In utero exposures to environmental organic pollutants disrupt epigenetic marks linked to fetoplacental development

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Abstract

While the developing fetus is largely shielded from the external environment through the protective barrier provided by the placenta, it is increasingly appreciated that environmental agents are able to cross and even accumulate in this vital organ for fetal development. To examine the potential influence of environmental pollutants on the placenta, we assessed the relationship between polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), 1,1-dichloro-2,2-bis(p-chlorophenyl) ethylene (DDE) and several epigenetic marks linked to fetoplacental development. We measured IGF2/H19 imprint control region methylation, IGF2 and H19 expression, IGF2 loss of imprinting (LOI) and global DNA methylation levels in placenta (n = 116) collected in a formative research project of the National Children’s Study to explore the relationship between these epigenetic marks and the selected organic environmental pollutants. A positive association was observed.
between global DNA methylation and total PBDE levels ($P < 0.01$) and between H19 expression and total PCB levels ($P = 0.04$). These findings suggest that differences in specific epigenetic marks linked to fetoplacental development occur in association with some, but not all, measured environmental exposures.

**Key words**: IGF2; H19; global DNA methylation; PBDE; PCB; DDE; environmental organic pollutants

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**Introduction**

The notable increase in recent years in the prevalence of chronic disorders, including neurodevelopmental disabilities, cancer and metabolic disorders, points to the contribution of non-genetic factors, including the increasing burden of environmental exposures [1–3]. The intrauterine period, in particular, is marked by a heightened sensitivity of the genome to environmental cues, informing both prenatal and postnatal development. Hence, health outcomes that manifest across the lifespan may be triggered by perturbations experienced as early as in utero development [4, 5].

The placenta is the principal organ that regulates the developmental trajectory of the fetus [6]. Appropriate transitioning through gestation requires the tightly coordinated orchestration of the placental epigenome [7], which serves as the interface between genes and the environment by enabling heritable and persistent changes in gene expression without altering the DNA sequence. Placental epigenetic regulatory elements include DNA methylation, histone modifications and non-coding RNAs [7], however DNA methylation is most commonly assessed, likely due to the technical feasibility of measuring this epigenetic mark. The DNA methylation profile of the human placenta includes unique global and site-specific DNA methylation patterns with respect to somatic tissues. Unlike the embryoblast, the trophoblast-derived placenta does not undergo extensive re-methylation following the wave of post-fertilization de-methylation, and, thereby, maintains its genome-wide hypomethylated state [8]. Although the role of global hypomethylation in fetoplacental development remains poorly understood, several studies have demonstrated the responsiveness of global DNA methylation patterns to environmental exposures. Altered placental DNA methylation patterns have been associated with exposures to folic acid [9], bisphenol A (BPA) [10], air pollution [11] and phthalates [12].

While the placenta maintains a hypomethylated state at a genome-wide level, site-specific epigenetic patterns are preserved at distinct loci. Imprinted genes are a subset of genes that undergo epigenetic programming during early development resulting in mono-allelic expression based on parent of origin. Although imprinting is initialized early in development, recent studies indicate that these marks continue to undergo re-modeling throughout the gestational period [13, 14]. The resulting dynamic intrauterine state of imprinting highlights the potential susceptibility of these marks to perturbations throughout gestation.

IGF2 and H19 are among the best-described imprinted genes. These two reciprocally imprinted genes co-localize as part of a cluster in the telomeric region of chromosome 11p15. IGF2 is a paternally expressed growth-promoting gene, involved in driving placental and fetal growth and the transfer of nutrients from mother to fetus, while H19 is a maternally expressed non-coding RNA that is located downstream of IGF2.

Various epigenetic elements may dictate the maintenance of IGF2/H19 imprinting, however, DNA methylation-mediated regulation of these loci is currently the best characterized. Differential methylation of the common imprint control region (ICR) located upstream of H19 determines access to shared enhancers and, thereby, facilitates the co-regulation of IGF2 and H19. Hence, the unmethylated ICR of the maternal allele allows access to the enhancers, thereby driving maternal expression of H19, whereas the methylated ICR on the paternal allele diverts the enhancers away from the H19 promoter towards the IGF2 promoter, driving paternal expression of the IGF2 allele [15].

Dysregulation of this carefully controlled phenomenon in utero has been linked with adverse health outcomes, particularly developmental defects. Additionally, these loci have also been shown to be responsive to various in utero exposures, including maternal nutrition [16, 17], tobacco [18], alcohol [19, 20], assisted reproductive technology [21, 22], BPA [23], phthalate/phenols [24], maternal infection [25], vinclozolin [26] and sodium fluoride [27, 28].

However, studies conducted thus far differ in the methods implemented to assess these loci, complicating the interpretability of the findings. Allele-specific expression is the most direct determination of imprint dysregulation, i.e. loss of imprinting (LOI), since focusing on the eventual endpoint of imprinting provides an assessment that is independent of the antecedent epigenetic mechanism. However, such assays can be tedious to conduct in a population-wide setting. Since allele-specific expression can be driven by differential DNA methylation levels at ICRs and can result in the alteration of overall expression levels, epidemiologic studies often rely on these more easily ascertained proxies, including ICR methylation and overall expression levels. However, the extent to which these proxies reflect alterations in imprinting remains unclear. In this study, we evaluate the impact of environmental pollutants on placental genome-wide methylation as well as the specific imprinting status of IGF2 and H19, using both direct and proxy measures of imprinting, in the National Children’s Study.

**Results**

The demographics of the study sample are shown in Table 1. The profiled placentas came from term pregnancies of roughly equivalent numbers of male and female infants. The relatively low levels of environmental pollutants detected in the placentas in our study fell within reported measurements from industrialized nations [29–31]. While multiple congeners of polybrominated diphenyl ether (PBDE) and polychlorinated biphenyl (PCB) were measured, given the unknown effects of the individual congeners, we focused our analysis on total congener levels to minimize loss of power due to multiple comparisons.

The distribution of each measured placental biomarker is shown in Table 2. This is the first study to report global DNA methylation levels as assessed by LUMA in human term placentas, with observed DNA methylation levels ranging from 48% to 66%. The ~50% IGF2/H19 DMR methylation levels observed in our study fall in line with previously reported placental levels [8]. We observed lower IGF2 expression levels, as indicated by the higher cycle threshold (C) values, than H19 expression levels, consistent with other reports [32]. Out of 116 profiled placentas,
LOI and IGF2 were reported [33–35].

Next, we assessed the correlation between IGF2/H19 LOI and IGF2 and H19 gene expression levels, IGF2/H19 ICR methylation levels, and global methylation levels (Fig. 1). As expected, a positive correlation was observed between the expression of the co-regulated H19 and IGF2 loci and global DNA methylation levels as well as methylation levels at the specific ICR shared between IGF2 and H19. A negative correlation was observed between IGF2 LOI and IGF2 expression levels, which is counter to the expected direction relating these two markers, although inconsistencies between LOI and expression have been previously reported [33–35].

Table 1. Characteristics of 116 study placentas from eight US counties

| Characters                  | N  | Median ± SD | Min–max |
|-----------------------------|----|-------------|---------|
| Gender (n)                  |    |             |         |
| Males                       | 57 |             |         |
| Females                     | 59 |             |         |
| Delivery method             |    |             |         |
| Vaginal                     | 95 |             |         |
| C-section                   | 21 |             |         |
| Collection time (hours)     | 116| 0.78 ± 0.52 | 0.12–1.97|
| Gestational age (weeks)     | 116| 39.5 ± 0.94 | 37.14–41.43|
| Birth weight (g)            | 116| 3493.5 ± 440.21| 2466–4760|
| 
| \(\sum_{10}^{32}\) PCB (pg/g)\a | 109| 197.4 ± 148.3 | 53.5–737.39|
| \(\sum_{10}^{32}\) PBDE (pg/g)\a | 109| 601.9 ± 333.7 | 215.4–1573.5|
| DDE (pg/g)                  | 109| 180.0 ± 466.9 | 76.2–4157.0|
| BPA (pg/g)                  | 63 | 264.9 ± 2880.6| 41.2–12391.9|

\(\sum_{10}^{32}\) PCBs: congeners 8, 28, 47, 66, 85, 99, 100, 101, 105, 114, 118, 126, 128, 138, 153, 156, 158, 166, 169, 170, 179, 180, 183, 187 and 189.

\(\sum_{10}^{32}\) PBDEs: congeners 28, 47, 66, 85, 99, 100, 153, 154, 183, 209; \(\sum_{10}^{32}\) PCBs: congeners 8, 28, 47, 49, 52, 60, 66, 70, 74, 77, 82, 87, 99, 101, 105, 114, 118, 126, 128, 138, 153, 156, 158, 166, 169, 170, 179, 180, 183, 187 and 189.

We noted no significant differences in the distribution of the analysed epigenetic marks based on gestational age. However, a trend towards higher global DNA methylation levels was observed among male infants compared with female infants (P = 0.05) (Fig. 2).

Environmental pollutant levels did not impact placental levels of IGF2/H19 LOI. However, total PBDE levels were positively associated with global methylation levels (P < 0.01) (Fig. 3), whereas total PCB levels were positively associated with (lower Ct) H19 expression level (P = 0.01) (Fig. 4). No significant associations were observed between any of the measured epigenetic marks and exposure to dichlorodiphenyldichloroethylene (DDE).

**Discussion**

We assessed the impact of environmental pollutants on a panel of epigenetic marks linked to fetoplacental development including IGF2 LOI, IGF2 expression, H19 expression, IGF2/H19 ICR methylation and global DNA methylation. While a number of studies have evaluated the variability of IGF2 and H19 in relation to various in utero exposures, including maternal nutrition [16, 17], tobacco smoke [18, 19] and alcohol consumption [20], most studies focused on the assessment of IGF2/H19 ICR methylation levels. Only a handful of studies have sought to clarify the relationship between ICR methylation levels and eventual expression levels [23, 27, 28], and even fewer considered actual measurements of LOI [33, 34]. This is the first study to assess the influence of organic environmental pollutants on IGF2 and H19 using both direct and proxy measures of imprinting in non-pathological human placenta. Similarly, associations between placental global DNA methylation levels and in utero exposures, including BPA [10], phthalate [12] and air pollution [36], have been reported, however, the influence of organic pollutants on placental global DNA methylation levels has not been described.

In our study, correlations between ICR methylation and allelic-specific expression of IGF2 were inconsistent. This is in agreement with the current literature, where establishing links between LOI and gene expression levels or ICR methylation levels has been largely ambiguous, with studies successfully establishing correlations mainly stemming from cancer studies focusing on assessments in differentiated tissues of adult populations [37–42]. Meanwhile, correlations between IGF2 LOI or IGF2 expression/ICR methylation have not been observed consistently in studies focusing on in utero assessments in the placenta [33, 34]. This suggests that while ICR methylation analysis likely captures perturbations in placental DNA methylation, this may not fully account for the allele-specific imprinted gene expression of these loci in the placenta. Furthermore, our findings, consistent with existing literature, support the hypothesis...
that assessing the overall expression of imprinted genes and LOI reflect two separate phenomena that independently respond to environmental stimuli.

We observed a trend suggesting gender-based differences in global DNA methylation levels ($P = 0.05$) consistent with findings from the Center for the Health Assessment of Mothers and Children of Salinas (CHAMACOS) study [43], in which global DNA methylation levels (assessed by LINE-1 and Alu repetitive elements) were also observed to be higher in male infants compared with female infants and was maintained in the assessments of the children at age 9. The effect size, a one percent difference in DNA methylation levels between the genders, was the same in both studies.

Exposure to environmental pollutants was associated with changes in placental expression of $H19$, but not with $IGF2$ LOI. The maintenance of placental imprinting in spite of perturbations observed in expression and methylation levels of associated genes has been reported in previous studies as well. An increase in placental $H19$ expression levels was observed among IVF/ICSI induced pregnancies compared with naturally conceived pregnancies, however no significant differences in $H19$ LOI levels was observed between the two groups [35]. Similarly, in an IUGR case-control study, differential placental expression of PEC10 was observed; however, no significant differences in PEC10 LOI status was observed [33]. These results suggest that while some variability in placentation expression/DNA methylation of loci is tolerated, a greater level of protection against environmental insults is conferred on the specific mechanism driving the imprinting status of the same loci, likely due to the importance of genomic imprinting in regulating fetal development.

This study adds to the literature on the impact of environmental toxicants on placentas assessed in a normal ‘non-pathological’ birth cohort. However, several limitations warrant caution in the interpretation of the reported findings. The determination of LOI requires informative heterozygous genetic variants, restricting the available sample size for analysis and thereby the power to detect meaningful differences. Furthermore, our measured outcomes of interest are putative environmentally responsive elements with potential phenotypic implications. However, as no health outcome data are available on our study population, the clinical relevance of our findings remains unclear. Similarly, as only a single time-point measurement is available at birth, our assessments reflect a snapshot in development that may not persist into adulthood. From a mechanistic perspective, it cannot be determined whether the observed variability in the markers indicates a pathologic or adaptive response to the environmental pollutants. Finally, while sampling of the placenta was restricted to villous tissue free from maternal decidua, it cannot be ruled out that heterogeneity...
in cell-type composition may have contributed to the observed variability of the assessed markers. Hence the findings reported here warrant replication in other population-based studies.

In summary, we report differences in epigenetic marks linked to fetoplacental development by organic pollutant exposure levels. These findings suggest the potential utility of these markers as in utero sensors of environmental exposures.

**Methods**

**Study Sample**

Placental samples were collected from 8 study locations across the USA (The Children’s Hospital of Philadelphia (Montgomery County, PA; Schuylkill County, PA), Icahn School of Medicine at Mount Sinai (Queens County, University of California, Davis (Orange County), University of North Carolina (Duplin County), University of Utah (Cache County, Salt Lake County), South Dakota State University (Brookings County)) that served as Vanguard Centers of the National Children’s Study (NCS). As shown in Fig. 5, 210 placentas were collected from singleton deliveries. We restricted our analysis to term deliveries (37–42 weeks gestation) with information available on infant gender, gestational age and delivery method and no reported pathological complications. Additionally, while collection times in the NCS ranged from 7 min to 121 h, we have established in a prior study that gene expression levels in these samples are stable within 6 h of delivery [44]. In keeping with the literature on placental gene expression studies [11, 45], we restricted our study to samples collected within 2 h of delivery (n = 116). The study protocol was reviewed and approved by the Office of Human Research Protections registered Institutional Review Board of each collection institution, and written informed consent was obtained from all participants.

**Sample Collection**

All placental specimens were excised from grossly normal areas of the villous parenchyma, excluding the decidua basalis and chorionic plate. All samples collected at participating hospitals were packaged in a standardized manner and shipped overnight to the University of Rochester’s Placental Processing Center (URPPC).

**Chemical Analysis**

Five to 10 g of collected villous tissue was stored in acid-washed, BPA-free 50-ml tubes using plastic (non-metallic) disposable for caps. Placental samples were stored on dry ice or at −80°C until shipped on dry ice to the URPPC, where they were stored at −80°C.

Environmental pollutants analysed in the current study include PCBs, PBDEs and DDE. Detailed procedures for the extraction, cleanup and instrumental analysis have been previously described [46, 47]. Briefly, placental tissues were freeze-dried, manually ground, and extracted using matrix solid phase dispersion. PCB congeners (including PCBs 8, 28, 37, 44, 49, 52, 60, 66, 70, 74, 77, 82, 87, 99, 101, 105, 114, 118, 126, 128, 138, 153, 156, 158, 166, 169, 170, 179, 180, 183, 187 and 189) and DDE were analysed using an Agilent 7890A gas chromatograph coupled to an Agilent 5973 mass spectrometer with electron capture negative ionization mode.

**Nucleic Acid Extraction**

Forty to 60 mg of villous trophoblast tissue was excised, rinsed with phosphate buffered saline, lightly macerated, placed in 5 ml of RNALater (Thermo Scientific, Waltham, MA), and stored at 4°C. Nucleic acids, including genomic DNA and total RNA, were isolated using an AllPrep DNA/RNA extraction kit (Qiagen, Germantown, MD) and quantified using a microplate spectrophotometer (Biotech, Winooiski, VT).

**cDNA Synthesis and Bisulfite-conversion**

Total RNA was reverse-transcribed into single-stranded cDNA using random primers in the AffinityScript cDNA synthesis kit (Agilent Technologies, Santa Clara, CA). Genomic DNA (500 ng) was bisulfite treated using EpiTect Bisulfite Kits (Qiagen, Hilden, Germany) and eluted in 20 ul elution buffer.

**IGF2 LOI Assay**

The conditions for measuring IGF2 LOI levels have been previously described [48]. Briefly, LOI was assessed using a two-step process. Individuals with heterozygous alleles for the IGF2 loci were first identified through polymerase chain reaction (PCR) amplification of genomic DNA using primers bracketing an area that contains a readout reporter polymorphism. Splitting the reverse-transcribed cDNA template into two equivalent batches, the relative expression abundance of the two alleles was then assessed by quantitative allele-specific PCR using two separate primer sets with the last base matching one of the two SNP alleles. Both steps were performed using a LightCycler480 (Roche, Indianapolis, IN).

**IGF2 and H19 Expression**

Gene expression levels were determined using quantitative RT-PCR following conditions previously described [33]. Briefly, cDNA was amplified in triplicate with primers targeting IGF2 (FWD: TTGTGCCCTCTCCTCTCCA; REV: CAAGGCCTCTGCGGAAACT) and H19 (FWD: ATTTTGACTAATGATTTGACG; REV: CAGTCACCCGGCCCAGAT) using a LightCycler480 (Roche).

**IGF2/H19 ICR Methylation**

The assayed IGF2/H19 ICR methylation region spanned chr11: 2021190–2021248 (GRCh37/hg19 built) which contains the hemi-methylated binding site for the CTCF transcriptional repressor.
Bisulfite-converted DNA was first PCR amplified and then sequenced (FWD: 5'-biotin-AGGGGTTTTTTCTATAGTATGGGT; REV: ACTCCAAATATTCTATCTCCTAACAAAATA; SEQ: TAAAATCCTATTGCGAAT) using a PyroMark Q24 (Qiagen). As one out of the six interrogated CpG dinucleotides contains a SNP (rs10732516) that disrupts methylation, the ICR methylation status was determined based on the mean methylation levels of the remaining five CpG sites.

Global Methylation

Global methylation levels were determined using the lumino-metric methylation assay (EUMA) [49]. This assay entails a two-step process of endonuclease digestion followed by polymerase extension. Genomic DNA was first digested with a methylation sensitive (HpaII) and a methylation-insensitive (MspI) restriction enzyme (New England Biolabs, MA), both targeting CCGG sites, in two separate reactions. Successful cleavage, generating CG overhangs, was quantified using bioluminometric polymerase extension with a PyroMark Q24 system (Qiagen). Percent methylation was calculated as the ratio between the successful extension of HpaII reactions and MspI reactions. 5-Aza-dC demethylated DNA and fully methylated Jurkat genomic DNA (New England Biolabs, MA) were included as internal controls.

Statistical Analysis

As the levels of the assessed epigenetic marks were not normally distributed, nonparametric tests were applied to evaluate differences by gender, delivery method, and gestational age. Mann–Whitney U Test was used to examine mean differences by gender and delivery method, and Spearman rank correlation was used to examine mean differences by gestational age. Spearman correlation analysis was also applied to determine the correlation among the placental markers of development.

As the congener’s likely co-occur and the biological function of individual congeners are unknown at this time, PBDEs and PCBs were analysed based on the concentration sums of their respective congeners (\(\sum_{10}\) PBDEs and \(\sum_{32}\) PCBs) to ensure sufficient statistical power. To allow flexible regression modeling of potentially nonlinear relationships, the relationship between the environmental pollutants and the selected epigenetic marks was analysed using generalized additive models (mgcv package [50]) adjusted for infant gender. Due to the small number of comparisons conducted, reported P values are not corrected for multiple comparisons to minimize the risk of inflating the type II error [51]. Figures were generated using ggplot2 [52]. All analysis was conducted using R 3.0.2 (http://www.r-project.org/). All statistical tests were two-sided, and \(P < 0.05\) was considered statistically significant.

Data availability

NIH is working now to make data and material collected by the NCS Vanguard Study available to researchers. The National Children’s Study Vanguard Data Repository is in development and is expected to become available to researchers beginning in late 2015.

Acknowledgements

We acknowledge the critical support of the National Children’s Study Project 18 Placenta Consortium Collection Teams: Jennifer Culhane, MD and her team from The Children’s Hospital of Philadelphia; Edward B. Clark, MD and his team at the University of Utah; James Swanson, PhD and his team at the University of California at Irvine; the collection team at the Icahn School of Medicine at Mount Sinai; the collection team at the University of North Carolina at Chapel Hill; Bonnie Specker, PhD, Natalie Thiex, PhD and their collection team at South Dakota State University. Also, we acknowledge with appreciation the review of the manuscript by Jeffrey Murray, MD, PhD. Finally this research would not have been possible without the support from the National Institutes of Health – National Children’s Study LOI-2-BIO-18.

Conflict of interest: None declared.

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