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Infant sex-specific placental cadmium and DNA methylation associations

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Ethics statement

The Swedish Medical Center Institutional Review Board for human subjects research reviewed and approved study protocols, prior to study conduct, and all participants provided written informed consent.

Disclosure of potential conflicts of interest

The authors have no conflicts of interest to disclose.

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Abstract

Background—Recent evidence suggests that maternal cadmium (Cd) burden and fetal growth associations may vary by fetal sex. However, mechanisms contributing to these differences are unknown.

Objectives—Among 24 maternal-infant pairs, we investigated infant sex-specific associations between placental Cd and placental genome-wide DNA methylation.

Methods—We used ANOVA models to examine sex-stratified associations of placental Cd (dichotomized into high/low Cd using sex-specific Cd median cutoffs) with DNA methylation at each cytosine-phosphate-guanine site or region. Statistical significance was defined using a false discovery rate cutoff (<0.10).

Results—Medians of placental Cd among females and males were 5 and 2 ng/g, respectively. Among females, three sites (near ADP-ribosylation factor-like 9 (ARL9), siah E3 ubiquitin protein ligase family member 3 (SIAH3), and heparin sulfate (glucosamine) 3-O-sulfotransferase 4 (HS3ST4) and one region on chromosome 7 (including carnitine O-octanoyltransferase (CROT) and TP5S target 1 (TP53TG1)) were hypomethylated in high Cd placentas. Among males, high placental Cd was associated with methylation of three sites, two (hypomethylated) near MDS1 and EVI1 complex locus (MECOM) and one (hypermethylated) near spalt-like transcription factor 1 (SALL1), and two regions (both hypomethylated, one on chromosome 3 including MECOM and another on chromosome 8 including rho guanine nucleotide exchange factor (GEF) 10 (ARHGEF10)). Differentially methylated sites were at or close to transcription start sites of genes involved in cell damage response (SIAH3, HS3ST4, TP53TG1) in females and cell differentiation, angiogenesis and organ development (MECOM, SALL1) in males.

Conclusions—Our preliminary study supports infant sex-specific placental Cd-DNA methylation associations, possibly accounting for previously reported differences in Cd-fetal growth associations across fetal sex. Larger studies are needed to replicate and extend these findings. Such investigations may further our understanding of epigenetic mechanisms underlying maternal Cd burden with suboptimal fetal growth associations.

Keywords

placenta; cadmium; DNA methylation; infant-sex; fetal growth

1. Introduction

Cadmium (Cd), a heavy metal that is widely used in industrial and agricultural settings, accumulates in the environment and has been associated with adverse health outcomes in

adults (Hellstrom et al., 2001; Menke et al., 2009). Also, a growing number of studies suggest that maternal Cd burden is associated with reduced fetal growth including lower birth weight (Ikeh-Tawari et al., 2013; Kippler et al., 2012a; Llanos and Ronco, 2009), shorter birth length (Ikeh-Tawari et al., 2013; Nishijo et al., 2004b; Zhang et al., 2004), and smaller head circumference (Ikeh-Tawari et al., 2013; Kippler et al., 2012a; Kippler et al., 2012b; Lin et al., 2011). Further, infant sex-specific differences in associations of maternal Cd burden with fetal growth indices have also been described (Kippler et al., 2012a; Kippler et al., 2012b). Investigators recently reported associations of higher maternal urine Cd with lower birth weight, smaller chest and head circumference in female infants only (Kippler et al., 2012a). These results parallel studies in adult populations that have described a higher body burden of Cd in females (Nishijo et al., 2004a) and end-stage kidney disease (Hellstrom et al., 2001) among females. Other investigators have reported Cd-related higher risk of cardiovascular diseases, cancer, and all-cause mortality among adult males (Menke et al., 2009). Mechanisms underlying observed differences in infant sex-specific associations of maternal Cd burden with fetal growth indicators are largely unknown.

During pregnancy, the placenta acts as an efficient, but partial, barrier to fetal Cd exposure, transferring as low as 10% of Cd (Kantola et al., 2000) from the mother to the fetus. Increasingly, investigators have suggested that the main mechanism by which maternal Cd burden influences fetal growth may be through its impact on the intrauterine environment (Henson and Chedrese, 2004; Kippler et al., 2010; Llanos and Ronco, 2009; Stasenko et al., 2010). Placental function disturbances subsequent to Cd exposure, including oxidative stress and disruption of endocrine function, may present a less than optimal intrauterine environment, influencing fetal programming, growth, and development (Almenara et al., 2013; Geary et al., 2003; Henson and Chedrese, 2004; Kantola et al., 2000; Kippler et al., 2010; Murphy et al., 2003; Ronco et al., 2009; Stasenko et al., 2010; Turgut et al., 2005; Vatten et al., 2002; Wang et al., 2012). DNA methylation, an epigenetic mechanism, may mediate the adverse consequences of maternal Cd on placental function and subsequent fetal growth (Cheng et al., 2012; Feil and Fraga, 2011). For example, there is growing evidence that DNA methylation profiles for placentas of growth restricted infants are distinct from profiles for placentas of appropriately grown infants (Hillman et al., 2014). Hypo- or hyper-methylation of placental DNA can lead to differences in expression of genes that are important in fetal growth, fetal growth restriction or other adverse developmental outcomes depending on the specific gene that is differentially methylated. To date, only one study, by Kippler et al., examined associations of maternal Cd burden, genome-wide DNA methylation, and infant birth weight and reported infant sex-specific associations of maternal peripheral blood and urine Cd and cord blood DNA methylation of several genes (Kippler et al., 2013).

To our knowledge, no previous study has investigated Cd-related DNA methylation in the placenta, a key component of the intrauterine environment. Therefore, our aim was to investigate infant sex-specific associations of placental Cd with genome-wide DNA methylation in the placenta. In a secondary analysis, we examined whether identified Cd-related placental DNA methylation was associated with infant birth weight.

2. Methods

2.1. Study setting and study population

The current study was conducted among participants of the Omega and Placental MicroArray studies (Enquobahrie et al., 2008; Meller et al., 2006). Briefly, the Omega study (1996–2008) is a prospective cohort study investigating risk factors of pregnancy complications among residents of the Pacific Northwest. Participants were recruited from women attending prenatal care clinics affiliated with Swedish Medical Center and Tacoma General Hospital. Women who initiated prenatal care before 20 weeks of gestation were eligible to participate. The Placental MicroArray study is a case-control study designed to examine differential placental gene expression that is related to pregnancy complications and included women who delivered at Swedish Medical Center.

Eligibility criteria for selection of participants (maternal-infant pairs) for the current study included the following: term delivery at Swedish Medical Center with available archived placental tissue, no history of chronic hypertension, or pre-pregnancy diabetes, singleton deliveries, and non-missing data for infant birth weight. For this pilot, a total of 24 participants were included, 8 participants randomly selected from the Omega study and 16 (all) controls from a previous preeclampsia gene expression study among Placental MicroArray study participants (Enquobahrie et al., 2008). The Swedish Medical Center Institutional Review Board for human subjects research reviewed and approved study protocols, prior to study conduct, and all participants provided written informed consent.

2.2. Data and sample collection

Trained personnel conducted medical records abstraction to obtain information on maternal socio-demographic and medical characteristics, and course and outcomes of pregnancy, including information on birth characteristics of the offspring. In addition, at-delivery, placenta samples were collected. Briefly, placenta specimens were double bagged, placed into coolers, and transported to a dedicated placenta-processing laboratory. After removing the chorionic plate and overlying membranes, biopsy samples (approximately 0.5 cm³ each) were collected from 16 sites (8 maternal and 8 fetal) adhering to a systematic technique to achieve uniformity and adequate sampling. Biopsy samples taken from the maternal side, that consist of villous tissue, uteroplacental arteries, and some decidua basalis, were evaluated for the current study. Abnormal morphology of these tissues has been associated with low birth weight and intrauterine growth restriction (Goldenberg et al., 2007; Sibley et al., 2005). Also, the placenta acts as a partial barrier to Cd leading to a potentially higher concentration of Cd on the maternal side (Esteban-Vasallo et al., 2012). Therefore, the Cd concentration of the maternal side of the placenta may be a more relevant measure of placental exposure to Cd and its effect. Biopsy samples were placed into cryotubes that contained RNAlater (Qiagen Inc, Valencia, CA) (Mutter et al., 2004), at 10 µL/mg of tissue and stored at –80°C until processing.

2.3. Placental Cd measurement

Briefly, 100 mg samples of placental tissue were thawed, partitioned, weighed and placed into 10 ml Teflon digestion vessels using acid-washed trace-metal free instruments and

vessels. Tissues were further prepared for Cd measurement by microwave-assisted closed vessel digestion with nitric acid (Krachler et al., 1997). Cd was quantified on an Agilent 7500 CE Inductively Coupled Plasma-Mass Spectrometer in helium mode using internal standard calibration. Terbium (Tb, 20 ng) was used as a recovery standard for digestion. Spike recovery was performed for 4 additional placental samples for quality control. The reporting limit of Cd for this procedure was 2 ng/g of placental tissue. Placental Cd measurements were conducted at the Environmental Health Laboratory and Trace Organics Analysis Center at the University of Washington.

2.4. DNA isolation and genome-wide DNA methylation profiling experiment

DNA was isolated using the QIAamp Tissue Kit (QIAGEN Inc. Chatsworth, CA). DNA purity was assessed by measuring OD260/280 and OD260/230 ratios. Samples with OD260/280 ratios >1.8 and OD260/230 ratios >2.0 were deemed of good quality DNA. DNA samples were archived at -80°C until further processing. DNA extraction procedures were conducted at the Center of Ecogenetics and Environmental Health Laboratories of the University of Washington. Isolated placental DNA samples were bisulphite-treated using EZ DNA Methylation Kit™, according to manufacturer's protocol (Zymo, D5001). Bisulphite-treated DNA was diluted with H₂O to 4 ul and hybridized to the Infinium HumanMethylation450KBeadChip (Illumina, San Diego, CA). Briefly, each array measures methylation of approximately 450K cytosine-phosphate-guanine (CpG) sites per sample and is comprehensive in genome-wide coverage, including 99% of RefSeq genes, and 96% of CpG islands with additional coverage in island shores and regions flanking them. Each probe is designed to interrogate individual CpG sites within a given DNA sample. Genome-wide DNA methylation profiling experiments were conducted at the University of Texas Southwest.

2.5. Data pre-processing

Data quality was checked using GenomeStudio™ Methylation Module software (2010.3). CpG sites (probes) with detection p-values > 0.01 in one or more samples were excluded. The raw data were imported using the Bioconductor minfi package. Raw intensity data were stratified by (type I or II), region (CpG Island, Shelf, Shore, or Other), and status (methylated or unmethylated) and sex, and then normalized using quantile normalization. After normalization, the intensity values were converted to β -values, which estimate the proportion of DNA that is methylated at each CpG site. Density bean plots (Kampstra, 2008) of methylation β -values were examined for data anomalies and abnormal skews. As a quality check, normalized data were visualized using a multi-dimensional scaling plot after excluding allosomes. Normalized intensities were converted to M values ($\log_2(\beta/(1-\beta))$) which can be interpreted as the log ratio between methylated and unmethylated probe intensities (Du et al., 2010).

2.6. Statistical analyses

Frequency distributions of maternal and infant characteristics for all participants and stratified by infant sex were examined. We defined an infant sex-specific placental Cd high/low cutoff value as greater than or equal to the median value specific to females or males. Two approaches were used to examine infant sex stratified associations of placental

Cd levels with placental genome-wide DNA methylation. The first approach used methylation of individual probes or CpG sites as the outcome while the second approach, a bump hunting method (Jaffe et al., 2012), used methylation over regions or clusters of probes as the outcome.

In the first approach, 482,422 separate ANOVA models were fit for each CpG site (stratified by infant sex) where methylation (M value) was the dependent variable, infant sex-specific Cd high/low status was the independent variable, and an indicator variable was included in the model for source population as an adjustment variable. FDR adjusted p-values (“q” values), to account for multiple testing, were obtained using the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). We used q value <0.10 as a cutoff to determine statistical significance.

The second or region analysis approach used the same infant sex stratified models as in the first set of analyses, with the M values as the dependent variable (averaged over probe clusters/regions with a maximum gap of 300 bases between contiguous probes within a region) and sex-specific Cd high/low status as the independent variable, and an indicator variable to adjust for source population. The Bioconductor bumpHunter package was then used to identify candidate peaks in the data that were consistent with differential methylation of a region. These candidate regions were then restricted to the 99th percentile of all such regions, and statistical significance was determined for each of the remaining candidate regions by permutation (1000 permutations). This second smoothed analysis approach utilizes the high correlation of methylation status for probes within the same region to minimize selection of a region based on a single probe.

In the secondary analyses, CpG sites or regions associated with Cd, with (FDR adjusted p-value) <0.1, were evaluated for their associations with infant birth weight. We first examined Spearman’s rank order correlation between methylation at each of these sites with birth weight, stratified by infant sex. Then we fit sex-stratified linear regression models examining associations of methylation at each site (or region) with birth weight. In these models, birth weight was the dependent variable, individual CpG site methylation or average methylation of all probes in the region (depending on the approach) was the independent variable, and models were adjusted for the source population (either the Omega study or the Placental MicroArray Study).

Analyses were conducted using Bioconductor and R packages.

3. Results

The majority of participants were non-Hispanic white (71%), married (83%), and nulliparous (63%) (Table 1). Mean (standard deviation) maternal age of participants was 31.3 (5.8) years. Median gestational age at delivery was 39 weeks. Mean birth weight was 3496 g for females and 3539 g for males. Median placental Cd was 5 ng/g (wet weight) for females and 2 ng/g for males. Placental Cd levels were <2 ng/g (the lower limit of detection) for 1 female infant and 6 male infants. In subsequent analyses, medians (5 ng/g for females and 2 ng/g for males) were used as cutoffs to determine high/low placental Cd groups.

Among female infants, in the individual probe analyses, we identified three differentially methylated CpG sites, all hypomethylated, in relation to high placental Cd levels. These sites were closest to ADP-ribosylation factor-like 9 (ARL9) (M value: -3.52 , false discovery rate (FDR) adjusted $p = 0.01$), siah E3 ubiquitin protein ligase family member 3 (SIAH3) (M value: -1.49 , FDR adjusted $p = 0.08$) and heparin sulfate (glucosamine) 3-O-sulfotransferase 4 (HS3ST4) (M value: -1.28 , FDR adjusted $p = 0.08$) genes (Table 2). In the genomic region analyses, among female infants, high placental Cd was associated with hypomethylation of one genomic region (region 86974674 to 86975244) on chromosome 7 which included genes carnitine O-octoyltransferase (CROT) and TP53 target 1 (TP53TG1) (FDR adjusted $p < 0.10$).

Among male infants, in the individual probe analyses, three CpG sites were differentially methylated in relation to placental Cd high/low levels. The two genes closest to the three CpG sites associated with high placental Cd in males were MDS1 and EVI1 complex locus (MECOM) (two hypomethylated sites, M values: -3.39 and -3.26 , FDR adjusted $p < 0.01$) and spalt-like transcription factor 1 (SALL1) (one hypermethylated site, M value: 2.34 , FDR adjusted $p = 0.08$). In the genomic region analyses, in male infants, high placental Cd was associated with hypomethylation of two genomic regions, one on chromosome 3 (region 169379554 to 169380078) and one on chromosome 8 (region 1792758 to 1792758), which included the MECOM and rho guanine nucleotide exchange factor (GEF) 10 (ARHGEF10) genes, respectively (FDR adjusted $p < 0.10$).

Among males, Spearman rank correlations (unadjusted) were marginally statistically significant for birth weight and methylation at CpG site and region related to MECOM (CpG site correlation -0.60 , $p = 0.04$, region correlation -0.64 , $p = 0.03$) and ARL9 (CpG site correlation 0.54 , $p = 0.07$), while respective correlations among female infants were weaker and not statistically significant (Table 3). However, among both male and female infants, Cd-related methylation at individual sites or genomic regions was not associated with infant birth weight.

4. Discussion

Our study provides suggestive evidence for infant sex-specific associations of placental Cd levels with placental DNA methylation. In female infants, high placental Cd (5 ng/g) was associated with hypomethylation of sites near ARL9, SIAH3, and HS3ST4, and regions including CROT and TP53TG1. In male infants, high placental Cd (2 ng/g) was associated with hypomethylation of two sites/regions near MECOM and ARHGEF10, and hypermethylation of a site near SALL1. Although we observed moderate correlations of methylation at some of these identified sites (e.g. MECOM and ARL9) and birth weight, particularly among male infants, associations were not statistically significant in this pilot study. Since the cutoffs for placental Cd were sex-specific and there was negligible overlap in the distributions of placental Cd in female and male infants, we were unable to contrast associations across infant sex.

To the best of our knowledge, this is the first study to investigate associations of Cd and DNA methylation in placental tissue, a target organ of Cd accumulation and toxicity and a

major component of the intrauterine environment. Kippler et al. investigated infant sex-specific associations of maternal Cd burden (characterized using measurements in urine collected at 8 weeks gestation and blood collected at 14 weeks gestation) with DNA methylation in mononuclear cells in cord blood among 127 maternal-infant pairs in Bangladesh (Kippler et al., 2013). In their study, the top 6 genes with Cd related-differential DNA methylation were G protein-coupled receptor 123 (GPR123)/tetratricopeptide repeat domain 40 (TTC40), solute carrier family 45, member 4 (SLC45A4) and general transcription factor IIA, 1 (GTF2A1), all hypomethylated, in female infants and tubulin folding cofactor D (TBCD), serine/threonine kinase 10 (STK10), and HRAS-like suppressor 2 (HRASLS2), all hypermethylated, in male infants. In that study, sex-specific pathway analyses suggested strongest associations of Cd with differential methylation for genes related to bone morphology and mineralization, and organ development in female infants and genes related to cell death among male infants. In the current study, identified genes among female infants included those that have been associated with cancer (HS3ST4 (Gorsi and Stringer, 2007), TP53TG1 (Takei et al., 1998), SIAH3 (Robbins et al., 2011)) while identified genes among male infants included those that have been associated with osteoporotic fracture (MECOM, (Hwang et al., 2013)) and kidney development (SALL1, (Nishinakamura et al., 2001)), outcomes or organs that have been related to Cd toxicity. However, genes identified in our study have not been previously linked to Cd, warranting future replication and/or mechanistic studies.

Some differences between the current study and the study by Kippler et al. deserve mention. We used maternal placental tissue for all investigations, whereas Kippler et al. examined Cd concentrations in maternal blood from 14 weeks gestation and DNA methylation of mononuclear cells from cord blood. Although Cd levels in maternal blood and placenta have been positively correlated, Cd concentration in placental tissue can be 10 times higher, since Cd accumulates in placenta (Kuhnert et al., 1982; Roels et al., 1978). Second, our assessment of DNA methylation in placental tissue may differ from the previous study's assessment of cord blood since DNA methylation can vary by tissue (Feil and Fraga, 2011). Third, evidence suggests that maternal Cd exposure was higher in the Kippler et al. study, where mean maternal urine concentration at 8 weeks gestation was 0.77 ug/L (5–95th percentiles: 0.25–2.40 ug/L) and among a subset of our study population, mean maternal urine concentration at 16 weeks gestation was 0.34 ug/L (5–95th percentiles: 0.13–0.63 ug/L). A study conducted among a similar population in Matlab, Bangladesh, reported median placental Cd of 20 ng/g wet weight, 4 times higher than our median placental Cd of 5 ng/g wet weight (Kippler et al., 2010). Kippler et al. noted that the higher maternal urinary Cd levels, compared to the much lower levels of urinary Cd reported by others (and similar to our current study) among young women the United States (geometric mean 0.27 ug/g creatinine) (Leventakou et al., 2014) and Europe (median, 0.31 ug/L) (García-Closas and Lubin, 1999), may be partially explained by the rice-based diet in Bangladesh, since rice tends to take up Cd from soil more readily than many other plants (Kippler et al., 2012a).

Sex-specific associations of Cd and fetal growth have been reported before. Kippler et al. reported that associations of higher maternal urine Cd (collected at 8 weeks gestation) and lower birth weight, head and chest circumference that were statistically significant ($p < 0.05$) in female infants only, although interaction p -values were not statistically significant

(Kippler et al., 2012a). The association of higher Cd with lower and higher birth length was observed for female and male infants, respectively (interaction $p = 0.11$). Romano et al., found associations of higher maternal urine Cd (collected at 16 weeks gestation) and lower birth length among female infants and higher birth length among male infants (p for interaction = 0.03) among 472 randomly selected maternal-infant pairs from the Omega study (unpublished, personal communication). Higher maternal Cd was also associated with higher ponderal index in females and lower ponderal index in males (p for interaction <0.01), similar to the observations reported by Kippler et al. In summary, these findings highlight the potential importance of evaluating sex-specific associations (and related mechanisms) of maternal Cd with fetal growth indices. While we only evaluated birth weight in the current study, future investigations of other indices of fetal growth are warranted.

While Cd-related DNA methylation changes in tissues, such as prostate and blood, have been described (Benbrahim-Tallaa et al., 2007; Kippler et al., 2013) whether sex-specific associations of placental Cd and fetal growth can be explained by epigenetic mechanisms is unknown. However, accumulating research supports the hypothesis that the placenta (a target organ of Cd accumulation and toxicity) mediates fetal programming and growth responses to environmental stressors in a sex dependent manner (Gabory et al., 2013). Studies suggest that placental responses to Cd that are associated with fetal growth, including placental 11β -hydroxysteroid dehydrogenase type 2 gene activity (Murphy et al., 2003; Yang et al., 2006) progesterone synthesis (Hartwig et al., 2013; Henson and Chedrese, 2004), and secretion of insulin like growth factors (Geary et al., 2003; Turgut et al., 2005; Vatten et al., 2002), may be sex dependent. Further, investigators have reported associations of cigarette smoke (Murphy et al., 2012) and arsenic (Pilsner et al., 2012) exposure and infant sex-specific differential methylation in cord blood. However, Cd and related sex-specific placental DNA methylation has not been investigated.

In the current study, we identified differential methylation of several target sites at or close to transcription start sites of novel genes whose functions play critical roles in cellular metabolism, growth, and development. MECOM encodes a zinc finger transcription factor that may be involved in hematopoiesis, cell cycle regulation, differentiation, proliferation, and embryogenesis, overexpression may be a predisposing factor of leukemia and osteoporotic fracture (Hwang et al., 2013; Wieser, 2007). The protein encoded by SALL1, also a zinc finger transcription factor, may regulate angiogenesis, vascularization, embryonic stem cell differentiation, and organogenesis, especially kidney development (Nishinakamura et al., 2001; Nishinakamura and Takasato, 2005; Yamamoto et al., 2010). ARL9 encodes a GTP binding member of the small GTPase protein family with a high degree of similarity to ARF proteins of the RAS superfamily. Genes SIAH3, HS3ST4, and TP53TG1 all regulate responses to cell damage (Gorsi and Stringer, 2007; Susini et al., 2001; Takei et al., 1998), which may have relevance to Cd-induced cell damage in placental tissue, although these genes have not been previously linked to Cd.

We performed follow-up analyses to evaluate associations of differentially methylated sites, identified by high/low Cd levels in our study, and genome-wide gene expression, using data from control participants ($n = 16$) of a previous preeclampsia gene expression study

(Enquobahrie et al., 2008), who also participated in the current study. For this set of models, we defined a 1Mb region around the CpG site in question (centered on the probe), and looked for genes within that region. Separate models examined associations of methylation status of the probe and gene expression. Increased methylation at three of the CpG sites that were associated with high (versus low) placental Cd in female infants were also associated with down-regulation of two genes: G protein-coupled receptor 160 (GPR160) (related to CpG-site-methylation near MECOM), HOP homeobox (HOPX) (related to CpG-site-methylation near ARL9) in placental tissue, $p < 0.05$ (Table 4). Of note Cd-related hypermethylation of a CpG site close to another member of the GPR gene family, G protein-coupled receptor 123 (GPR123), was the top hit among female infants in the study by Kippler et al. (Kippler et al., 2013). Related proteins that are encoded by the GPR gene family and facilitate transmission of signals across cell membranes and promote GDP-GTP exchange on the alpha subunit of G-proteins (Bertheleme et al., 2013). In males, expression of KIAA1211 (down-regulated, $p < 0.05$) was associated with CpG site methylation near ARL9. Little is known about the function of this gene.

We evaluated cross-sectional associations between placental cadmium and DNA methylation, therefore, we are limited to infer a causal relationship from the current study. Other study limitations that should be considered when interpreting our results include limited statistical power to detect sex-specific associations of Cd with genome-wide DNA methylation and fetal growth, due to sample size constraints. Since the cutoffs for placental Cd were sex-specific and there was negligible overlap in the distributions of placental Cd in female and male infants, we did not examine sex interactions. The clinical relevance of the placental Cd levels we observed in the current study is not clear. We are unaware of any guidelines that have been published for an acceptable or normal level for placental Cd. Authors of a review paper reported that for 46 studies of placental Cd conducted between 1976 and 2011, concentrations ranged from 1.2 ng/g to 53 ng/g wet weight (Esteban-Vasallo et al., 2012). Medians and arithmetic means were mostly < 20 ng/g. Based on this limited evidence, it seems that the Cd levels observed in our study were lower than or consistent with previous reports of placental Cd. There may be confounding from placental exposure to other heavy metals that have been previously related to reduced fetal growth, such as arsenic (Llanos and Ronco, 2009), mercury (Foldspang and Hansen, 1990), and lead (Xie et al., 2013) and that also have been correlated with placental Cd (Al-Saleh et al., 2011). Since most participants ($n = 19/24$) had placental mercury levels below the limit of detection (< 2 ng/g tissue), placenta mercury was not included in subsequent analyses. Therefore, we cannot completely rule out confounding by placental exposure to other xenobiotics or other potential factors. We did not examine associations of DNA methylation with other indices of fetal growth, such as birth length or head circumference, which have been related to maternal Cd burden previously (Ikeh-Tawari et al., 2013; Kippler et al., 2012a; Kippler et al., 2012b; Lin et al., 2011; Nishijo et al., 2004b; Zhang et al., 2004). Birth weight was highly correlated with birth length in female (Spearman $\rho = 0.75$, $p = 0.01$) and male (Spearman $\rho = 0.65$, $p = 0.02$) infants in our study. Future studies of associations of Cd related methylation and other fetal growth indices are warranted. Our study of mostly non-Hispanic white, married participants who initiated prenatal care early in pregnancy, may not generalize to women of other race/ethnicity or socioeconomic status.

Our study has several strengths. The placenta, a target organ of Cd accumulation and toxicity, plays a central role in the association between maternal Cd burden and fetal growth. Therefore, measurements of placental Cd are of particular relevance. Second, DNA methylation is tissue specific (Feil and Fraga, 2011) and use of placental tissue for genome-wide DNA methylation is pertinent for exploring mechanisms related to alterations to the intrauterine environment. We used a systematic sampling technique to collect placenta tissue from the maternal side to determine maternal Cd exposure since studies suggest that Cd may not be uniformly distributed across the placenta (Esteban-Vasallo et al., 2012). Fourth, we used two approaches to examine associations of placental Cd and genome-wide methylation (i.e. individual probes and genomic regions). Finally, we had an opportunity to examine associations between placental Cd-related DNA methylation and gene expression in a subset of the study population, allowing us to assess downstream effects of DNA methylation.

5. Conclusions

In summary, we found that among females, high Cd was associated with hypomethylation of SIAH3, HS3ST4, and TP53G1, genes that have been linked to response to cell damage (Gorsi and Stringer, 2007; Robbins et al., 2011; Takei et al., 1998). Among male infants, high Cd was associated with hypomethylation of MECOM, and hypermethylation of SALL1, genes which have been linked to cell differentiation (Hwang et al., 2013), angiogenesis, and organ development (Nishinakamura et al., 2001; Nishinakamura and Takasato, 2005). Our study provides suggestive evidence for sex-specific associations of placental-Cd with -DNA methylation of several genes. Larger studies, that are able to examine possible sex-interactions, may enhance our understanding of epigenetic mechanisms that potentially contribute to infant sex-specific associations and differences in associations of maternal Cd burden and fetal growth.

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Abbreviations

Cd	Cadmium
ARL9	ADP-ribosylation factor-like 9
SIAH3	siah E3 ubiquitin protein ligase family member 3
HS3ST4	heparin sulfate (glucosamine) 3-O-sulfotransferase 4
CROT	carnitine O-octanoyltransferase
TP53TG1	TP5S target 1
MECOM	MDS1 and EVI1 complex locus

SALL1	spalt-like transcription factor 1
ARHGEF10	rho guanine nucleotide exchange factor (GEF) 10
CpG	cytosine-phosphate-guanine
FDR	false discovery rate
GPR123	protein-coupled receptor 123
TTC40	tetratricopeptide repeat domain 40
SLC45A4	solute carrier family 45, member 4
GTF2A1	general transcription factor IIA, 1
TBCD	tubulin folding cofactor D
STK10	serine/threonine kinase 10
HRASLS2	HRAS-like suppressor 2
GPR160	G protein-coupled receptor 160
HOPX	HOP homeobox

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Highlights

- We examine sex-specific placental-Cd and -genome-wide DNA methylation associations.
- In females, associated sites were at/near genes involved in cell damage response.
- In males, associated sites were at/near angiogenesis and organ development genes.
- Our study supports infant sex-specific placental Cd-DNA methylation associations.

Table 1

Characteristics of mother-infant pairs, stratified by infant sex.

Characteristic	Female Infants ^a (n = 12)	Male Infants ^a (n = 12)
Maternal characteristics		
Non-Hispanic white race/ethnicity ^b	10 (83.3)	7 (58.3)
Maternal age (years)	33.3 ± 5.4	29.3 ± 5.7
Unmarried status	0 (0.0)	4 (33.3)
Nulliparous	8 (66.7)	7 (58.3)
Pre-pregnancy Body Mass Index (kg/m ²)	22.9 (20.2–39.5)	22.6 (18.0–38.7)
Urinary creatinine (g) ^b	82.0 (44.0–89.0)	57.0 (47.0–115.0)
Urinary Cd (ug/g creatinine) ^b	0.5 (0.2–1.0)	0.6 (0.2–0.8)
Maternal placental Cd (ng/g) ^c	5.0 (<2.0–7.0)	2.0 (<2.0–5.0)
Infant characteristics		
Gestational age at delivery (weeks)	39 (38–41)	39 (37–41)
Birth weight (g)	3496.4 ± 474.7	3538.8 ± 534.1

^aValues are shown as mean ± SD; median (range); or n (%).^bMaternal race missing for 1 male infant. Maternal urinary Cd and creatinine missing for 7 female and 7 male infants^cPlacental Cd includes 1/12 females and 6/12 males with levels below the lower limit of detection (<2 ng/g).

Table 2

Cadmium-high/low status and DNA methylation, stratified by infant sex.

CpG site or genomic region	M value ^a	FDR adjusted p-value	CHR	Closest gene or genes within region symbol/name
Female Infants				
cg04528060	-3.52	0.01	4	ARL9/ADP-ribosylation factor-like 9
cg00613224	-1.49	0.08	13	SLAH3/siah E3 ubiquitin protein ligase family member 3
cg03884018	-1.28	0.08	16	HS3ST4/heparan sulfate (glucosamine) 3-O-sulfotransferase 4
86974674-86975244	-1.82	0.06	7	CROT/carnitine O-octanoyltransferase TP53TG1/TP53 target 1 (non-protein coding)
Male Infants				
cg15958576	-3.39	<0.01	3	MECOM/MDS1 & EVII complex locus
cg10903116	-3.26	<0.01		
cg09728607	2.34	0.08	16	SALL/spalt-like transcription factor 1
169379554-169380078	-3.04	0.03	3	MECOM/MDS1 & EVII complex locus
1792758-1792758	-4.57	0.07	8	ARHGEF10/Rho guanine nucleotide exchange factor 10

Abbreviations: FDR, False Discovery Rate; CHR, Chromosome

^aM values can be interpreted as the log ratio between methylated and unmethylated probe intensities.

Table 3

Cadmium-related differential DNA methylation and infant birth weight, stratified by infant sex.

CpG site or genomic region	CHR	Closest gene	Methylation birth weight association β^a	p-value	Rho	p-value
Female Infants						
cg04528060	4	ARL9	-78.10	0.33	-0.06	0.86
cg00613224	13	SIAH3	-185.00	0.32	-0.13	0.70
cg03884018	16	HS3ST4	-291.00	0.17	-0.12	0.72
cg15958576	3	MECOM	-8.45	0.92	-0.33	0.30
cg10903116	3	MECOM	-4.48	0.95	-0.12	0.72
cg09728607	16	SALL1	-36.74	0.74	0.22	0.49
86974674-8697522	7	CROT/TP53TG1	-0.05	1.00	-0.15	0.65
Male Infants						
cg15958576	3	MECOM	-64.80	0.36	-0.48	0.12
cg10903116	3	MECOM	-66.40	0.37	-0.60	0.04
cg09728607	16	SALL1	70.20	0.48	0.27	0.39
cg04528060	4	ARL9	2.50	0.98	0.54	0.07
cg00613224	13	SIAH3	326.00	0.20	-0.11	0.73
cg03884018	16	HS3ST4	151.00	0.36	0.49	0.11
169379554-169380078	3	MECOM	-43.30	0.38	-0.64	0.03
1792758-1792758	8	ARHGEF10	-40.60	0.27	-0.30	0.34

Abbreviations: CHR, Chromosome

^a β = fold change in gene expression

Table 4

DNA methylation and gene expression, stratified by infant sex.

Gene/name	Gene ontology (molecular function)	CpG Site	Female Infants		Male Infants	
			β^a	p-value	β^a	p-value
HOPX/HOP homeobox	Sequence-specific DNA binding and transcription factor activity	cg04528060	-1.84	0.04	0.10	0.95
KIAA1211/KIAA121		cg04528060	-5.86	0.28	-9.65	0.04
CPB2/Carboxypeptidase B2	Metalloproteinase activity, zinc ion binding	cg00613224	0.58	0.05	-1.28	0.09
		cg15958576	-4.88	0.02	-0.86	0.74
GPR160/G protein-coupled receptor 160	G-protein coupled receptor activity	cg10903116	-5.38	0.03	-0.10	0.97
SEC62/SEC62 (S. cerevisiae)	Protein transporter & receptor activity	cg10903116	3.52	0.05	5.16	0.07

^a β = log2 fold change in gene expression for every two-fold increase in methylation