847	0.02	0.46	Body;Body	ARID5B	0.92	0.90	-0.02	0.91	-0.01	0.92	0.01	Body;Body	0.03	0.42	0.10	0.04	1.05	-1.81	-2.30
cg22782105	0.01	0.40	Body	ARID5B	0.90	0.85	-0.04	0.87	-0.03	0.87	-0.02	Body	0.03	0.42	0.10	0.04	1.05	-1.81	-2.30
cg24495062	0.01	0.39	Body	ARID5B	0.76	0.76	0.01	0.72	-0.04	0.73	-0.03	Body	0.03	0.42	0.10	0.04	1.05	-1.81	-2.30
cg26002628	0.03	0.48	TSS1500	ARID5B	0.06	0.06	0.00	0.06	0.00	0.06	0.00	TSS1500	0.03	0.42	0.10	0.04	1.05	-1.81	-2.30
cg04987499	0.02	0.46	TSS1500;TSS1500	CYB561	0.50	0.51	0.01	0.48	-0.02	0.50	0.00	TSS1500;TSS1500	0.05	0.91	0.06	0.03	1.09	1.09	-1.43
cg11442173	0.04	0.49	5'UTR;5'UTR;5'UTR	CYB561	0.97	0.97	0.00	0.97	0.01	0.96	0.00	5'UTR;5'UTR;5'UTR	0.05	0.91	0.06	0.03	1.09	1.09	-1.43
cg13118906	0.04	0.48	TSS1500;TSS1500	CYB561	0.44	0.47	0.03	0.43	-0.02	0.44	0.00	TSS1500;TSS1500	0.05	0.91	0.06	0.03	1.09	1.09	-1.43
cg11521888	0.05	0.50	5'UTR;TSS1500	H6PD	0.71	0.73	0.02	0.74	0.03	0.75	0.04	5'UTR;TSS1500	0.03	0.33	0.22	0.03	1.19	-1.41	-2.45
cg14094793	0.04	0.49	Body	H6PD	0.85	0.87	0.02	0.85	0.01	0.87	0.02	Body	0.03	0.33	0.22	0.03	1.19	-1.41	-2.45
cg16256342	0.02	0.46	3'UTR	H6PD	0.71	0.73	0.02	0.71	0.00	0.71	0.00	3'UTR	0.03	0.33	0.22	0.03	1.19	-1.41	-2.45
cg17805199	0.01	0.42	Body	H6PD	0.57	0.58	0.00	0.59	0.02	0.61	0.03	Body	0.03	0.33	0.22	0.03	1.19	-1.41	-2.45
cg25190094	0.02	0.44	Body	H6PD	0.85	0.88	0.03	0.86	0.01	0.89	0.04	Body	0.03	0.33	0.22	0.03	1.19	-1.41	-2.45
cg27266479	0.01	0.40	5'UTR;1stExon	H6PD	0.19	0.19	0.00	0.20	0.01	0.22	0.02	5'UTR;1stExon	0.03	0.33	0.22	0.03	1.19	-1.41	-2.45
cg06711418	0.01	0.39	3'UTR	MT2A	0.91	0.93	0.01	0.91	-0.01	0.92	0.01	3'UTR	0.01	0.95	0.54	0.00	1.47	11.37	80.07
cg04173992	0.01	0.43	5'UTR	PDGFRB	0.78	0.79	0.00	0.79	0.01	0.82	0.04	5'UTR	0.01	0.03	0.42	0.10	1.85	1.06	-1.86
cg05526146	0.04	0.49	5'UTR	PDGFRB	0.71	0.73	0.02	0.72	0.01	0.74	0.03	5'UTR	0.01	0.03	0.42	0.10	1.85	1.06	-1.86
cg26247309	0.01	0.41	Body	PDGFRB	0.20	0.18	-0.02	0.19	-0.01	0.19	-0.01	Body	0.01	0.03	0.42	0.10	1.85	1.06	-1.86

ID	pval	qval	Location	UCSC_RefGene_Name	Avg Cont Beta	Avg 0.5 Beta	Beta Diff 0.5 v Cont	Avg 1 Beta	Beta Diff 1 v Cont	Avg 3 Beta	Beta Diff 3 v Cont	UCSC_RefGene_Group	pval (Tx)	pval (0.5 vs. Cont)	pval (1 vs. Cont)	pval (3 vs. Cont)	Avg Fold Change 0.5 As	Avg Fold Change 1 As	Avg Fold Change 3 As
cg02976543	0.04	0.49	TSS1500	PER2	0.06	0.06	0.00	0.07	0.01	0.06	0.00	TSS1500	0.00	0.86	0.02	0.00	1.17	-2.14	-5.19
cg04117792	0.04	0.49	Body	PER2	0.84	0.86	0.02	0.84	0.00	0.84	0.00	Body	0.00	0.86	0.02	0.00	1.17	-2.14	-5.19
cg06174546	0.03	0.48	Body	PER2	0.92	0.94	0.02	0.93	0.01	0.92	0.00	Body	0.00	0.86	0.02	0.00	1.17	-2.14	-5.19
cg20146792	0.00	0.34	Body	PER2	0.87	0.86	0.00	0.84	-0.02	0.83	-0.04	Body	0.00	0.86	0.02	0.00	1.17	-2.14	-5.19
cg02904344	0.04	0.49	Body;1stExon;5'UTR	SGK1	0.74	0.76	0.02	0.76	0.03	0.79	0.06	Body;1stExon;5'UTR	0.03	0.06	0.53	0.14	-1.46	1.05	1.91
cg04060943	0.02	0.44	Body	SGK1	0.88	0.90	0.01	0.89	0.00	0.87	-0.02	Body	0.03	0.06	0.53	0.14	-1.46	1.05	1.91
cg04905719	0.03	0.47	Body	SGK1	0.16	0.16	0.00	0.16	0.00	0.12	-0.04	Body	0.03	0.06	0.53	0.14	-1.46	1.05	1.91
cg15680307	0.05	0.50	Body	SGK1	0.77	0.76	-0.02	0.80	0.03	0.80	0.02	Body	0.03	0.06	0.53	0.14	-1.46	1.05	1.91
cg17519444	0.03	0.47	Body;Body;ExonBnd;ExonBnd;ExonBnd;ExonBnd;ExonBnd;Body;Body;Body	SGK1	0.05	0.05	-0.01	0.06	0.01	0.05	-0.01	Body;Body;ExonBnd;ExonBnd;ExonBnd;ExonBnd;ExonBnd;Body;Body;Body	0.03	0.06	0.53	0.14	-1.46	1.05	1.91
cg20731573	0.00	0.33	Body	SGK1	0.88	0.90	0.02	0.90	0.02	0.90	0.02	Body	0.03	0.06	0.53	0.14	-1.46	1.05	1.91
cg03979695	0.04	0.49	Body;Body	SLC22A5	0.86	0.86	0.00	0.89	0.03	0.86	0.00	Body;Body	0.04	0.27	0.31	0.05	1.27	-1.15	-1.51
cg06687184	0.05	0.50	Body;Body	SLC22A5	0.45	0.45	0.00	0.45	-0.01	0.41	-0.04	Body;Body	0.04	0.27	0.31	0.05	1.27	-1.15	-1.51
cg16022017	0.01	0.41	Body;Body	SLC22A5	0.76	0.78	0.02	0.77	0.00	0.74	-0.02	Body;Body	0.04	0.27	0.31	0.05	1.27	-1.15	-1.51
cg16647868	0.05	0.50	Body	SLC22A5	0.20	0.19	-0.01	0.19	-0.01	0.19	-0.01	Body	0.04	0.27	0.31	0.05	1.27	-1.15	-1.51

ID	pval	qval	Location	UCSC_RefGene_Name	Avg Cont Beta	Avg 0.5 Beta	Beta Diff 0.5 v Cont	Avg 1 Beta	Beta Diff 1 v Cont	Avg 3 Beta	Beta Diff 3 v Cont	UCSC_RefGene_Group	pval (Tx)	pval (0.5 vs. Cont)	pval (1 vs. Cont)	pval (3 vs. Cont)	Avg Fold Change 0.5 As	Avg Fold Change 1 As	Avg Fold Change 3 As
cg19040266	0.03	0.46	Body	SLC22A5	0.78	0.79	0.01	0.76	-0.02	0.75	-0.03	Body	0.04	0.27	0.31	0.05	1.27	-1.15	-1.51
cg19157439	0.03	0.47	Body;Body	SLC22A5	0.83	0.83	0.00	0.81	-0.02	0.80	-0.02	Body;Body	0.04	0.27	0.31	0.05	1.27	-1.15	-1.51
cg19173554	0.04	0.49	1stExon;1stExon;TSS1500	SLC22A5	0.49	0.49	-0.01	0.48	-0.01	0.49	0.00	1stExon;1stExon;TSS1500	0.04	0.27	0.31	0.05	1.27	-1.15	-1.51
cg23083514	0.03	0.47	1stExon;1stExon;TSS200	SLC22A5	0.40	0.39	-0.01	0.40	-0.01	0.38	-0.02	1stExon;1stExon;TSS200	0.04	0.27	0.31	0.05	1.27	-1.15	-1.51
cg26647941	0.03	0.48	Body;Body	SLC22A5	0.84	0.84	0.00	0.85	0.02	0.86	0.02	Body;Body	0.04	0.27	0.31	0.05	1.27	-1.15	-1.51
cg03116948	0.05	0.50	5'UTR	SPSB1	0.91	0.92	0.01	0.90	0.00	0.93	0.02	5'UTR	0.04	0.32	0.10	0.01	1.21	1.17	2.58
cg03355970	0.01	0.40	5'UTR	SPSB1	0.88	0.91	0.04	0.89	0.01	0.91	0.04	5'UTR	0.04	0.32	0.10	0.01	1.21	1.17	2.58
cg08732275	0.01	0.42	5'UTR	SPSB1	0.45	0.45	-0.01	0.44	-0.02	0.42	-0.03	5'UTR	0.04	0.32	0.10	0.01	1.21	1.17	2.58
cg09146084	0.00	0.35	Body	SPSB1	0.84	0.85	0.01	0.85	0.01	0.86	0.02	Body	0.04	0.32	0.10	0.01	1.21	1.17	2.58
cg11413763	0.00	0.26	5'UTR	SPSB1	0.79	0.82	0.03	0.79	0.00	0.81	0.02	5'UTR	0.04	0.32	0.10	0.01	1.21	1.17	2.58
cg12458866	0.05	0.50	5'UTR	SPSB1	0.06	0.07	0.00	0.06	0.00	0.07	0.01	5'UTR	0.04	0.32	0.10	0.01	1.21	1.17	2.58
cg22436957	0.04	0.48	5'UTR	SPSB1	0.77	0.79	0.02	0.78	0.01	0.81	0.04	5'UTR	0.04	0.32	0.10	0.01	1.21	1.17	2.58
cg25906315	0.02	0.44	5'UTR	SPSB1	0.80	0.83	0.03	0.81	0.01	0.82	0.02	5'UTR	0.04	0.32	0.10	0.01	1.21	1.17	2.58
cg26360004	0.01	0.40	5'UTR	SPSB1	0.92	0.94	0.02	0.94	0.02	0.95	0.02	5'UTR	0.04	0.32	0.10	0.01	1.21	1.17	2.58
cg00599708	0.01	0.42	5'UTR	ZHX3	0.73	0.73	0.00	0.71	-0.03	0.68	-0.05	5'UTR	0.05	0.09	0.79	0.16	1.18	-1.18	-1.52
cg22294908	0.02	0.44	1stExon;5'UTR	ZHX3	0.87	0.90	0.03	0.89	0.02	0.89	0.02	1stExon;5'UTR	0.05	0.09	0.79	0.16	1.18	-1.18	-1.52

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