



RESEARCH ARTICLE

Placental lncRNA expression associated with placental cadmium concentrations and birth weight

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Abstract

Heavy metal exposures, such as cadmium, can have negative effects on infant birth weight (BW)—among other developmental outcomes—with placental dysfunction potentially playing a role in these effects. In this study, we examined how differential placental expression of long non-coding RNAs (lncRNAs) may be associated with cadmium levels in placenta and whether differences in the expression of those lncRNAs were associated with fetal growth. In the Rhode Island Child Health Study, we used data from Illumina HiSeq whole transcriptome RNA sequencing ($n = 199$) to examine association between lncRNA expression and measures of infant BW as well as placental cadmium concentrations controlled for appropriate covariates. Of the 1191 lncRNAs sequenced, 46 demonstrated associations ($q < 0.05$) with BW in models controlling for infant sex, maternal age, BMI, maternal education, and smoking during pregnancy. Furthermore, four of these transcripts were associated with placental cadmium concentrations, with *MIR22HG* and *ERVH48-1* demonstrating increases in expression associated with increasing cadmium exposure and elevated odds of small for gestational age birth, while *AC114763.2* and *LINC02595* demonstrated reduced expression associated with cadmium, but elevated odds of large for gestational age birth with increasing expression. We identified relationships between lncRNA expression with both placental cadmium concentrations and BW. This study provides evidence that disrupted placental expression of lncRNAs may be a part of cadmium's mechanisms of reproductive toxicity.

Key words: prenatal; cadmium; fetal growth; lincRNA; expression

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Introduction

Increasing scientific understanding of the intrauterine environment has highlighted the importance of prenatal conditions and environmental exposures in development and lifelong health (1). Maternal exposures to cadmium during pregnancy have been linked to infant and early childhood health outcomes including low birth weight (BW), reduced growth, immune system dysfunction, and cognition (2). Cadmium exposure occurs through multiple pathways, including contact with air and dust contaminated by industrial pollution, phosphate fertilizers, gasoline and diesel combustion and particles released by tire wear (3, 4). The highest levels of exposure, however, typically result from tobacco smoking and dietary consumption of contaminated foods such as mushrooms, shellfish, liver, and plant products (5).

Importantly, cadmium has been implicated to exert its toxic effects on pregnancy outcomes through placental damage (6), including impacts on cellular oxidative stress (7, 8), impaired calcium metabolism (9), endocrine disruption (10), interference with placental transfer of elements such as copper, iron, zinc, manganese and selenium to the fetus during pregnancy (11), and variations in gene-expression or DNA methylation, impacting or representing altered placental function (12–15). Given that the placenta is the critical interface between mother and fetus, performing the functions of the lung, liver, gastrointestinal tract, kidney and endocrine organs (16), exposures which can impact placental vascularization, growth, transport activity, metabolism and hormone production, can result in newborn outcomes which can have long-term implications in child health (17, 18). The placenta actively transports essential elements such as calcium, copper, zinc, and iron to the fetus (19), yet cadmium is partially sequestered to the placenta via increases in placental metallothionein (20, 21), which binds cadmium and limits its access to the fetal bloodstream.

Long non-coding RNAs (lncRNAs) are actively synthesized and regulated RNA transcripts that play cellular roles in homeostasis and pathogenesis throughout the body (22). Prior to the genomic era one of the only known examples of active lncRNA was *XIST*, an RNA transcript implicated in the dosage inactivation of one of the two X chromosomes in female cells (23), but now over 100 000 lncRNAs have been described in humans alone (24). In general, lncRNA transcripts measure >200 nucleotides with a median size of 500 (25), generally smaller than messenger RNAs (mRNAs). Around 98% are spliced, with 80% having 2–4 exons, and are polyadenylated like some mRNAs, though most show a relative absence of open reading frames >100 nt (26). lncRNAs are far more tissue-specific and expressed at lower levels than mRNAs (27), and can be divided into a number of different sub-classes based on their transcriptional locations and physical structure (28). Some lncRNAs bind histone modifiers or assemble with protein complexes at their sites of transcription, allowing for regulation of cell cycles, apoptosis, and identity (29–31). The largest sub-class of lncRNAs is long intergenic non-coding RNAs (lincRNA), which are >200 nucleotides in length and do not overlap with the coding regions of known coding genes; although the specific functions of most individual lincRNAs are still uncharacterized, lincRNAs overall are thought to be involved in tissue-specific gene-expression and may have widespread regulatory functions (32).

Within the placenta, some of the more well-characterized lincRNAs (*H19* and *LINC-HELLP*) have been shown to be involved in the regulation of trophoblast proliferation and placental growth (33–35), and a number of lncRNAs have been shown to

be differentially expressed in association with pregnancy complications or growth restriction (35). The capacity for toxic exposures to modulate lncRNA levels, including a number of lincRNAs, across the body has been demonstrated for numerous chemicals (36), thus differential expression of lincRNAs in the placenta may serve as indicators of toxicological response, be involved in the trophoblast alterations linked to cadmium exposures and adverse reproductive outcomes, and warrants further investigation in relation to prenatal exposures. To begin to examine the role that prenatal exposures can play in regulating the expression of lincRNAs in the placenta and the potential for variation in their expression to impact growth, this study examined the relationship between lincRNA expression levels, BW, and cadmium concentrations in human placental tissues.

Methods

The Study Population

The Rhode Island Child Health Study is a cohort of mother–infant pairs recruited from Women and Infants Hospital of Providence, RI, and oversampled for small for gestational age (SGA, ≤ 10 th percentile of BW) and large for gestational age (LGA, ≥ 90 th percentile of BW) infants based on a priori interest in fetal growth and development. Infants were matched to appropriate for gestational age newborns (AGA, 10% < BW percentile < 90%) on sex, gestational age (± 3 days) and maternal age (± 2 years) (13). Only singleton, term infants born at ≥ 37 weeks gestation without pregnancy complications and lacking congenital or chromosomal abnormalities were included; all mothers were over the age of 18 and spoke English as a language of consent (12). Data on gestational weeks, infant sex, and BW (g) were obtained via medical records abstraction. BW percentiles were calculated based on the Fenton growth charts accounting for gestational age and infant sex (37). An interviewer administered questionnaire was used to collect self-reported socio-demographic, lifestyle, and medical history data, followed by structured medical records review to collect anthropometric and clinical data. Placental RNA sequencing (RNA-seq) was performed on 200 placenta—with one sample excluded from analysis for sex mismatch—and placental metal concentrations were measured for 259 placenta, with an overlap of 85 placenta having both measures (12). All participants provided written informed consent, approved by the Institutional Review Boards at Women and Infants Hospital and at Emory University.

Placental Sampling

Full-thickness sections of placenta were obtained within 2 h of birth from the fetal side, 2 cm from the umbilical cord insertion site from four quadrants, cleaned of maternal decidua and were immediately placed in RNAlater TM (Applied Biosystems, Inc., AM7020) and held at 4°C. After at least 72 h at this temperature, samples were blotted dry and snap-frozen in liquid nitrogen, followed by homogenization and storage at -80°C until analysis. For metals determination, placenta samples were stored immediately at -80°C following collection. Details of placental cadmium quantification are described elsewhere (38). Briefly, metals quantification was performed at the Dartmouth Trace Elements Analysis Core with inductively coupled plasma mass spectrometry and none of the placental samples yielded non-detectable Cd concentrations.

RNA Sequencing

Total RNA was isolated from placental samples using the RNeasy Mini Kit (Qiagen, Valencia, CA) and stored in RNase-free water at -80°C (12). Yields were quantified using a Qubit Fluorometer (Thermo Scientific, Waltham, MA) and an Agilent Bioanalyzer was used to assess integrity (Agilent, Santa Clara, CA). Ribosomal RNA was removed using a Ribo-Zero Kit (39), with the remaining RNA converted to cDNA using random hexamers (Thermo Scientific, Waltham, MA). The HiSeq 2500 platform (Illumina, San Diego, CA) (40) was used to assess transcriptome-wide 50 bp single-end RNA-seq, which was conducted in three sequencing batches and samples were randomized across batches; 10% of samples within each batch were run in triplicate.

QC Filtering and Normalization

Raw RNA-seq data were quality assessed for both read length and GC content using the FastQC software, with quality reads mapped to the hg19 human reference genome using the Spliced Transcripts Alignment to a Reference aligner (41). This technique masked single nucleotide polymorphisms (SNPs) in the reference genome prior to alignment, and transcripts with counts <1 per million across 30 or more samples were filtered out (12). The EDASeq R package (42) was used to adjust for GC content, and the calcNormFactors function from the edgeR R package (43) subsequently used for Trimmed Mean of M-values (TMM) correction to adjust for differences in library sizes across samples. The limma R package's voom function (44) was used to transform the data into \log_2 counts per million (logCPM) values. Duplicate samples were removed. Next, any logCPM-normalized counts below 2 on a \log_2 scale in a minimum of 30 samples were excluded. The final filtered, normalized data set included 12 135 transcripts, of which around 3000 were unique non-coding transcripts and 1191 were classified as lincRNAs using Ensembl (hg19) which were selected for this study.

Differential Expression of lincRNA with BW

The association between placental lincRNA expression and BW was assessed using linear models and the Benjamini-Hochberg false discovery rate (FDR) to control for multiple testing. Grams of BW were regressed on log-transformed lincRNA expression ($n=199$), while controlling for gestational age, infant sex, maternal age, maternal BMI and maternal smoking during pregnancy (45–47). Figures were generated using the ggplot2 package (48). All analyses were conducted within R 3.4.1 (49).

Transcripts that were differentially expressed with BW (FDR $< 5\%$) were also modeled using logistic regression to determine their associations with the extremes of BW. We estimated the odds of SGA and LGA compared to AGA (the referent), adjusted for known factors associated with BW outcomes that are not utilized in the derivation of the Fenton classifications: maternal age, highest education level—as an indicator of socioeconomic status—maternal pre-pregnancy BMI and smoking status during pregnancy. We then tested whether including a three-level batch variable altered our observed associations.

Differential Expression of lincRNA with Cadmium

Amongst the transcripts demonstrating an association with BW (FDR, $q < 0.05$), we then tested the associations between lincRNA

expression and log-transformed placental cadmium ($n=85$) using limma while controlling for potential confounders including maternal smoking status—as the primary source of cadmium exposure during pregnancy (50)—and infant sex, again using the Benjamini-Hochberg FDR to control multiple testing. Maternal education was highly correlated with smoking status within the cohort and thus removed from models due to collinearity issues. We then tested whether including a three-level batch variable altered our observed associations.

Correlations of lincRNA Expression with Placental Co-expression Modules

To gain insights into the potential roles that the BW- and cadmium-associated lincRNAs may play in placental functions, we tested the correlations between the expression levels of these lincRNAs with the eigengenes from our previously published gene co-expression network (12). We used Spearman's rho to test for correlations, and constructed a pairwise correlation network of all correlations that yielded P -values < 0.05 with visANT (51).

Results

Study Population

The demographics of the population with available lincRNA data and those with placental metals are shown in Table 1. Average gestational age was consistent across all BW categories around 39 weeks [mean (standard deviation): LGA = 39.4 (0.8); SGA = 39.1 (1.1); AGA = 39.4 (1.0)]. Consistent with existing literature (52), a greater percentage of LGA infants in the cohort were male (0.64) and a greater proportion of SGA infants were female (0.77), with AGA infants close to evenly represented across sexes. The majority of participants (76%) were born to Caucasian mothers, though SGA births were more common among black mothers (17%), which is consistent with previous literature (53). The distributions of these demographic characteristics were largely consistent between the 199 samples with RNA-seq and with the 85 samples in which placental cadmium concentrations were also measured.

lincRNA Expression Associations with BW and Cadmium

Of the 1191 lincRNAs tested, 46 demonstrated associations with BW at an FDR, $q < 0.05$, while controlling for infant sex and maternal age, BMI, education level, and smoking during pregnancy (Table 2). For the majority of these lincRNAs, 40, higher expression was associated with larger BW (Fig. 1). The magnitudes of associations varied widely among these 46 lincRNAs, with a fold change in MIR22HG on chromosome 17 associated with the largest decrease in BW (-462.7 g) and JPX on the X chromosome associated with the largest increase in BW (558.7 g). The results from all 1191 linear models of BW and lincRNA expression are available in Supplementary Table S1. Additional adjustment for batch effects did not alter the observed estimates of differential expression at the 46 identified lincRNAs (Supplementary Fig. S1A). We also examined how the expression of these lincRNAs associated with the extremes of BW by estimating the odds of LGA and SGA births, relative to AGA births, in relation to higher expression of the lincRNAs that were significantly associated with continuous BW (Supplementary Fig. S1). Higher expression of eight lincRNAs significantly increased the odds ($P < 0.05$) of LGA while three lincRNAs were significantly associated with

Table 1: Demographic characteristics of the study populations across birth size categories for analyses of lincRNA vs BW and lincRNA vs cadmium

	lincRNA vs BW (n = 199)			lincRNA vs Cd (n = 85)		
	SGA ^a (n = 30)	AGA ^b (n = 110)	LGA ^c (n = 59)	SGA ^a (n = 15)	AGA ^b (n = 38)	LGA ^c (n = 32)
Continuous	Mean (SD)			Mean (SD)		
BW (g)	2577 (368.1)	3394 (363.0)	4270 (241.2)	2663 (463.3)	3314 (386.6)	4251 (245.8)
Gestational age (weeks)	39.1 (1.1)	39.4 (1.0)	39.4 (0.8)	39.1 (1.3)	39.2 (1.0)	39.3 (0.8)
Maternal age (years)	31.5 (5.8)	31.1 (4.7)	31.4 (4.0)	29.3 (6.7)	30.8 (5.5)	30.7 (4.1)
Maternal BMI (kg/m ²)	25.6 (6.9)	25.6 (6.0)	28.2 (6.7)	24.8 (5.5)	24.4 (6.1)	28.8 (6.8)
Categorical	N (%)			N (%)		
Infant sex						
Female	23 (77)	57 (52)	21 (36)	12 (80)	18 (47)	12 (38)
Male	7 (23)	53 (48)	38 (64)	3 (20)	20 (53)	20 (62)
Maternal ethnicity						
Caucasian	16 (53)	88 (80)	50 (85)	7 (47)	27 (71)	25 (78)
Black	5 (17)	3 (3)	3 (5)	2 (13)	2 (5)	3 (9)
Other/unknown	9 (30)	19 (17)	6 (10)	6 (40)	9 (24)	4 (13)
Maternal education						
≥ Junior college	25 (83)	93 (85)	52 (88)	11 (73)	29 (76)	28 (87)
≤ High school	5 (17)	17 (15)	7 (12)	4 (27)	9 (24)	4 (13)
Smoking during pregnancy						
Yes	3 (10)	7 (6)	7 (12)	1 (7)	4 (11)	3 (9)
No	27 (90)	103 (94)	52 (88)	14 (93)	34 (89)	29 (91)

^aSmall for gestational age.

^bAverage for gestational age.

^cLarge for gestational age.

SD, standard deviation; Cd, cadmium.

odds of SGA. Only one lincRNA was significantly associated with both SGA and LGA, AP000766.1 located on chromosome 11, in which higher expression was associated with decreased odds of SGA [OR = 0.52, 95% CI = (0.30, 0.92)] and with increased odds of LGA [OR = 2.04, 95% CI = (1.23, 3.27)]. Overall, the greatest odds of LGA was associated with AC008543.1 on chromosome 19 [OR = 2.34, 95% CI = (1.25, 4.39)], while the lowest odds of LGA was associated with ERVH48-1 on chromosome 21 [OR = 0.38, 95% CI = (0.18, 0.81)] (Supplementary Fig. S2). However, the greatest odds of SGA was associated with OSER1-AS1 on chromosome 20 [OR = 3.19, 95% CI = (1.30, 7.82)] while lowest odds of SGA was associated with HCG11 on chromosome 6 [OR = 0.05, 95% CI = (0.01, 0.21)].

We aimed to reduce the multiple testing burden for the analyses with cadmium for which we had a smaller sample size (n = 85) and thus the 46 BW-associated lincRNAs were subsequently examined for their associations with placental cadmium concentrations. These models identified four lincRNAs (MIR22HG, AC114763.2, ERVH48-1, and LINC02595) that were nominally significantly ($P < 0.05$) associated placental cadmium concentrations (Table 3). Estimates for these associations ranged from -1.54 to 0.47 log fold change expression per log cadmium increase, with MIR22HG exhibiting the strongest association ($\beta = 0.40$, $P = 0.002$, FDR, $q = 0.074$). The results from all 46 models assessing the relationships between lincRNA expression and placental cadmium concentrations are provided in Supplementary Table S2, while we additionally highlight the odds of SGA and LGA associated with the increasing expression of these lincRNAs (Fig. 2). Additional adjustment for batch effects did not alter the observed estimates of differential expression at the four Cd-associated lincRNAs (Supplementary Fig. S1B).

Correlations of LincRNAs with Placental Gene-Expression Modules

Finally, we aimed to determine the potential cellular pathways and functional networks impacted by lincRNA variation that may play roles in growth regulation and responsiveness to prenatal environmental exposures such as cadmium. Thus, we examined the relationships between lincRNA expression levels (MIR22HG, AC114763.2, ERVH48-1, and LINC02595) with the activities of placental gene co-expression modules from our lab's prior work (12). These four lincRNAs, that exhibited differential expression associated with both cadmium and BW, demonstrated moderate to strong correlations with a number of the eigengene values representing previously reported placenta co-expression modules (Fig. 3, Supplementary Table S3). The strongest correlations were observed between LINC02595 with the gene module involved in regulation of RNA splicing ($\rho = 0.66$, P -value < 0.00001) and with the gene module involved with cellular respiration ($\rho = -0.58$, P -value < 0.00001). The top cadmium-associated lincRNA (MIR22HG) was most strongly correlated with the cellular respiration module ($\rho = 0.46$, P -value < 0.00001) and with the gene module involved in organ development ($\rho = -0.39$, P -value < 0.00001).

Discussion

In this study, we explored the relationships between placental lincRNAs and BW, as well as a potential molecular mechanism underlying the relationship between *in utero* cadmium exposures and BW. We found that the expression levels of 46 placental lincRNAs were significantly associated with BW, the majority of which (40 of 46) were associated with increased growth relative to expression, indicating growth promotion as the predominant role for these placental lincRNAs. While all of

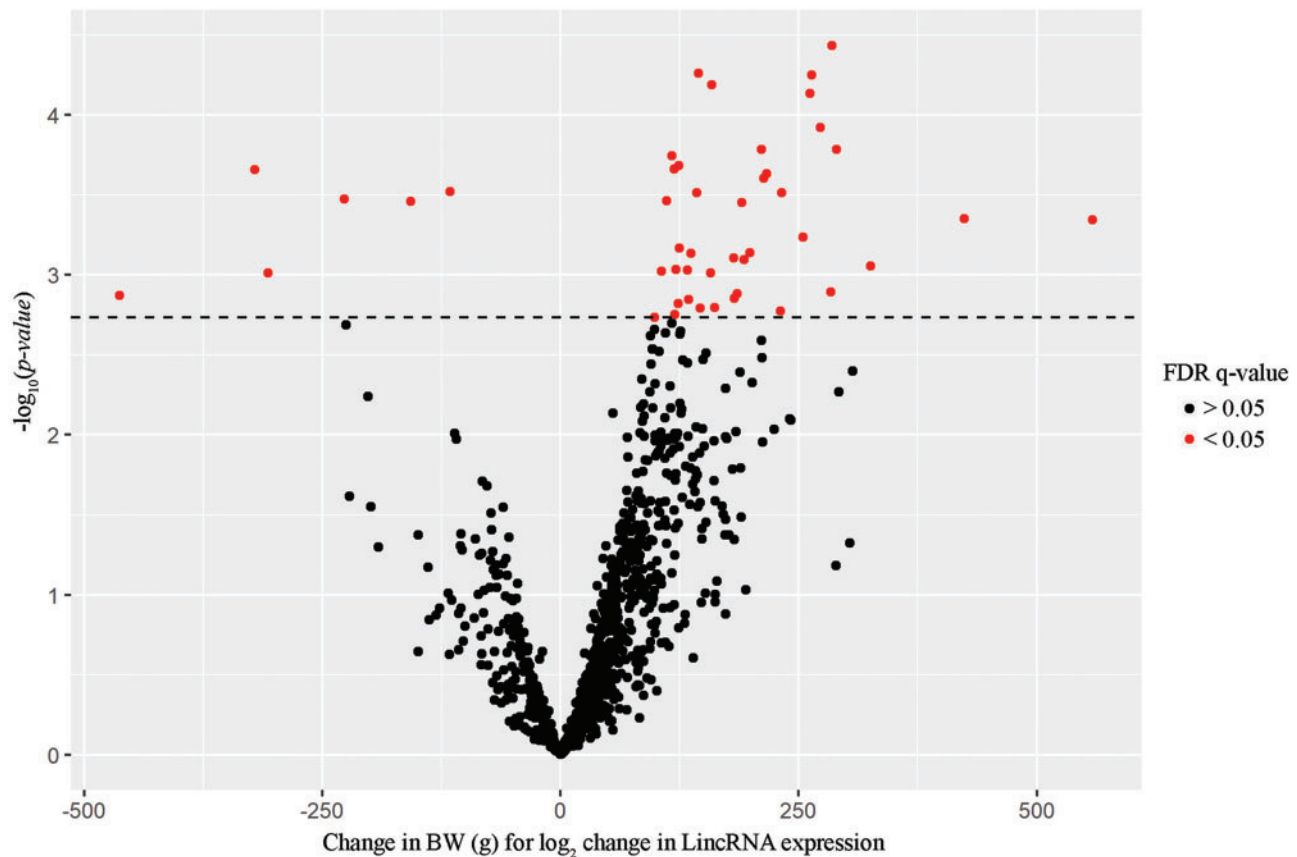


Figure 1: Volcano plot of the regression coefficients for the relationships between lncRNA expression and BW, adjusted for gestational age, infant sex, maternal age, maternal BMI, maternal education and smoking status during pregnancy, with an FDR significance threshold set at 5% (red)

these lncRNAs have been classified as long intergenic non-coding RNAs via Ensembl and therefore are annotated to chromosomal locations, only a few have been characterized beyond nomenclature. *JPX*, which yielded one of the strongest associations with BW is an Xist activator involved in X chromosome inactivation, thereby mediating dosage control of X-linked genes; *JPX* deletion was lethal in X/X-phenotype embryonic stem cells (54). *MIR22HG*, which exhibited the strongest growth-restrictive association, is an early-responding indicator of chemically induced cell stress in human-induced pluripotent stem cells (55), is down-regulated in ovarian cancer tissues when compared to ovarian surface epithelium (56), and appears to play a role as a tumor suppressor by down-regulating cell-cycle genes in lung cancer (57). Of note, we also found that *MIR22HG* expression was up-regulated with higher placental cadmium concentrations. Thus, our findings for this lncRNA as being potentially involved in growth-regulation and responsive to chemical exposures, are consistent with previously recognized functions. Another cadmium-associated transcript, *ERVH48-1*, was negatively associated with BW, which is a human endogenous retroviral (HERV) transcript involved in syncytialization (spontaneous cell fusion) repression via binding of the syn1 receptor protein (58). The localization of most HERV transcripts exclusively to the placenta indicates the importance of this anti-fusion property in controlling placental growth, and their expression was found to be reduced in cases of pregnancy-induced hypertension (59). In our study, the strongest associations with both BW and cadmium were observed with *MIR22HG*, *AC114763.2*, *LINC02595* and *ERVH48-1*; these four lncRNAs may

be involved in cadmium-mediated variations in BW or provide insights into the placental functions that are disrupted by prenatal cadmium exposure. An alternative example of lncRNA expression as a potential toxicological marker for cadmium, though not for prenatal exposures, is highlighted by Dempsey and Cui (36), where levels of the lncRNA ENST00000414355 in the lungs and blood positively correlated with respiratory and urinary levels of cadmium, respectively and increased expression of ENST00000414355 appeared to be an effect-biomarker for DNA damage, which is a known mechanisms of toxicity for cadmium. Thus, lncRNA expression levels may have some utility as markers of cadmium toxicity in different tissues and different exposure paradigms.

In the placenta, *MIR22HG* and *ERVH48-1* expression were associated with higher concentrations of cadmium and with smaller birth size. In contrast, *AC114763.2* and *LINC02595* expression were associated with larger birth size and these lncRNAs exhibited lower expression with higher cadmium levels. Thus, measurement of placental levels of these lncRNAs could potentially lend insights into whether placental cadmium concentrations are contributing to fetal growth restriction. It should be noted that although the specific functions of most lncRNAs are currently unknown, lncRNAs can interact with multiple different mRNA and thus perform numerous regulatory functions. Thus, dysregulation of a single lncRNA can impact multiple functional pathways, which may result in widespread impacts on biological function. To gain some insights into the potential functional consequences of the observed differential lncRNA expression from our study, we

Table 2: Linear associations between lincRNAs with continuous BW (FDR, 5%) while adjusting for infant sex and maternal age, BMI, education level and smoking during pregnancy

Annotation			Regression results				
Ensemble ID	Gene name	Chr.	β Est.	LL	UL	P-value	FDR
ENSG00000197332	AC008543.1	19	285	152.8	417.1	<0.001	0.018
ENSG00000260585	AL359851.1	X	144.6	75.9	213.2	<0.001	0.018
ENSG00000233237	LINC00472	6	263.9	138.4	389.4	<0.001	0.018
ENSG00000260645	AL359715.3	6	159	82.7	235.3	<0.001	0.018
ENSG00000261326	LINC01355	1	262	135.3	388.7	<0.001	0.018
ENSG00000221949	LINC01465	12	272.7	136.6	408.7	<0.001	0.02
ENSG00000230262	MIRLET7DHG	9	289.9	142.1	437.6	<0.001	0.02
ENSG00000231566	LINC02595	X	211.1	103.5	318.7	<0.001	0.02
ENSG00000225298	LINC00113	21	116.8	56.9	176.7	<0.001	0.02
ENSG00000239828	AC063944.1	3	124.2	59.9	188.6	<0.001	0.02
ENSG00000256972	AP000462.3	11	119.2	57.2	181.2	<0.001	0.02
ENSG00000233056	ERVH48-1	21	-320.9	-487.8	-154	<0.001	0.02
ENSG00000260317	AC009812.4	8	216.6	103.4	329.7	<0.001	0.02
ENSG00000261098	AP000766.1	11	213.3	101.3	325.2	<0.001	0.02
ENSG00000229642	AC087857.1	3	-116.1	-178	-54.3	<0.001	0.02
ENSG00000227060	LINC00629	X	142.9	66.7	219	<0.001	0.02
ENSG00000152931	PART1	5	232	108.4	355.6	<0.001	0.02
ENSG00000225077	LINC00337	1	-226.9	-348.6	-105.2	<0.001	0.02
ENSG00000230569	AC114763.2	2	111.3	51.4	171.1	<0.001	0.02
ENSG00000247095	MIR210HG	11	-157.1	-241.6	-72.6	<0.001	0.02
ENSG00000260971	AC119674.1	1	190.3	87.8	292.7	<0.001	0.02
ENSG00000228223	HCG11	6	424.1	191.5	656.7	<0.001	0.023
ENSG00000225470	JPX	X	558.7	251.9	865.6	<0.001	0.023
ENSG00000230590	AL353804.1	X	254.4	111.9	396.9	0.001	0.029
ENSG00000236532	LINC01695	21	125	54.1	196	0.001	0.032
ENSG00000236871	LINC00106	X	198.5	85.2	311.8	0.001	0.032
ENSG00000237713	AC011287.2	7	136.7	58.6	214.7	0.001	0.032
ENSG00000257496	AC025031.1	12	181.7	77.4	286.1	0.001	0.033
ENSG00000229989	MIR181A1HG	1	192.6	81.8	303.5	0.001	0.033
ENSG00000246067	RAB30-AS1	11	325.5	136.7	514.4	0.001	0.033
ENSG00000235813	AL672032.1	X	121.4	50.7	192.2	0.001	0.033
ENSG00000235139	AC003984.1	7	133.4	55.6	211.1	0.001	0.033
ENSG00000250064	AC097480.1	4	106.2	44.2	168.1	0.001	0.033
ENSG00000259715	AC022087.1	15	157.7	65.4	249.9	0.001	0.033
ENSG00000223891	OSER1-AS1	20	-307	-486.5	-127.4	0.001	0.033
ENSG00000188185	LINC00265	7	283.8	113.7	453.9	0.001	0.042
ENSG00000249258	AC079193.2	8	185.2	73.9	296.5	0.001	0.042
ENSG00000186594	MIR22HG	17	-462.7	-741.5	-183.9	0.001	0.042
ENSG00000253385	AP003696.1	8	182.4	72.1	292.7	0.001	0.042
ENSG00000253143	AC022730.3	8	134.7	53.1	216.3	0.001	0.042
ENSG00000230250	LINC01753	1	123.5	48.3	198.7	0.002	0.044
ENSG00000240770	C21orf91-OT1	21	161.5	62.6	260.4	0.002	0.045
ENSG00000257083	AC073530.1	12	146.6	56.8	236.4	0.002	0.045
ENSG00000132204	LINC00470	18	230.8	88.9	372.7	0.002	0.045
ENSG00000254263	AC022973.3	8	120.1	45.9	194.4	0.002	0.047
ENSG00000234675	AL445123.1	6	98.5	37.4	159.7	0.002	0.048

Chr., chromosome; β Est., parameter estimate from linear model; LL, lower limit of the 95% confidence interval; UL, upper limit of the 95% confidence interval; FDR, false discovery rate q-value.

examined whether the expression levels of our BW- and cadmium-associated lincRNAs were correlated with the placental co-expression modules identified in our lab's prior work (12). Interestingly, one of the most highly correlated eigengenes, particularly for MIR22HG and LINC02595, represented a co-expression module involved in cellular respiration. Mitochondrial electron transport, a critical biochemical process in cellular respiration, has been shown to be disrupted by exposure to cadmium (60), and disruptions to the electron transport

chain can promote reactive oxygen species formation and increase oxidative stress (61), a well-recognized mechanism of cadmium toxicity.

Esteban-Vasallo et al. (62) reviewed a large number of studies examining cadmium, mercury and lead measurement within the placenta, and reported cadmium concentrations ranging from 1.2 to 53 ng/g worldwide. Our study occupied the lower end of that range of placental cadmium concentrations, 1.1–16.4 ng/g. In addition, only 19 of the 74 studies included in

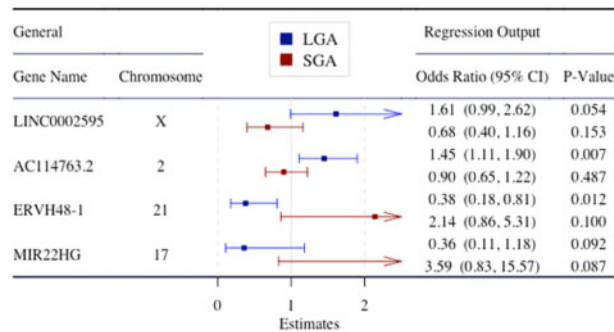


Figure 2: Odds of LGA and SGA associated with lincRNA expression (among those associated with both BW and cadmium), adjusted for maternal age, highest education level—as an indicator of socioeconomic status—BMI and smoking status during pregnancy

Table 3: Linear relationships between lincRNA expression and placental cadmium concentrations that yielded nominally significant associations, adjusted for smoking during pregnancy and infant sex

Annotation			Regression results		
Ensembl ID	Gene name	Chromosome	logFC	P-value	FDR q-value
ENSG00000186594	MIR22HG	17	0.4	0.002	0.074
ENSG00000230569	AC114763.2	2	-1.54	0.009	0.202
ENSG00000233056	ERVH48-1	21	0.47	0.029	0.444
ENSG00000231566	LINC02595	X	-0.65	0.039	0.444

LogFC, log fold change; FDR, false discovery rate.

lnc RNA genes and eigengenes correlations

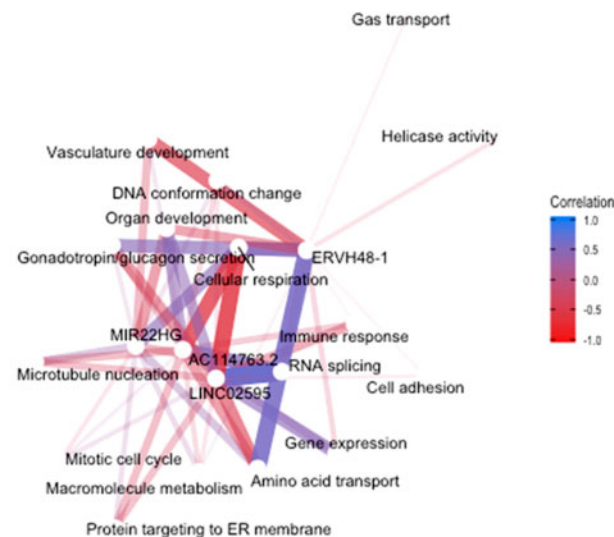


Figure 3: Spearman correlation network of the relationships between lincRNAs (MIR22HG, AC114763.2, ERVH48-1 and LINC02595) with eigengenes from a previously published study of placental co-expression networks; network nodes are labeled with the growth and developmental processes attributed to those modules via gene-enrichment analyses (12). LincRNAs are connected to a module if they achieved a Spearman correlation P-value < 0.05, while the thickness of the connections and brightness of the colors increase with increasing ρ -values

that review had >100 subjects (62), which highlights the issue of small sample sizes that have reduced statistical power and may be less generalizable. Consequently, this is also a limitation for

our study, which was restricted to 85 samples for analyses testing the relationships between cadmium and lincRNA expression, and 199 samples for analyses of relationships between BW and lincRNAs. We did employ multiple testing adjustments, such as FDR, to reduce the likelihood of false positives. However, the associations between cadmium and lincRNA expression were not within the 5% FDR threshold, and only MIR22HG (q -value = 0.07) was within a less conservative FDR of 10%; thus, false positives are a possibility. In addition, the cross-sectional design prevents us from distinguishing whether reverse causation is playing a role in these associations, and it is possible that the expression levels associated with larger BW are downstream consequences or markers of other growth promoting processes rather than upstream drivers of growth promotion. The small sample size for our study also limited our ability to observe modest associations, particularly those between cadmium and lincRNAs, and thus similar studies with larger samples would be encouraged to not only validate our findings, but potentially observe associations that our design would have been underpowered to detect. However, this was the largest study to date on placental lincRNA expression levels and their associations with BW and toxic metals, and these findings can help to guide how future investigations examine similar research questions.

Despite these limitations, our study had numerous strengths that allowed us to identify multiple interesting relationships between placental lincRNAs with BW and with cadmium concentrations. These included our assumption of a lack of homogenous metal deposition, which was addressed via homogenization of multiple samples from a single placenta; previous studies frequently would treat a single sample as representative of the entire placenta (62). In addition, both our metal and transcriptome measurements included stringent quality control and storage procedures aimed at RNA preservation, increasing the likelihood of accurate metal and transcript measurements and consistency of measurements across samples. Further analysis within larger cohorts will be required in the future to characterize the role of lincRNAs in fetal growth, and whether they play a role in mediating developmental disease phenotypes due to prenatal environmental exposures.

Conclusions

In utero exposure to cadmium has been found to impair fetal development, particularly BW, yet the toxicological mechanisms of these effects are unclear. One possible factor includes the partial sequestration of cadmium to the placenta, which we hypothesized may contribute toward diminished function of this essential organ via influencing placental lincRNA expression. We found that numerous lincRNAs were associated with changes in BW, and that placental cadmium was associated with increased or decreased placental expression of some of these lincRNAs known to control cellular and/or placental growth and regulate exogenous chemical response. This study provides evidence that disrupted placental regulation of lincRNAs may contribute to cadmium's reproductive toxicity, and further research is needed to validate the observed relationships and to characterize the functions of the lincRNAs highlighted.

Supplementary data

Supplementary data are available at *EnvEpiG* online.

Data Availability

The raw fastq files for placental RNA-seq measurement are available in the NCBI database for Genotypes and Phenotypes (dbGaP) under accession number phs001586.v1.p1.

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