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Inorganic Arsenic as an Endocrine Disruptor: Modulation of the Glucocorticoid Receptor Pathway in Placental Cells via CpG Methylation

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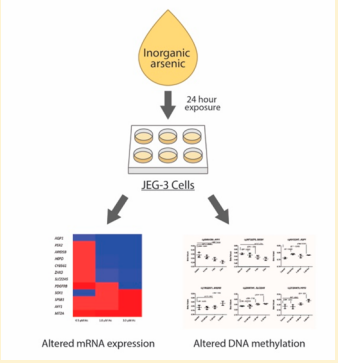
Supporting Information

ABSTRACT: Prenatal exposure to inorganic arsenic (iAs) has been associated with adverse developmental and reproductive outcomes. These outcomes may be tied to altered functionality of nuclear transcription factors such as the glucocorticoid receptor (GR) in the placenta and associated gene expression. The GR pathway is integral for proper fetal and placental development, and perturbations in this pathway may underlie observed associations between prenatal iAs exposure and adverse birth outcomes. We therefore set out to investigate whether iAs modulates the GR signaling pathway in placental cells. JEG-3 trophoblasts were exposed to environmentally-relevant doses of iAs, and mRNA expression assessed. To examine the links between iAs exposure, the GR signaling pathway, and epigenetic modification, DNA methylation levels were also quantified. Treatment with iAs altered the expression of 12 GR-genes that play a role in fetal and placental development. Furthermore, at a gene-specific level, mRNA abundance was associated with changes in DNA methylation patterning in JEG-3 cells, suggesting that the effects of iAs are mediated by epigenetic mechanisms. The identified target genes have been associated with prenatal iAs exposure, placental physiology, and fetal development. This study provides further evidence for iAs as an endocrine disruptor and provides insight as to the mechanisms by which prenatal iAs exposure may induce adverse birth outcomes.



INTRODUCTION

Inorganic arsenic (iAs) is a ubiquitous contaminant, and high levels of exposure represent a worldwide health concern. Over 100 million individuals worldwide are exposed to potentially harmful levels of iAs in drinking water that exceeds the World Health Organization (WHO) limit of 10 μg/L.[1] In the United States, iAs has been quantified in drinking water at levels that exceed 800 μg/L.[2] High level exposure is a significant public health concern as iAs has been associated with skin lesions, diabetes mellitus, cancer, and high blood pressure in adult populations.[1,3] Previously, iAs has been shown to act as a potential endocrine disruptor.[4] Specifically, iAs influences glucocorticoid receptor (GR)-associated gene expression, although the specific molecular mechanisms are not fully understood. However, it is well established that epigenetic modifications, such as DNA methylation, may underlie the iAs-associated modulation of biological pathways associated with adverse health outcomes.[4−7]

The gene that encodes for GR (*NR3C1*) is highly transcribed in the placenta, and signaling of the GR-pathway plays an important role during pregnancy.[8] GR mediates the effects of stress on the developing fetus and drives formation of the heart, brain, lungs, kidneys, and maturation of the fetal hypothalamic−pituitary−adrenal (HPA) axis.[8,9] Modulation of the GR pathway in the placenta and fetal tissues have therefore been associated with fetal abnormalities and potentially lifelong adverse health effects such as neurobehavioral dysfunction, cardiovascular disease, cancer, and inflammatory disease.[10−17]

The placenta is a multifaceted organ that is responsible for maintaining a healthy pregnancy to term. Depending on the toxicant, the placenta can serve as a mediator for environmental exposures including toxicants in the maternal circulation as well as the maternal immune system.[18,19] Additionally, aberrations in placental gene expression and DNA methylation have been associated with early and later life outcomes as well as exposure to environmental toxicants.[20−22] Thus, the placenta can function as a "biosensor" for environmental exposures that impact developmental health.[22] Importantly, the placenta acts as an endocrine organ during fetal development to maintain proper fetal and placental development by producing hormones key to development such

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as cytokines and growth factors.[23] It regulates fetal and maternal homeostasis and provides adequate nutrition for the fetus.[24−29]

Of concern, iAs is able cross to the human placenta, and prenatal exposure to iAs is associated with adverse reproductive and developmental outcomes such as preterm birth, low birth weight, and susceptibility to infection.[1,30,31] At high levels, iAs has been associated with spontaneous abortion, low birthweight, stillbirth, and infant mortality.[32,33] A potential molecular mechanism that may mediate these health outcomes is iAs-induced epigenetic modifications, such as gene methylation, in both the placenta and the fetus, as exposure to iAs has been associated with altered DNA methylation states within the placenta as well as the fetal epigenome.[5,20,34] Additionally, previous studies have demonstrated that iAs accumulates in placental tissue after crossing the placenta.[35]

Given the evidence that iAs modulates the GR pathway and the integral role that the GR pathway plays in fetal and placental development, there exists a clear need to understand the specific effects of iAs on GR signaling in the placenta. In the present study, we investigated whether iAs exposure modulates GR signaling in trophoblasts by measuring mRNA targets of the GR pathway. We hypothesized that iAs would alter GR-dependent transcription in trophoblasts consistent with the biphasic response observed in other cell types and that these changes would be associated with alterations in DNA (CpG) methylation patterns.

EXPERIMENTAL PROCEDURES

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 Gibco Minimum Essential Medium, supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1 mM sodium pyruvate at 37 °C in 50% CO_2. Cells were plated at 5×10^6 cells per 25 cm^3 flask and incubated under standard conditions until achieving 80−90% confluence. Caution: iAs is a hazardous substance and should be handled carefully. To investigate the effects of iAs *in vitro*, JEG-3 cells were seeded in a six-well culture plate at 0.5×10^6 cells per well and incubated for 24 h prior to treatment. On the day of treatment, iAs was dissolved in deionized water, added to cell culture medium, and vortexed to create final concentrations of 0.5 μM, 1 μM, and 3 μM iAs. Cells were incubated in the presence or absence of iAs for 24 h and then harvested for DNA and RNA isolation.

mRNA Expression Assessment by Quantitative Real-Time Polymerase Chain Reaction (RT-PCR). Treated and untreated JEG-3 cells were harvested in 350 μL of buffer RLT and placed in a QIAcube (Qiagen, Valencia CA) for RNA and DNA extraction using the AllPrep DNA/RNA/miRNA Universal Kit (#80224) according to the manufacturer's protocol. RNA was quantified using 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. To analyze gene expression, extracted RNA was converted to cDNA. Following this, mRNA expression was analyzed with the RT^2 Profiler PCR Array (real-time RT qPCR) for Human Glucocorticoid Signaling, which uses real-time RT qPCR and previously validated primers to quantify the expression of 84 genes that are involved in the GR-signaling pathway (Qiagen, Valencia CA). Real-time RT-qPCR Ct values were then normalized against the housekeeping gene β-actin (*ACTB*), and fold changes in expression were calculated based on the ΔΔCT method.[36]

DNA Methylation Analysis. Extracted DNA was quantified using a Nanodrop 1000 spectrophotometer (Thermo Scientific). To analyze placental CpG methylation, extracted DNA was sent to Wayne State (Detroit, MI), 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. Using the *minfi* package in R, methylation levels were calculated from .idat files, and data were quantile normalized, background corrected, and filtered based on detection *p*-values. β-values were then generated for analysis.[37]

Western Blotting. Cells were harvested following a 24 h treatment with 0.5 μM, 1 μM, and 3 μM iAs. Whole cell lysates were harvested in 150 μL of RIPA buffer (Fisher Scientific, New Hampshire). Protein quantity was determined using the Pierce BCA assay kit according to the manufacturer's protocol (Thermo Scientific, Waltham, MA), and whole cell protein expression was measured by Western blot. For this procedure, samples containing 20 μg of protein were combined with 2× Laemmli sample buffer supplemented with 5% β-mercaptoethanol and loaded onto a 10% mini SDS-page gel. Unless otherwise stated, all Western blot materials came from Biorad (Hercules, CA). The protein was then transferred to a mini polyvinylidene difluoride membrane and blocked in 5% nonfat milk dissolved in 1× Tris-buffered saline with Tween 20 for 30 min at 37 °C degrees. The membrane was then incubated overnight at 4 °C using a monoclonal GR antibody from Cell Signaling (Danvers, MA) (#12041) or β-actin antibody from Cell Signaling (#4970) at a 1:1000 dilution. Following overnight incubation, the membrane was washed and incubated in secondary Cell-signaling HRP-linked antibody (#7074) at a 1:5000 dilution for 1 h at room temperature on a plate shaker. Antibody−protein complexes were then visualized using the Supersignal West Femto Maximal Sensitivity Reagent (Thermo Scientific) and a CCD Imager (BioRad Versadoc 4000MP) with Quantity One software (Version 4.6.9). Western blot images were semiquantified using ImageJ Software.

Cytotoxicity Assay. For the cytotoxicity analysis, JEG-3 cells were seeded at 1.0×10^4 cells per well in 96-well culture plates and incubated overnight. To assess the cytotoxicity of the iAs treatments, cells were treated as described above and incubated for 24 h. Resazurin dye (10 μg/L final concentration) was then added to the culture and plates were incubated for an additional 2.5 h at 37 °C, during which viable cells convert resazurin to the fluorescent resorufin product. Following incubation, fluorescence was measured using a Promega spectrophotometer microplate reader at an excitation wavelength of 560 nm and an emission wavelength of 590 nm according to the manufacturer's protocol. The fluorescence of each well is proportional to the number of metabolically responsive, viable JEG-3 cells present in the population.

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, mRNA expression, and protein levels between exposed and unexposed cells were analyzed using a one-way analysis of variance (ANOVA) in Partek Genomics Suite Software (St. Lewis, MO). To investigate direct comparisons, a post hoc Tukey test was utilized. Differentially methylated sites were identified using the dmpFinder tool in *minfi*.[37] Quality control was performed and probes with greater than 5% poor detection *p*-value probes were removed. Data were then subsequently quantile normalized and an F-test was used to determine categorical difference between exposed and unexposed groups. Finally, variance shrinkage was applied due to small sample size.[37] For all tests, statistical significance was set at a Bonferroni-corrected $p < 0.05$.[38]

RESULTS

iAs Modulates Glucocorticoid-Receptor Mediated Gene Expression in a Dose-Dependent Manner. Prior to treating the JEG-3 cells with iAs to explore gene expression alterations, a resazurin cytotoxicity assay was performed. The data (not shown) demonstrate that 0.5 μM iAs, 1 μM iAs, and 3 μM iAs did not induce cytotoxicity in the JEG-3 cells. To gain insight as to whether noncytotoxic doses of iAs modulate the expression of genes involved in the GR-signaling pathway,

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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 using real-time RT qPCR. The results demonstrated iAs exposure induced a significant alteration ($p < 0.05$) in the expression of 12 GR-signaling related genes (Figure 1).

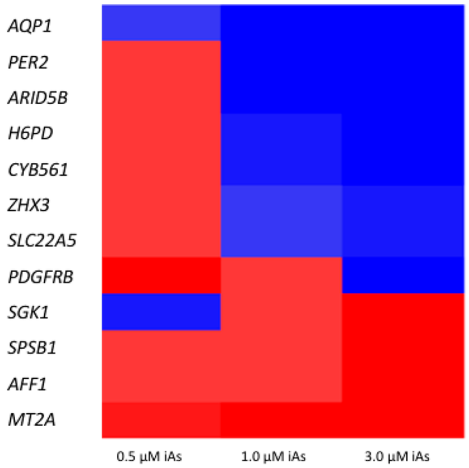

Figure 1. Total of 12 genes displayed a significant alteration ($p < 0.05$) in mRNA expression with iAs treatment relative to controls. Red coloring denotes an increase in expression while blue coloring indicates a decrease in expression.

Specifically, three genes displayed a dose-dependent increase in expression: namely, metallothionein 2A (*MT2A*), AF4/FMR2 family, member 1 (*AFF1*), and SplA/ryanodine receptor domain and SOCS box containing 1 (*SPSB1*). Conversely, Aquaporin 1 (*AQP1*) displayed a dose-dependent decrease in expression across all treatment groups relative to controls. Serum/GC-regulated kinase 1 (*SGK1*) demonstrated a biphasic dose−response relationship where 0.5 μM iAs was associated with a decrease in mRNA expression, while at 1−3 μM iAs, mRNA expression increased. An additional seven genes displayed a biphasic response in mRNA expression following iAs treatment where low doses (0.5 μM iAs) increased mRNA expression, while higher doses (3 μM iAs) decreased mRNA expression. These genes were Period Homologue 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*). In addition to these results, it is interesting to note that the mRNA expression of *NR3C1* was not significantly ($p < 0.05$) altered by iAs treatment (Supplemental Table 1).

iAs Does Not Alter Whole Cell GR Protein Expression. A possible mechanism by which iAs exposure may modulate the GR-signaling pathway in placental cells is through altering the expression of whole cell GR protein. To investigate if whole cell expression of GR was altered following treatment with iAs, GR protein was assessed by Western blot and semi-quantified using ImageJ software. Figure 2 demonstrates our results, which show that iAs treatment did not change whole cell GR protein expression. Furthermore, these results highlight that alterations in mRNA expression of GR-signaling genes may be due to downstream nuclear signaling events as opposed to direct alterations in GR expression.

iAs Alters DNA Methylation Patterns of GR-Pathway Related Genes. To determine if noncytotoxic doses of iAs alter the expression of GR-signaling pathway associated genes in trophoblasts by an epigenetic mechanism, genome-wide DNA methylation was quantified using the 850k Illumina platform. When DNA methylation data were integrated with the expression data of the 12 significant ($p < 0.05$) identified genes from the GR-signaling array, 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 dose-responsive changes in DNA methylation levels.

AFF1, *SBSB1*, *AQP1*, *ARID5B*, *SLC22A5*, and *H6PD* had at least one probe site that displayed dose-dependent methylation patterning across all treatment groups and represented in Figure 3. 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 dose-responsive DNA methylation patterning across some but not all treatments. In

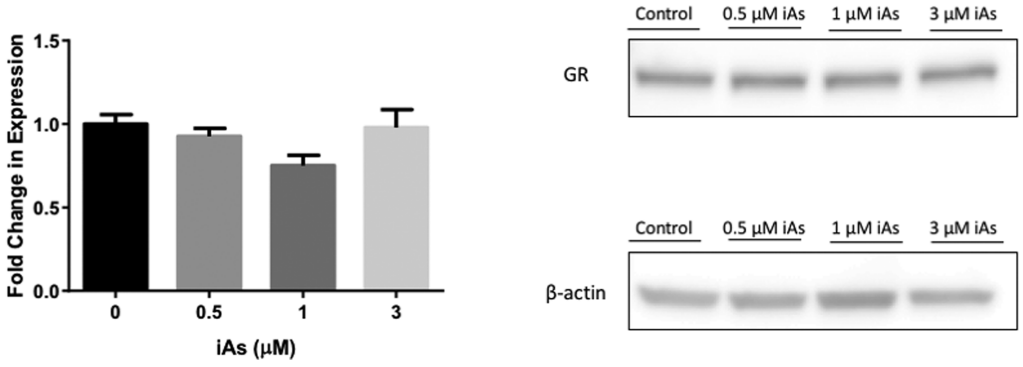

Figure 2. Results of whole cell GR protein level in response to iAs treatment alone compared to control. Whole cell protein was extracted following 24-h iAs treatments and quantified with Western blot.

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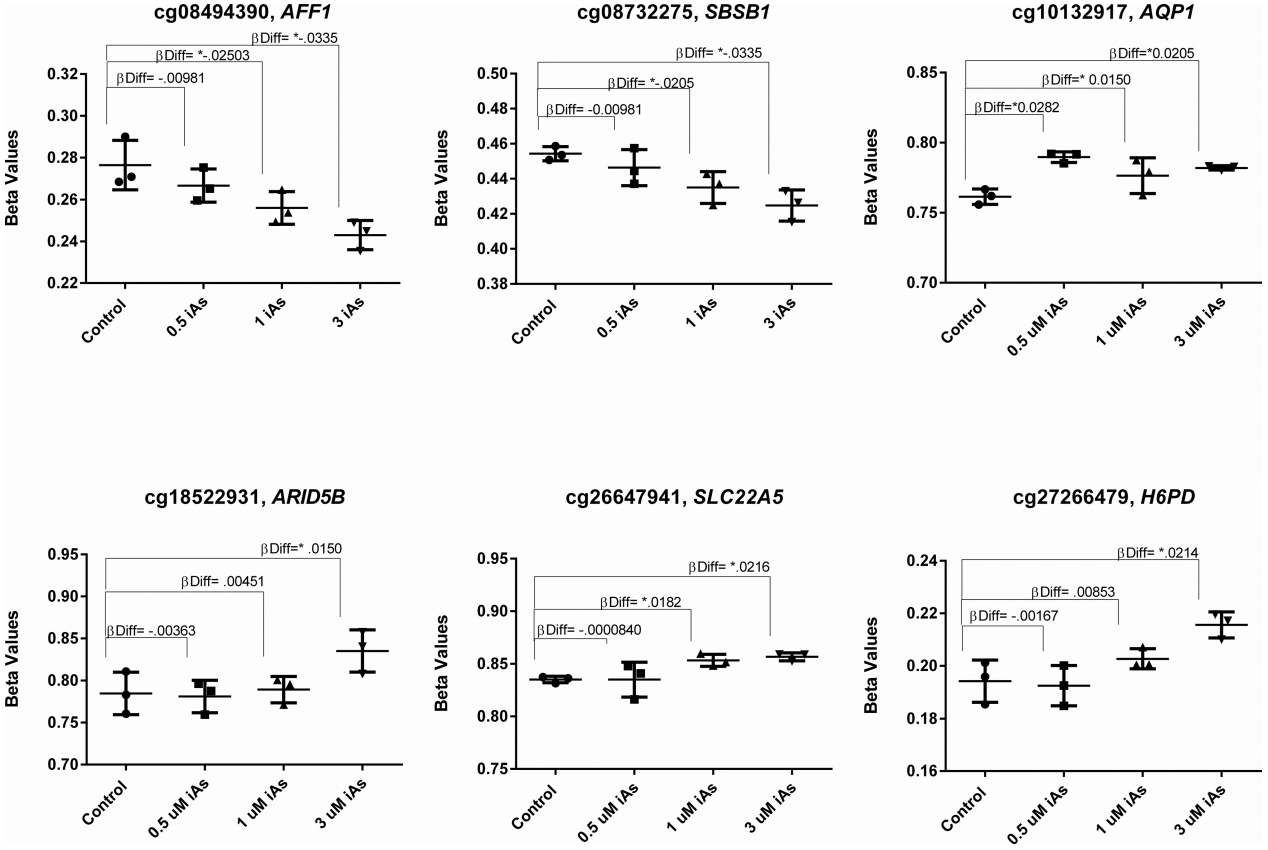

Figure 3. Six identified genes that displayed iAs-dependent alterations in DNA methylation across all treatment groups. Significant differences in β differences are indicated with an asterisk.

summary, half of the 12 identified genes had probes that displayed dose-responsive changes in DNA methylation and mRNA expression patterning across all treatment groups, signifying a relationship between placental DNA methylation alterations and mRNA expression in GR-signaling genes. β differences as well as β values for all 12 identified genes that displayed significant (Bonferroni-corrected $p < 0.05$) alterations in methylation and mRNA expression can be found in Supplemental Table 2.

DISCUSSION

Exposure to iAs is a significant health concern as iAs can act as an endocrine disruptor that has been shown to interfere with the GR-signaling pathway.[11,39] In this study, we investigated the capacity of noncytotoxic doses of iAs to modulate GR-target gene expression in trophoblasts either through altered protein expression of the GR or through altering CpG methylation of GR targets. We demonstrate that iAs modulates the mRNA expression of select GR pathway genes in trophoblast cells with known involvement in placental growth and transport.[40−44] Importantly, in contrast to our *a piori* hypothesis, these gene expression effects were observed without any change in the overall cellular abundance of the GR-protein itself. Interestingly, these data show that iAs alters GR-gene specific CpG methylation in a manner consistent with changes observed at the transcriptional level. Overall, this study provides further evidence that iAs can interfere with the GR pathway in placental cells that is in part mediated by epigenetic modification.

Previous literature has demonstrated that iAs alters glucocorticoid receptor-target gene expression at environmentally-relevant doses.[45,46] GR target gene transcription in EDR3 cells, a human hepatocyte cell line, was increased at 0.5 μM and decreased at 3 μM doses of iAs, consistent with the data in trophoblasts presented here.[46] Specifically, our results demonstrated this biphasic dose−response relationship with seven GR-associated genes. These genes included *AQP1*, *MT2A*, *AFF1*, *SGK1*, among others, that are known play a role in placental cell growth and fetal growth and development.[40,41] Specifically, *AQP1*, a maternally-expressed gene that encodes for the water channel protein Aquaporin 1, is involved in placental growth and development.[40] Dysregulation of *SGK1*, a key regulator of sodium transport in human tissues, has been shown to result in errors in embryonic implantation and cell survival.[41] *PDGFRB* regulates 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.[44] Additionally, *MT2A* and *SLC22* regulate the trans-placental transfer of micronutrients critical for fetal development such as L-carnitine.[42,43] Importantly, the observed changes in GR-pathway associated gene expression were not a result of altered expression of the GR mRNA or protein as determined by RT-PCR and Western blot, respectively. Nevertheless, it is highly plausible that iAs-induced alteration of the GR-related genes may be due to downstream events such as nuclear translocation of GR, CpG methylation, or binding of GR to promoter regions. Future research will aim to investigate whether iAs treatment impacts these processes.

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We hypothesized that iAs potentially altered the expression of GR-associated genes through epigenetic modification. This hypothesis was based upon two key factors. First, iAs-induced alterations in DNA methylation influence mRNA gene expression of many genes in placental tissue and may mediate iAs-induced adverse health outcomes.[4,5,7,34] Second, iAs is known to impact the expression of the GR pathway in liver cells by affecting the ability of the GR to act as a transcription factor.[46] As a hypothesis related to this, when the transcription factor is bound, DNA methyltransferase cannot gain access to DNA, thereby influencing CpG methylation patterning and subsequent gene expression.[47,48] In support of an epigenetic mechanism mediating GR-associated gene expression, changes in DNA methylation levels at 65 CpG sites representing 12 GR-target genes were altered after iAs treatment. Interestingly, in three of the six genes that displayed a biphasic dose response in mRNA expression following iAs treatments, a biphasic dose response was also observed for DNA methylation. Specifically, for *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 increases in methylation relative to controls. It must be noted that the specific effect of DNA methylation on gene transcription may be site-dependent with some methylation marks being associated with increased expression, decreased expression, or no correlation with expression.[49,50] More 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.[6,51] In this study, iAs-induced changes in CpG methylation could be driven by either altered GR binding to promoter regions, as described above, or by direct inhibition of DNA methyltransferase by iAs, which has been previously reported.[47,52] Taken together, these findings suggest that DNA methylation plays a role in the biphasic dose−response observed of iAs-induced modulation of GR-signaling genes.

While this study is among the first to show that iAs alters GR-associated gene transcription through epigenetic modifications in trophoblasts, it is not without limitations. The JEG-3 trophoblast model cannot fully recapitulate tissue microenvironments. As such, the influence of other placental cells such as placental macrophages (Hofbauer cells) and fetal endothelial cells was not assessed. To confirm the specific biomolecular mechanisms of iAs toxicity proposed here, future studies will employ comprehensive models such as *ex vivo* placental explants and 3D *in vitro* cocultures. Furthermore, specific isoforms of GR were not investigated for their respective iAs responsiveness, and further studies will be needed to elucidate the effect of each isoform on the iAs transcriptional response.

In summary, this work contributes to the understanding of the mechanisms that underlie iAs-associated placental toxicity. We have shown that iAs alters the transcription of GR-associated genes in trophoblasts and that these effects may be driven by iAs-induced epigenetic modifications. Those genes responsive to iAs treatment have been previously associated with placental growth, nutrient transport, and hormone production, which together may have further implications toward fetal development. This work builds upon prior reports that iAs negatively impacts placental homeostasis and proposes that this is mediated by changes in GR signaling. Overall, these data highlight the ability of iAs to alter a crucial pathway involved in fetal and placental development and posits that adverse health effects associated with exposures to iAs during gestation may be mediated by modulation of the GR pathway in trophoblasts.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.8b00352.

All 85 genes on GR-signaling array and their associated *p*-values, fold change values, and standard error values; β differences and β values for all 12 identified genes that displayed significant ($p < 0.05$) alterations in DNA methylation and mRNA expression; full Western blot image of glucocorticoid receptor protein and β-actin loading control (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

ACTB, β-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 3 C member (glucocorticoid receptor); *PDGFRB*, platelet-derived growth factor receptor, beta; *PER2*, period homologue 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

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