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Prenatal Arsenic Exposure Associated with microRNA Expression in the Placenta

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Abstract

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Proper placental function is critical in fetal development and successful birth outcomes. Dysregulation of the expression of placental microRNA, post-transcriptional regulators of gene expression, can lead to the disruption of normal placental functionality and the potential programming of adverse birth and long-term health outcomes. These miRNA have also been shown to be sensitive to environmental stressors including trace toxic metals. Arsenic is an extremely common environmental toxicant that easily crosses the placental interface, where *in utero* arsenic exposure is associated with various adverse pregnancy outcomes. The molecular mechanisms underlying this association, however, are largely unexplored. To develop a better understanding of the influential role prenatal arsenic exposure plays in shaping placental microRNA expression and placental functionality, we performed miRNA sequencing and trace metal analysis data to study microRNAs in placentae from the Rhode Island Child Health Study (RICHS) (n=115). MicroRNA counts were regressed on $\log_2$-transformed placental arsenic using negative binomial generalized models, with miRNAs deemed to be differentially expressed with respect to arsenic at a False Discovery Rate of <0.10. We identified three placental miRNAs whose expression was significantly associated with $\log_2$-transformed placental arsenic concentrations. Utilizing bioinformatic target prediction software, we identified potential mRNA targets associated with these arsenic-sensitive placental miRNAs. Downstream pathway enrichment analyses identified some of these miRNA:mRNA targets as having potential roles in Rho-GTPase signaling as well as TNF binding. Overall, a robust association between placental miRNA expression and placental arsenic was identified, suggesting dysregulation of placental microRNAs may play a role in the developmental programming of adverse birth outcomes and lifelong risk of chronic disease.

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1. Introduction

The Developmental Origins of Health and Disease (DOHaD) hypothesis posits that exposure to environmental stressors during prenatal development, a time of crucial cell differentiation and tissue formation, has the potential to program long term health [1,2]. Early research in the DOHaD field utilized epidemiological data to identify links between both gestational malnutrition and low birth weight with adverse long-term health outcomes, including cardiovascular diseases, metabolic syndromes, and Type 2 diabetes, [3,4,5]. However, recent studies have sought to define associations between various prenatal chemical exposures and long-term health outcomes in support of the DOHaD hypothesis.

Arsenic is a naturally occurring contaminant of ground water and is one of many ubiquitous environmental chemicals that is easily transferred from mother to fetus throughout gestation by crossing the placental interface [6]. *In utero* arsenic exposure is associated with increased risk of adverse pregnancy and newborn health outcomes, including low birth weight and spontaneous abortions [7]. Perigestational arsenic exposure is also associated with long-term health outcomes of individuals exposed during pregnancy, such as liver, bladder, lung, and laryngeal cancers, cardiovascular disease, and respiratory diseases [7,8,9].

There is a growing body of evidence that has suggested perturbations to the epigenetic landscape during gestation may contribute to developmental plasticity. This epigenetic variation may, in turn, be responsible for the developmental programming of late onset disease [10]. These molecular disruptions are primarily facilitated by changes to epigenetic modes of gene regulation, such as DNA methylation, histone modifications, microRNA (miRNA) expression, and long non-coding RNA (lncRNA) expression [2,10]. High levels of exposure to arsenic *in utero* is known to alter epigenetic profiles associated with the expression of genes critical toward

fetal development, cell signaling, and neurodevelopment [11] and these molecular alterations may be implicated in the observed long term health outcomes associated with gestational arsenic exposure.

1.1 Role of the Placenta in Fetal Development:

Serving as the interface between fetal and maternal environments, the placenta plays an essential role in regulating fetal growth and metabolism and monitors the gestational environment to offer protection against potentially harmful gestational exposures [12]. The placenta continually develops throughout gestation and regulates many facets of fetal development through the expression of growth hormones, cytokines, and other signaling molecules. The vital nature of the placenta allows it to serve as a unique tissue by which to study the role of various environmental stressors during pregnancy and how they may influence both newborn and long-term health outcomes [13,14].

Environmental toxicants and heavy metals have the potential to perturb normal placental functionality, as well as the placental epigenetic landscape [13]. Additionally, changes in placental epigenetic profiles during early development are associated with alterations and impairments to placental function [15,16,17]. These environmentally-induced alterations or impairments to placental physiology may function to program adverse birth and long-term health outcomes as suggested by the DOHaD hypothesis [13]. Developing a better understanding of how placental epigenetic profiles respond to various environmental exposures, such as arsenic, and how these responses elicit changes to placental functionality could provide critical insight into the molecular mechanisms underlying the developmental origins of complex diseases [14].

1.2 Role of Placental miRNA in Fetal Development:

The alteration of placental microRNA (miRNA) expression is one epigenetic mechanism by which exposure to environmental toxicants can affect fetal development and potentially manifest as disease later in life [18,19]. MiRNA are small, noncoding RNA molecules that are capable of regulating mRNA expression by base pairing to the 3' untranslated region (UTR) of a target mRNA, resulting in the degradation or translational repression of that transcript. Alterations to placental miRNA expression are associated with adverse cell proliferation, growth and development changes, as well as disruption of normal placental functionality, and consequently, miRNA expression in the placenta is thought to play a vital role in regulating proper fetal development [18,20]. Circulating miRNAs with placental origins in maternal serum have been implicated as useful, accessible biomarkers of fetal congenital heart defects, suggesting the importance of miRNA as a tool when studying variability in gestational environments as it relates to pregnancy outcomes [21]. Studying the regulation of miRNA in the placenta may thus give critical insight into molecular mechanisms influencing fetal development and pregnancy outcomes.

Associations between placental miRNA dysregulation and various environmental exposures such as Bisphenol-A (BPA), an endocrine disrupting compound, and heavy metals including lead, and mercury have been identified, suggesting that miRNA dysregulation may occur in response to changes in gestational environments [16, 22, 23]. Various pregnancy outcomes, such as birth weight, are known to be sensitive to changes in the gestational environment. Additionally, our group has recently described a robust association between birth weight percentile and placental miRNA expression [31]. Thus, studying miRNA as they relate to

exposures in the gestational environment may provide insights into mechanisms underlying the environment's impacts on fetal development.

1.3 Arsenic and Placental miRNA:

Exposure to arsenic at low doses is extremely common, and it is estimated that the average American takes in 50 μg of organic arsenic and 2.5 μg of inorganic arsenic per day. Exposure primarily occurs through inhalation or the consumption of contaminated food and drinking water [24]. *In utero* arsenic exposure, even at low concentrations, has the potential to induce changes in placental functionality via multiple epigenetic mechanisms, including placental miRNA expression [25]. Recent studies conducted in Bangladesh, a country with historically high levels of arsenic contamination in drinking water, have found that *in utero* arsenic exposure has a strong effect on placental miRNA expression and is associated with extreme birthweight outcomes [26]. Significantly associated miRNAs were found to regulate the expression of genes regulating cell proliferation, and Insulin-like Growth Factor (IGF) signaling cascades among other biological pathways implicated in fetal growth [26]. This growing body of evidence suggests arsenic as an environmental toxicant that may alter placental miRNA expression profiles and induce changes in fetal development, ultimately contributing to the developmental programming of long-term health outcomes.

1.4 Study Objectives:

In this study, high-throughput data analysis was used to identify placental miRNAs whose expression is associated with placental arsenic concentrations. Placental miRNA sequencing data, as well as trace metal analysis, were collected from 115 mother-infant pairs from the Rhode Island Child Health Study (RICHS) cohort. Regression analyses were used to define associations

between placental miRNAs and placental arsenic [27]. Further analysis using bioinformatic target prediction software was conducted to identify potential mRNA targets of differentially expressed miRNAs [28]. Following target prediction, gene ontology and pathway enrichment analyses were performed to understand the potential biological pathways disrupted by changes in expression of arsenic-associated miRNAs. Characterizing placental miRNAs with arsenic-associated expression profiles will provide novel insight into the mechanisms by which prenatal arsenic exposure affects placental functionality and fetal development [16].

2. Methods

2.1 Study Population

The Rhode Island Child Health Study (RICHS) is a birth cohort of singleton, term pregnancies from the Women & Infants Hospital in Providence, Rhode Island enrolled between September 2010 and February 2013. Written informed consent was provided by all participants, and all protocols were approved by the Institutional Review Boards at the Women & Infants Hospital of Rhode Island and Emory University. Mothers enrolled in the RICHS cohort were all over the age of 18, healthy with no life-threatening illnesses or conditions, and had delivered infants at ≥37 weeks gestation with no congenital/chromosomal abnormalities [30]. Infants born small for gestational age (SGA) ($\leq 10^{th}$ BW percentile) and large for gestational age (LGA) ($\geq 90^{th}$ BW percentile) were oversampled, and infants adequate for gestational age (AGA) (between the 10^{th} and 90^{th} BW percentile) that matched on sex, gestational age (± 3 days) and maternal age (± 2 years) were coincidentally enrolled [29]. Placental miRNA abundance data (n=230) and data for trace metals analysis (n=115) were taken from a subset of the mother-infant pairs recruited in the RICHS cohort [30].

Sociodemographic and lifestyle data for the RICHS cohort, as well as anthropometric and medical history data, were collected from interviewer-administered questionnaires and structured medical record reviews, respectively. Medical records were also reviewed for data on infant sex, birth weight, intrauterine growth restriction status, and gestational age at birth. SGA, LGA, and AGA classifications for size at birth were calculated as birthweight percentiles via the Fenton growth chart, accounting for gestational age, infant sex, birthweight (g), head circumference (cm), and birth length (cm) [31,32].

2.3 Placental Tissue Collection

Full-thickness sections of placental tissue samples free of maternal decidua were collected 2cm from the umbilical cord insertion site within two hours of delivery; sections were then immediately placed in RNAlater™ at 4℃ (Applied Biosystems, Inc., AM7020). After at least 72 hours at this temperature, samples were blotted dry, snap-frozen in liquid nitrogen, and then homogenized and stored at -80℃ until analysis [30]. Samples not treated in RNAlater and instead immediately frozen were used for trace metal analysis by ICP-Mass Spectrometry at the Dartmouth Trace Elements Analysis Core [33].

2.4 microRNA Isolation, Sequencing, Processing, and Quality Control

The Qiagen miRNeasy Mini Kit with on-column DNAse digestion and TissueLyserLT (Qiagen, Germantown, MD, USA) were used to extract total RNA from pulverized, homogenized placenta placenta tissue samples. Quantification and quality checks of the RNA were performed using a NanoDrop (Thermo Fisher, Waltham, MA, USA) and the Agilent RNA 6000 Nano kit (Agilent, Santa, Clara, CA, USA), respectively. After removal of ribosomal RNA

[34], and conversion to cDNA [35], transcriptome-wide sequencing of the single-end RNA was performed using the HiSeq 2500 platform [36]. Raw miRNA reads were trimmed after initial quality control with FASTQ software, and raw miRNA counts were obtained using *miRDeep2* [29, 37].

2.5 Data Normalizing and Filtering

Raw miRNA counts data were imported into *DESeq2* to be normalized for differential expression analysis [27]. The raw counts were filtered to include only miRNA that had at least 1 count per million in at least 10% of samples. 800 of the 2,656 miRNA transcripts loaded into *DESeq2* remained after filtering [27].

The filtered miRNA raw counts were then normalized by estimated dispersion and size factors. Normalized counts were then exported from *DESeq2* for Surrogate Variable Analysis (SVA). The Variance Stabilizing Transformation (VST) was used on the filtered, normalized miRNA counts to yield approximately normalized and $\log_2$ transformed abundances.

2.6 Statistical Analyses

2.6.1 Surrogate Variable Analysis

Potential unmeasured sources of variation were adjusted for using the *sva* package [38, 39, 40]. The filtered and normalized raw microRNA counts were imported into the *svaseq* function, which uses an iteratively re-weighted least squares algorithm to estimate surrogate variables based on control transcripts [30]. A full and a null model were used in the *svaseq* function, where the full model included the log2-transformed arsenic, gestational age, birth weight (g), infant race (coded as white or non-white), infant sex, flow cell lane number and RNA

integrity number (RIN). The null model was composed of all covariates from the full model while excluding placental arsenic. Three surrogate variables were generated from the normalized microRNA counts and utilized as covariates in the differential expression analysis to adjust for unknown sources of confounding.

2.6.2 Differential Expression Analysis

Negative Binomial Generalized Linear Models were constructed in *DESeq2* and were used to define associations between normalized miRNA transcript abundances and $\log_2$ transformed placental arsenic concentrations [27]. The normalized miRNA counts were regressed on $\log_2$-transformed placental arsenic, adjusting for covariates including gestational age (wks), birth weight (g), RIN, flow cell lane number, and three surrogate variables. MicroRNAs were considered to be differentially expressed with respect to placental arsenic concentrations at a false discovery rate (Benjamini-Hochberg adjusted) less than 10%.

2.6.3 Bioinformatic Target Prediction

Potential mRNA targets of arsenic-associated miRNAs were predicted using the miRNA Data Integration Portal (mirDIP), an online human microRNA-target prediction database which provides nearly 152 million human miRNA-target predictions from 30 different sources [28,30,41]. mirDIP generates an integrated score for each mRNA target prediction which combines confidence scores from all available predictions, and serves as an estimate of the potential of an miRNA:mRNA interaction [28,41]. Only gene targets identified to be expressed in RICHS placental mRNA sequencing data, with an integrated score greater than 0.4, were further utilized in pathway enrichment analyses.

2.6.4 Pathway Enrichment Analysis

mRNA targets that passed initial filtering were utilized in pathway enrichment over-representation analyses using the Consensus Path Data Base (CPDB). CPDB uses 12 separate pathway analysis databases to aggregate consensus data and predict biological pathways over-represented from user input gene lists [42]. Gene symbols from the mirDIP analysis were entered into CPDB, from which associated pathway-based sets as defined by pathway databases were determined. A gene library derived from parallel RICHS mRNA sequencing was used as a gene background for pathway analysis [32]. Only those genes expressed at 1 cpm in at least 10% of samples were included in this background library. Pathways were considered to be significantly overrepresented with a q-value<0.1 [42].

3. Results

The demographic characteristics of the 115 mother-infant pairs from the RICHS cohort with both miRNA sequencing and trace metals analysis data are summarized in Table 1. Placenta tissue samples analyzed in this study were collected from healthy mothers over the age of 18 who had full-term pregnancies (≥37 weeks gestation) and infants with no congenital or chromosomal abnormalities. The majority of mothers and infants in this subset of the RICHS cohort were white (72% of mothers and 64% of infants), and 53% of infants were male. The mean placental arsenic concentration in this subset of samples is 0.413 µg/L, ranging from 0.0 µg/L to 2.765 µg/L (Table 1).

3.1 Differential Expression Analysis

To assess the associations between placental miRNA expression and placental arsenic, differential expression analysis was performed. Negative binomial generalized linear models were constructed in *DESeq2* [29], in which normalized microRNA transcript abundances were

regressed on log_2-transformed placental arsenic, controlling for gestational age (wks), birth weight (g), RNA integrity number (RIN), flow cell lane number, and three surrogate variables. Of the 800 miRNA transcripts remaining after initial filtering and normalization, three microRNAs were found to be significantly associated with log_2-transformed placental arsenic at a False Discovery Rate (FDR) of <0.10 (hsa-miR-33a-5p, hsa-miR-24-5p, and hsa-miR-2277-5p) (Figure 1). miR-33a-5p and miR-24-5p had positive estimated log_2 fold changes of 0.497 and 0.145 respectively, while miR-2277-5p had a negative estimated log_2 fold change of -0.528 (Table S1).

Sensitivity analyses were performed to adjust for other potential covariates, including infant race (coded as white or non-white), infant sex, and both infant race and sex. The inclusion of race and sex in the model did not drastically change the effect size estimates of the arsenic associated miRNAs, suggesting the robustness of the associations and effect size estimates outlined in the original model (Figure S1).

3.2 Bioinformatic Target Prediction

The potential mRNA targets of the three placental arsenic associated microRNAs were bioinformatically predicted using the microRNA Data Integration Portal (mirDIP) [42, 43]. mirDIP initially predicted 2,563 mRNA targets of miR-33a-5p and miR-24-5p, but miR-2277-5p did not produce potential mRNA targets. Considering bioinformatic target prediction is prone to generating false positives because miRNA:mRNA interactions do not require perfect complementarity, we utilized parallel RICHS RNA sequencing data to further filter the predicted targets to only those mRNAs expressed within this dataset. Additionally, predicted targets were required to hold an integrated score >0.4 to be included in downstream pathway analyses. The

integrated score is statistically inferred from predicted confidence scores aggregated from numerous resources within miRDIP, and thus provides more accurate predictions of miRNA:mRNA interaction potential [28]. 296 of these predicted targets were found to be expressed in RICHS mRNA sequencing while also having an integrated score greater than 0.4. These 296 mRNA targets were then further utilized for pathway enrichment analyses (Table S2).

3.3 *Pathway Enrichment Analysis*

Pathway enrichment analysis of the 296 predicted mRNA targets of miR-33a-5p and miR-24-5p was performed using the Consensus Path Data Base (CPDB) [32,44]. Pathways related to Rho-GTPase inactivation, signaling transduction, and proinflammatory signaling were found to be over-represented (q-value<0.1) (Table 2).

4. Discussion

Prenatal arsenic exposure is extremely common and is associated with numerous adverse birth outcomes as well as various chronic cardiovascular, metabolic, and respiratory diseases [7,8,9]. Understanding the molecular mechanisms underlying these associations may provide important insight into the etiology of complex disease. This study sought to identify associations between placental arsenic concentrations and placental microRNA expression. This particular perturbation of the placental epigenetic landscape would represent one mechanism by which prenatal arsenic exposure could impact fetal development and the developmental programming of adverse birth and long-term health outcomes through the disruption of normal placental functionality.

Here, we have utilized high-throughput microRNA sequencing data from the RICHS cohort to identify placental miRNAs whose expression is associated with placental arsenic

concentrations. The expression of three miRNAs were found to be significantly associated with log_2-transformed placental arsenic, miR-33a-5p (log_2 fold change=0.497, FDR=0.035), miR-24-5p (log_2 fold change=0.145, FDR=0.035), and miR-2277-5p (log_2 fold change=-0.528, FDR=0.035) (Figure 1, Table S1). Both miR-33a-5p and miR-24-5p have been implicated in the pathogenesis of diverse cardiovascular, cerebrovascular, and metabolic diseases. miR-33a-5p is associated with the pathogenesis of dyslipidemia and atherosclerosis, and miR-24-5p is associated with the pathogenesis of insulin resistance, diabetes, myocardial infarction, heart failure, as well as preeclampsia [43, 44]. However, the functional role of all three significantly associated miRNAs in placental functionality and physiology have been critically understudied.

There are several mechanisms by which arsenic may affect placental miRNA expression. Arsenic is known to inhibit complex I in the electron transport chain of the mitochondria, leading to excessive ROS generation, lipid peroxidation, and opening of the mitochondrial transition pore, subsequently leading to further mitochondrial damage and apoptotic effects [45]. It has previously been shown that arsenic holds this ROS-generating ability in the placenta, which highlighted an increase in intracellular H_2O_2 levels and upregulated expression of antioxidative enzymes in placental choriocarcinoma cells in response to inorganic arsenic exposure [46]. Other work has also outlined miRNAs whose expression was dysregulated by ROS, specifically those generated in response to arsenic exposure [47, 48]. Thus, a potential mechanism of action by which dysregulation of placental miRNA expression is mediated by arsenic exposure is through the generation of excess ROS throughout gestation, however, follow-up- analyses should be conducted to further elucidate this relationship within RICHS placentae specifically.

Pathway overrepresentation analyses of the potential mRNA targets of arsenic-associated miRNAs largely suggested involvement in the regulation of Rho-GTPases CDC42 and RAC1, as

well as binding of pro-inflammatory TNFs to their physiological receptors. (Table 2). CDC42 and RAC1 are known modulators of actin cytoskeleton development and dysregulation of their expression is associated with the impairment of placental development and several critical processes in placental physiology and functionality such as angiogenesis, vasculogenesis, and trophoblast proliferation [49]. Pro-inflammatory TNFs play an important role in inflammation response, cellular growth and development-related cascades within the placenta, and their regulation is thus critical in normal placental and fetal development [50]. Dysregulation of CDC42, RAC1, and TNFs are each associated with various adverse birth and pregnancy outcomes, such as preeclampsia, preterm birth, intrauterine growth restriction, low birth weight, and spontaneous abortion, further highlighting the suggested contributions of the miRNAs and mRNA targets identified in this study to these arsenic-associated health outcomes. [51, 52, 53].

Placental arsenic concentrations have been found to inhibit angiogenesis, vasculogenesis, and has been implicated in numerous adverse birth outcomes such as preeclampsia, preterm birth, low birth weight, and spontaneous abortion [54, 55, 7]. Furthermore, pre-and-postnatal arsenic exposure is associated with RAC1, CDC42, and TNF-α signaling and expression in various assays, corroborating the suggested role of placental arsenic concentrations and dysregulation of associated placental miRNAs in Rho-GTPase and pro-inflammatory signaling pathways [54, 55]. Thus, adverse birth outcomes and the development of chronic diseases associated with prenatal arsenic exposure may, in some part, be due to the effects of RAC1, CDC42, and TNF signaling pathway dysregulation, potentially initiated by the dysregulation of placental miRNAs

Here we have identified a novel association between the expression of miR-33a-5p, miR-24-5p, and miR-2277-5p in the placenta, and placental arsenic concentrations. However, limitations in this study must be accounted for when interpreting these results. Although the specificity of

arsenic exposure is a strength in this study, the potential dysregulation of placental miRNA by other environmental toxicants, and potential interaction between these toxicants and arsenic, were not studied. Additionally, although the consistently low arsenic exposure in all participants may be representative of general population exposure, it does limit the exposure contrast examined. Larger variation in placental arsenic concentrations may have allowed for the identification of additional differentially expressed placental miRNAs that may be more sensitive to prenatal arsenic exposure. Furthermore, although associations between arsenic exposure and the upregulation of miRNAs in cord blood have previously been reported, suggesting the plausibility of the dysregulated miRNA expression observed in this study, changes to miRNA expression in response to arsenic exposure have only recently been described in a placental context [57]. The cohort in this study only includes live, full term pregnancies, excluding severe or terminal effects of arsenic that are ultimately excluded and could bias results. Additionally, given the nature of human-based placenta research, temporality and causality between placental arsenic concentrations and placental miRNA expression cannot be established from these results.

5. Conclusion

Dysregulation of placental microRNA, post-transcriptional regulators of gene expression, can lead to the disruption of normal placental functionality, and the programming of adverse birth and long-term health outcomes. Environmental toxicants have the potential to perturb the placental epigenetic landscape by dysregulating placental miRNA expression, thereby contributing to the onset of adverse birth outcomes and chronic disease risk. In this study, the expression of three placental miRNAs were found to be robustly associated with placental arsenic concentrations in term placentae. Pathway over-representation analyses identified a

potential role of mRNA targets of these arsenic-sensitive placental miRNAs in RAC1, CDC42 signaling and TNF binding, suggesting the influence of these targets in placental development and functionality. Arsenic may thus contribute to adverse birth, pregnancy, and long-term health outcomes via dysregulation of placental miRNA.

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Tables and Figures

Table 1: RICHS Cohort Demographics (n=115)

Maternal Characteristics	
Race (white)- *% (n)*	72% (83)
Mean Maternal Age (yrs)- (*range)*	30 (18-40)
Delivery Method (vaginal)- *% (n)*	47% (54)
Infant Characteristics	
Race (white) -% (*n)*	64% (74)
Sex (male) -% *(n)*	53% (61)
Mean Gestational Age (wks) - (*range)*	39 (37-41.3)
Mean Birthweight (g)- *(range)*	3610 (2160-5465)
Mean Placental As (µg/L)- *(range)*	0.413 (0-2.765)
Birthweight Group	
Small for Gestational Age- *% (n)*	16% (18)
Average for Gestational Age- % (n)	45% (52)
Large for Gestational Age- % (n)	39% (45)

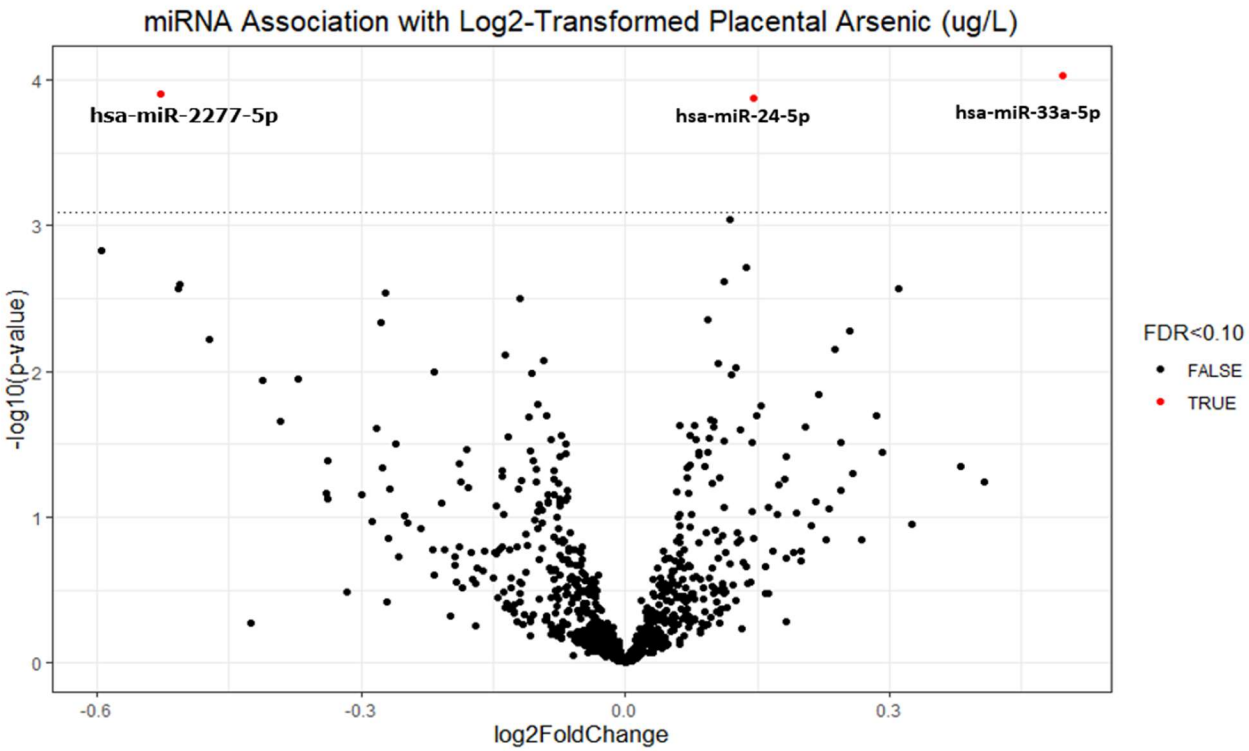


Figure 1: Placental miRNA is associated with log_2-transformed placental arsenic concentrations (µg/L). Volcano plot representing the results of the differential expression analysis. Normalized miRNA counts regressed on log_2-transformed placental arsenic and controlled for gestational age (wks), birth weight (g), and technical and surrogate variables. The x-axis displays the effect estimates of the microRNA transcript abundance per doubling of placental arsenic in units of log_2 fold change, and the y-axis displays the -log10(p-value) associations of each microRNA with placental arsenic. The dashed line represents the Benjamini-Hochberg adjusted FDR significance threshold (FDR = 10%). 3 microRNAs were found to be significantly (FDR<0.10) associated with log_2-transformed placental arsenic.

Table 2: Target Pathway Set Over-Representation Analysis Results

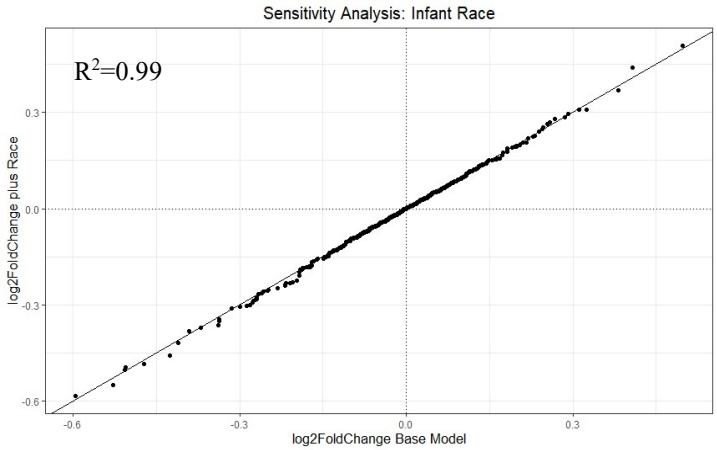
Enriched Pathway-based Set Name	Set size (Effective Set Size)	Candidate Genes Contained (%)	p-value	q-value	Pathway Source
Signal Transduction	2647 (1768)	60 (3.4%)	7.88E-05	0.040	Reactome
Axon guidance	358 (310)	18 (5.8%)	1.05E-04	0.040	Reactome
Inactivation of CDC42 and RAC1	8 (7)	3 (42.9%)	3.11E-04	0.067	Reactome
TNFs bind their physiological receptors	29 (17)	4 (23.5%)	3.80E-04	0.067	Reactome
cGMP-PKG signaling pathway - Homo sapiens (human)	163 (131)	10 (7.6%)	4.67E-04	0.067	KEGG
CDC42 signaling events	71 (67)	7 (10.4%)	5.23E-04	0.067	PID

Supplemental Materials

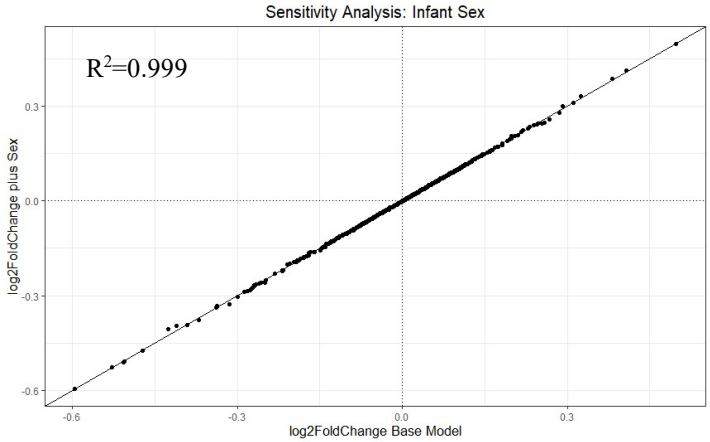
Table S1: Differential Expression Analysis Output (not shown)

Table S2: mRNA Targets Predicted by mirDIP (not shown)

(A)



(B)



(C)

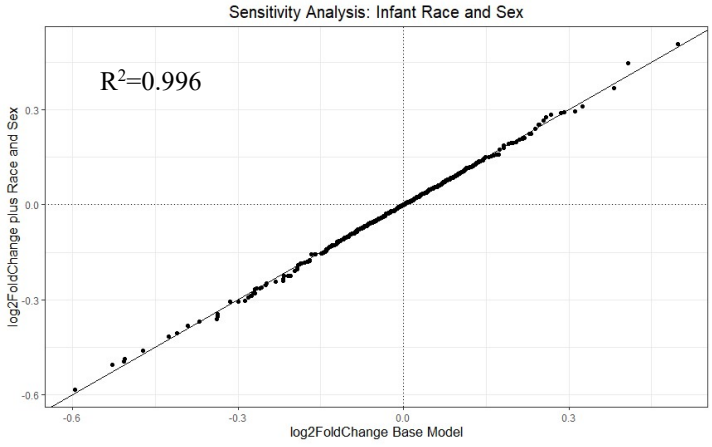


Figure S1: Effect estimates are highly robust on a transcriptome-wide scale. Scatter plots depicting the coefficient of determination (R^2) to emphasize the relationship of effect size estimates between the original (base)

model and various models which correct for additional covariates. (A) Effect size estimates are highly robust to the inclusion of infant race as a covariate (R^2=0.99) (B) infant sex (R^2= 0.999) (C) as well as both infant race and sex simueltaneously (R^2=0.996).