PERINATAL DEHP EXPOSURE INDUCES SEX- AND TISSUE-SPECIFIC DNA METHYLATION CHANGES IN BOTH JUVENILE AND ADULT MICE

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Abstract

Di(2-ethylhexyl) phthalate (DEHP) is a type of phthalate plasticizer found in a variety of consumer products and poses a public health concern due to its metabolic and endocrine disruption activities. Dysregulation of epigenetic modifications, including DNA methylation, has been shown to be an important mechanism for the pathogenic effects of prenatal exposures, including phthalates. In this study, we used an established mouse model to study the effect of perinatal DEHP exposure on the DNA methylation profile in liver (a primary target tissue of DEHP) and blood (a common surrogate tissue) of both juvenile and adult mice. Despite exposure ceasing at 3 weeks of age (PND21), we identified thousands of sex-specific differential DNA methylation events in 5-month old mice, more than identified at PND21, both in liver and blood. Only a small number of these differentially methylated cytosines (DMCs) overlapped between the time points, or between tissues (i.e. liver and blood), indicating that DEHP exposure may not be an appropriate surrogate tissue to estimate the effects of DEHP exposure on liver DNA methylation. We detected sex-specific DMCs common between 3-week and 5-month samples, pointing to specific DNA methylation alterations that are consistent between weanling and adult mice. In summary, this is the first study to assess the genome-wide DNA methylation profiles in liver and blood at two different aged cohorts in response to perinatal DEHP exposure.
exposure. Our findings cast light on the implications of using surrogate tissue instead of target tissue in human population-based studies and identify epigenetic biomarkers for DEHP exposure.

**Key words:** DNA methylation; DEHP exposure; liver; blood; perinatal; mouse model; bisulfite sequencing

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**Introduction**

Di(2-ethylhexyl) phthalate (DEHP) is a plasticizer (1) often found in polyvinyl chloride products, including medical equipment, car upholstery, food and beverage containers, and building materials (2). DEHP does not covalently bond to polymer chains in these products and is therefore likely to spread into the environment after repeated usage (3). Due to the large quantity and wide variety of products containing DEHP that people interact with frequently, the estimated range of human exposure to DEHP is 3-30 μg/kg/day (4).

DEHP has been detected in tissues such as blood, amniotic fluid, umbilical cord blood, and breast milk in humans (5–8), indicating that exposure starts as early as the fetal developmental stage (9). As a lipophilic compound, DEHP can be absorbed through dermal exposure, inhalation, and oral ingestion (6, 7, 10). The estimated human oral absorption varies from 25% to 50% depending on the dosage (11, 12), while absorption in rodents can be as high as 58% of the oral dose, but 50% is assumed on average (13). The biological action of DEHP is very similar to a group of chemicals called peroxisome proliferators (PPs), and liver is a primary target organ for the effects of DEHP and other PPs (14). Prenatal DEHP exposure in rodents results in elevated fatty acid metabolism, as well as peroxisome proliferation and the accumulation of lipofuscin granules which are implicated in hepatocarcinogenesis (15, 16). There are multiple modes of action of DEHP in hepatocytes, including activation of peroxisome proliferator-activated receptors (PPARs), induction of cell proliferation, suppression of apoptosis, oxidative DNA damage, and inhibition of gap junctional intercellular communication (14). Once absorbed, DEHP and its metabolites are distributed throughout the body in the blood promoting its endocrine disrupting properties.

Epigenetic modifications, such as DNA methylation, define and control cell and tissue development by regulating gene expression (17). For example, DNA methylation patterning of imprinted genes is crucial for embryonic development (18). Previous animal and human studies have reported that perinatal exposure to DEHP is associated with altered DNA methylation in offspring that were developmentally exposed to 5 mg/kg/day, assuming that preg- nant females were exposed during gestation and lactation.

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**Methods**

**Animals and Dosing Paradigm**

Mice utilized in this study were obtained from a colony maintained for over 230 generations with the Avy allele passed through the male line, resulting in forced heterozygosity on a genetically invariant background with 93% identity to C57BL/6j (27). Virgin a/a females (6–8-weeks old) were mated with virgin a/a males (7–9-weeks old), and randomly assigned to receive a control or DEHP-exposed diet through consumption of chow (28, 29). DEHP was dissolved in corn oil and mixed with chow (AIN-93G, TD.95092, Harlan Teklad) to achieve 7% corn oil and 25 mg DEHP per kg of chow. Control animals received the same chow with 7% corn oil. Dams began consumption of control or DEHP diets 2 weeks prior to mating, and exposure was continued during gestation and lactation.

DEHP was mixed into corn oil from Envigo to create a stock solution, and the stock solution was sent back to Envigo where it was mixed with the corn oil used to produce custom 7% corn oil chow in order to achieve uniform distribution of phthalates within the chow. The DEHP exposure level was selected based on a target maternal dose of 5 mg/kg-day, assuming that pregnant and nursing female mice weigh approximately 25 g and eat approximately 5 g of chow per day. This target dose was selected based on literature demonstrating obesity-related phenotypes in offspring that were developmentally exposed to 5 mg/kg-day of DEHP (30, 31). The resulting exposure level is estimated to fall within the range of exposures experienced by humans (32). This is based on amniotic fluid levels of phthalates found in humans (ranging from <LOD to 100.6 ng/mL) and a study in rodents that orally ingested 11 mg/kg-day of phthalates resulting in amniotic fluid levels of 68 ng/mL (33–40).
After weaning (PND21), pups were weighed, and all animals received DEHP-free chow for the remainder of the study (Fig. 1A). Approximately 1–2 male and 1–2 female offspring per litter were followed until 5 months of age (the total number of animals per treatment group is between 5 and 7, and the details can be found in Table 1). All animals had access to food and drinking water ad libitum throughout the experiment and were housed in polycarbonate-free cages. This study protocol was approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC). All the experiments in this study were conducted according to procedures established by the NIEHS TaRGET II Consortium.

Tissue Collection

Upon euthanasia at both PND21 and 5 months of age, blood and liver samples were collected according to protocols established by the TaRGET II Consortium (26). Briefly, blood was collected by cardiac puncture into tubes with EDTA anticoagulant, centrifuged, and plasma was removed. Liver tissue was weighed and then separated into individual lobes, and the left lobe of the liver was cryo-pulverized and suspended in Buffer RLT for nucleic acid extraction (Qiagen, cat # 1053393). Blood and liver DNA/RNA extraction was performed using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen #80224).

Enhanced Reduced Representation Bisulfite Sequencing (ERRBS)

A total of 50 ng of genomic DNA was digested using restriction enzyme Mspl, and the digested DNA was then purified using phenol: chloroform extraction and ethanol precipitation in the presence of glycogen, before blunt-ending and phosphorylation. A single adenine nucleotide was next added to the 3' end of the fragments in preparation for the ligation of the adapter duplex with a thymine overhang. The ligated fragments were cleaned, then processed for size selection on agarose gel. Selected
fragments were treated with sodium bisulfite to convert unmethylated cytosines to uracils, which are then replaced with thymines during PCR amplification. These libraries were next cleaned up with AMPure XP beads (Product #A63880; Beckman Coulter), quantified using the Agilent TapeStation genomic DNA kit (Catalog #G2991AA; Agilent) and Qubit High Sensitivity dsDNA (Catalog #Q32850; Invitrogen). ERRBS samples were multiplexed, and sequencing was performed with Illumina HiSeq 4000, generating single-end, 50-bp reads for each library.

RNA-Seq Analysis

After RNA extraction, library preparation was conducted in two batches using KAPA mRNA Hyper Prep Kit with Dual Indexing Adapters (PND21 samples) and Illumina TruSeq stranded mRNA Library Prep Kit (5-month samples). These libraries were cleaned up and quantified using the Agilent 2200 TapeStation. Sequencing on these samples was carried out on the NovaSeq S2 flow cell and the Illumina HiSeq 4000, generating single-end, 50-bp reads for each library.

ERRBS Data Analysis

Sequence quality per sample was first assessed with FastQC (v0.11.3), then Trim Galore! (v0.4.5) was used for adapter and quality trimming. More specifically, low-quality bases (quality score lower than 20), adapter sequences (required overlap of 6bp), and end-repair bases from the 3’ end of reads were trimmed, and all reads shorter than 20bp after trimming were discarded. Bismark (41) was used for alignment and methylation calling, where reads were aligned to mm10 genome using Bowtie2 and methylation calls were reported for all nucleotides with a read depth of at least 5.

Differentially methylated cytosines (DMCs) and differentially methylated regions (DMRs) of 50 bp windows were identified using the methylSigDSS function of the methylSig R package (42, 43), with significance cutoff of FDR < 0.05 and an absolute difference in methylation of > 10%. The number of DMCs and DMRs was similar across conditions, so we used DMCs for all of the following results but enrichment analysis. Sufficient sequencing coverage for a minimum of four samples from each treatment group was required for the test. Batch effect was taken into consideration by using run number as a covariate, and sex was used as a covariate for combined analysis of both females and males. Sex-stratified tests were also performed for each tissue and time point. The annotation was performed with the annotate_regions function of the annotatr R Bioconductor package (44). Gene set enrichment testing on DMRs was performed using the nearest TSS locus definition in the ChIP-Enrich R Bioconductor package to find enriched pathways, and pathways with an FDR < 0.05 were considered significant (45).

Annotation of DMCs to CTD and Imprinted Genes

Genes having previous evidence of correlating with DEHP exposure were downloaded from the Comparative Toxicogenomics Database (CTD) (46), resulting in a list of 1186 genes from 1903 literature entries. A list of 316 mouse imprinted genes was generated by retrieving imprinted gene sets from two publications (47, 48), followed by removal of the redundant genes.

RNA-Seq Data Analysis

Data quality checks and gene feature summarization were conducted using the TaRGET II RNA-seq pipeline set by the Consortium Data Coordinating Center (DCC). Genes with >1 CPM in at least 10 samples across all the consortium samples were used for normalization and differential analysis, which was conducted by the DCC using the RUVr function in RUVSeq (49) (k = 3) separately for each institution’s data and DESeq2 (50), respectively. As determined to be optimal by the DCC, differentially expressed genes were identified with absolute fold change > 1.5 and adjusted P-value < 0.001. Cell type deconvolution was conducted on the RNA-seq data of all blood samples at weaning (PND21) and adult (5 month) using CIBERSORTx (51). The differences of cell portions by treatment were tested with t-test.

Results

Genome-Wide Differential Methylation Analysis for PND21 and 5-month Mice

To identify epigenetic changes in liver and blood induced by perinatal DEHP exposure in our mouse model, we focused on the effect of DNA methylation by conducting enhanced reduced representation bisulfite sequencing (ERRBS). All of the 99 samples (see Fig. 1B for individual group sizes) resulted in sufficient

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**Table 1: sex-stratified differentially methylated cytosines (DMCs) consistent in both blood and liver at PND21 and 5 months**

| Group | # of DMCs | Direction in DEHP | Location | Gene | Annotation | Methylation difference % (blood; liver) |
|-------|-----------|--------------------|----------|------|------------|----------------------------------------|
| PND21 female | 1 | Hypermethylated | chr2:32672381 | Eng | Promoter; 1to5kb; exon | 30.59; 32.98 |
| PND21 male | 0 | | | | | |
| 5-month female | 3 | Hypermethylated | chr4:153333737 | Intergenic | 12.63; 16.47 |
| | | Intergenic | chr6:132173514 | Intergenic | 17.33; 16.54 |
| | | Hypermethylated | chr9: 43151371 | Pou2f3 | Intron | -13.50; -20.93 |
| | | Hypermethylated | chr6:12402190 | Intergenic | 10.85; 53.92 |
| | | Hypermethylated | chr6:114408371 | Intergenic | 16.27; 11.60 |
| | | Hypermethylated | chrX : 37950454 | Rhox11-ps2-201 | 1to5kb | -10.05; -25.61 |
| | | Hypermethylated | chr13:53564366 | Intergenic | -12.63; -23.13 |
| | | Hypermethylated | chr14:17868149 | Slc6a9; Cdc24 | Exon; 3’UTR | -14.86; -17.66 |
| | | Hypomethylated | chr16:93981703 | Cldn14 | Intron | -15.15; -14.91 |

Genomic locations and genic annotations are provided. The percentage of methylation difference was calculated as DEHP—Ctrl in blood and liver samples respectively. The gene marked in bold indicates prior correlation with DEHP exposure from CTD.
DEHP induced DNA methylation changes in mice | 5

To examine how much inter-individual variation can be explained by methylation, we calculated the methylation level of the sex-specific DMCs identified above for all samples. The majority of individual animals could be clearly distinguished as PND21 or 5 months of age on the basis of methylation percentage of DMCs in both blood and liver (Fig. 3E and F). More specifically, within blood samples, PND21 and 5-month animals separate clearly in PC1, with significantly more heterogeneity among the 5-month animals (Fig. 3E, Supplementary Fig. S3A). This separation is not as obvious in liver samples in the first three principal components, due to the even higher variation among 5-month livers (Fig. 3F, Supplementary Fig. S3B). The PND21 subjects do not separate well based on treatment either in blood or in liver, while there is trend of separation between DEHP and control groups in blood samples at 5 months.

We identified a total of 1215 DMCs that mapped to gene promoters at PND21 (219 DMCs mapped to 149 genes) and 5 months (996 DMCs mapped to 715 genes) (Supplementary Table S2). We further prioritized these DMCs by overlapping the promoter annotated genes with genes shown to be correlated with DEHP exposure, as indicated in the Comparative Toxicogenomics Database (CTD). Out of 2024 PND21 and 10312 5-month sex-specific DMCs, 74 (3.66%) and 358 (3.37%) were mapped on CTD genes, respectively. The number of DMCs that overlapped with CTD genes can be found in Supplementary Table S3, while a complete list of DMCs with their corresponding locations and their relevance to CTD genes are displayed in Supplementary Table S4. Interestingly, DEHP was found to affect the mRNA expression of the GNAS gene [53], an imprinted gene in the G protein-coupled receptors and Ras signaling pathway. We identified promoter hyper-methylation of GNAS in the DEHP-exposed group in both male liver and blood at PND21, and both female liver and blood at 5 months. The prioritized gene that had the highest methylation change in female blood at PND21 was Ucp2, which encodes a mitochondrial uncoupling protein, and was found to be both hypermethylated (53.89% methylation difference) and DEHP-relevant in CTD with opposite direction of gene expression. We also identified three hypomethylated sites.

Figure 2: overview of the number of DMCs between DEHP exposure and control groups. The bar plot shows the number of hypermethylated and hypomethylated DMCs in DEHP separately for each comparison at both PND21 and 5 months.

Annotating these sex-specific DMCs to genes and CpG islands revealed that at PND21, more DMCs occurred in CpG islands and shores in female blood compared with the other groups, and slightly higher proportion of DMCs were annotated to introns and exons in female samples than male samples (Fig. 3A and B). At 5 months, a similar but more significant trend was observed for higher rates of CpG island and shore DMCs in female blood (z-test P-value < 10^{-5}), although there was a higher proportion of DMCs annotated to introns in liver than in blood in female samples (Fig. 3C and D).

quality of data covering 5% of all CpG sites across the mouse genome. The total alignment percentage ranged from 58.5% to 72.4%, and the average bisulfite conversion rate was 99.9%.

When comparing differential methylation between the DEHP exposed group and the control group at each time point, including sex as a covariate, we found fewer changes at PND21 (an average of 39 DMCs) than at 5 months (an average of 453 DMCs) of age for both blood and liver. The number of DMCs detected at 5 months was approximately an order of magnitude higher than those at PND21, despite DEHP exposure stopping at PND21 for the F1 offspring (Supplementary Table S1).

The majority of the covered CpG sites (82.75% for females and 81.04% for males) are overlapped among the four respective groups (PND21 blood, PND21 liver, 5-month blood, 5-month liver), indicating the CpG sites were covered comprehensively among all comparisons. Larger numbers of DMCs were consistently identified across time and tissues using sex-specific analyses, again with more significant DMCs at PND21 blood, PND21 liver, 5-month blood, and 5-month liver, Supplementary Fig. S1), indicating the CpG sites were covered comprehensively among all comparisons. Larger numbers of DMCs were consistently identified across time and tissues using sex-specific analyses, again with more significant DMCs at PND21 than at 5 months.

Supplementary Fig. S2). Compared to the overall, sex-combined analyses, we observed higher numbers of both hypo- and hyper-methylated sites with DEHP exposure, with similar numbers of hypermethylated and hypomethylated CpGs (Fig. 2).

We next tested for sex-specific methylation changes, since endocrine disruptors often have sex-specific effects [52]. The majority of the covered CpG sites (82.75% for females and 81.04% for males) are overlapped among the four respective groups (PND21 blood, PND21 liver, 5-month blood, 5-month liver, Supplementary Fig. S1), indicating the CpG sites were covered comprehensively among all comparisons. Larger numbers of DMCs were consistently identified across time and tissues using sex-specific analyses, again with more significant DMCs at PND21 than at 5 months.

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in the DEHP group in the promoter region of the 
Esr1 (estrogen receptor 1) gene in female liver of 5 months.

Pathways Enriched with Differential Methylation in 
DEHP Exposure

In addition to individual genes, we were interested in identifying sex-specific pathways enriched with differential methylation at PND21 and/or 5 months as a result of DEHP exposure. We found more enriched GO terms for 5 months (n = 408) than for PND21 (n = 16) (Supplementary Fig. S4A and B). After combining related GO terms, organ growth and morphogenesis in thymus, muscle, eye, heart, and embryos were enriched in female blood at 5 months, together with cell–cell adhesion. In female liver, cellular response to estrogen stimulus, DNA binding and intracellular transport were significant. For male-specific pathways, cell adhesion molecule binding was enriched in blood, while muscle contraction, cholesterol homeostasis, and organ morphogenesis were enriched in liver.

Consistent Differential Methylation Marks across 
Tissues and Time Points

To address the TaRGET II consortium’s aim of determining the extent to which a surrogate tissue (blood) reflects the changes observed in a target tissue (liver), we compared the DMCs from liver and blood at both PND21 and 5 months. We identified only one overlapped DMC in female for PND21 samples, which was near the promoter region of the Eng gene. Eng encodes the endothelin protein which plays an important role in the regulation of angiogenesis (Table 1). A total of 12 and 12 overlapped DMCs were detected for 5-month female and 5-month male, respectively, but only 3 and 7 of these were changed in the same direction for both tissues (Table 1). The only gene showing prior correlation with DEHP exposure from CTD was a glycine
DNA methylation differences between blood and liver are relevant for determining the extent to which Cpg can be used as a surrogate for biomarkers of exposure. We investigated the methylation level at Cpg islands, shores, and shelves for all the mice tested at PND21, and the result showed that levels were significantly higher in blood than in liver for all these regions, especially in Cpg shores and shelves (Fig. 4A). No clear sex-specific difference was found. Upon replicating the comparison for 5-month mice, we found an even more drastic difference between the methylation levels of blood and liver (Fig. 4B). In both cases, no clear overall difference in methylation levels was observed between DEHP exposure and the control group. These large baseline methylation differences in blood compared to liver may contribute to the lack of consistent changes observed due to DEHP exposure.

DEHP Exposure Impacts DNA Methylation of Imprinted Genes

Previous publications suggest that exposure to DEHP and other endocrine disruptors can profoundly alter epigenetic marks in imprinted genes (53, 55, 56); however, the effect of perinatal DEHP in blood and liver specifically remains unknown. Here, we compared the methylation level of mouse imprinted genes at Cpg islands, shores, and shelves, and discovered that the effect of DEHP on imprinted genes is both sex and tissue specific (Fig. 5A and B). For instance, male liver showed slight increased methylation at Cpg shores with DEHP exposure at both PND21 and 5 months, while male blood had decreased methylation at Cpg shelves at PND21. In general, changes in imprinted gene methylation levels by DEHP exposure were more obvious in liver than in blood, reinforcing the sensitivity of liver as a target tissue of DEHP exposure.

We found multiple mouse imprinted genes with DMCs in their promoter at both time points. For instance, the imprinted gene we found with the most DMCs was Gnas, which had 11 DMCs with DEHP hypermethylation in PND21 male blood and liver, as well as 5-month female blood, female liver, and male liver (Supplementary Table S5). Another important imprinted gene, Runx1, which functions in hemopoiesis, was found to have promoter DEHP hypomethylation in female blood at PND21, but with DEHP hyper-methylation in female blood at 5 months. A similar pattern was found for Zrsr1, a maternally imprinted gene, which showed both DEHP hyper- and hypomethylation in its promoter region at both time points in female liver. Despite lack of averagemethylation change on the gene body, Igef2r was found with several DMCs in both directions of female blood, female liver, and male blood. A complete list of imprinted genes with more than two DMCs mapped on them can be found in Supplementary Table S5.

DEHP Exposure Has Greater Effects on Gene Expression at PND21 than at 5 months

To examine whether the differential methylation associated with DEHP exposure had an impact on gene expression at the same time points, RNA-seq data were generated using RNA from the same animals. Results showed a subtle overall effect, with only a few differentially expressed genes identified at 5 months. At PND21, a total of 5 and 70 significant genes are in male and female blood, respectively, and 52 and 706 in female and male liver (the total number of differentially expressed genes in each group are listed in Fig. 6A, and a complete list of these genes and their corresponding details are in Supplementary Table S6). By comparing differential gene expression with previously identified DMCs, we did not observe any genes with both altered DNA methylation and change in gene expression at 5 months. However, Zfhx3, Sh3pxd2a, and Plec were all upregulated in the DEHP exposure group at PND21 in female liver, and these genes also harbored hypomethylation on the gene body in the respective group. On the other hand, downregulated gene Col4a2 was found with hypermethylation on exon 23 in male blood. In PND21 male liver, a total of 20 differentially expressed genes were found to harbor 24 DMCs, with 1 of these genes being hypermethylated and downregulated,
and 9 being hypomethylated and upregulated (Fig. 6B, Supplementary Table S7). Two of the genes, Dnmt3a and Abca2, were DEHP-relevant genes in CTD with opposite direction between gene expression and methylation.

Discussion

In this study, we used a mouse model with pre- and peri-natal DEHP exposure to assess resultant genome-wide methylation responses at two ages (PND21 and 5 months) using ERRBS. We identified sex-specific DNA methylation changes in both juvenile and adult mouse liver and blood linked with the DEHP exposure. We found a few hundred genes harboring DMCs in their promoter regions, though only one (Spata13) showed significant gene expression change of the opposite direction from DNA methylation. For instance, the gene Esr1 was found to harbor three hypomethylated sites upon DEHP exposure in the promoter region in livers of 5-month-old females. Interestingly, although previous studies demonstrated both positive and negative correlations between DEHP exposure and change in Esr1 gene expression (57–60), the two studies of mouse liver both suggested that DEHP exposure is correlated with an increase in Esr1 expression. Our finding of hypomethylation, which occurred in liver but not blood, is consistent with the fact that the previously-observed effects of DEHP on Esr1 expression were liver-specific and not observed in other tissues (60).

Other interesting finding lies with the imprinted gene GNAS, which displayed promoter hyper-methylation in the DEHP group in both male liver and blood at PND21, and both female liver and blood at 5 months. As an important component in G protein-coupled receptors, GNAS plays a role in development of obesity and regulation of energy balance (61). Notably, GNAS was previously found to have differential methylation in response to many types of environmental exposures, including bisphenol A (62), lead (54), folic acid depletion (63), gases/fumes (64), and prenatal maternal stress (65), suggesting the GNAS imprinted cluster may be an especially labile region, sensitive to many environmental exposures. Although there was prior evidence from CTD showing the impact of DEHP on DNA methylation levels of imprinted genes Igf2r, Peg3, and H19 in mouse

Figure 4: sex- and tissue-stratified genome-wide DNA methylation levels at CpG islands, shores, and shelves at (A) 3 weeks (PND21) and (B) 5 months. Blood shows higher DNA methylation levels than liver in CpG shores and shelves for both sexes, and with tissue differences increasing with mouse age.
oocytes (18), we did not observe any significant changes in DNA methylation at these genes in either blood or liver. Most of the differential methylation marks identified in this study were sex-specific, which is related to the fact that DEHP interacts with the androgen (AR), estrogen (ER), and PPARs, with a negative impact on testosterone production (66). Another explanation lies with the fact that reprogramming of post-fertilization methylation and in primordial germ cells occur with different kinetics and mechanisms between males and females (67). Multiple direct effects at the molecular level of both DEHP and its metabolites were observed in vitro by using a reporter assay with human cell lines on the estrogen receptor (ESR1) and on AR (68, 69). In our study, Esr1 demonstrated sex-specific differential methylation at its promoter region, while AR also showed sex-specific differential methylation, but in the gene body region. PPARs also regulate the expression of UCPs, including Ucp2, which harbored a hypermethylated site with 53.89% methylation difference, and previous research showed DEHP can result in decreased expression of Ucp2 (70).

The differential methylation results also revealed that the number of DMCs at 5 months were almost 3-5 times the number of DMCs at PND21, indicating DEHP exposure early in life is correlated with more significant altered DNA methylation at a later stage of mouse development. Interestingly, significantly greater heterogeneity in methylation was observed among the 5-month animals than PND21 counterparts in both blood and liver. One explanation could be increased cell type methylation heterogeneity with age after DEHP exposure, which has been reported in mice of various ages (71–73), but we are not able to distinguish this hypothesis from the effect of a few outlier individuals without single-cell analysis. Another possibility lies with 'environmental deflection', a toxicant-mediated shift away from the baseline rates of age-related methylation (74), where the deflection of DEHP could shift the methylation process during.

Figure 5: sex- and tissue-stratified genome-wide DNA methylation levels of imprinted genes at CpG islands, shores, and shelves at (A) 3 weeks (PND21) and (B) 5 months. Blood shows higher DNA methylation levels than liver in CpG shores and shelves for both sexes, and slightly higher level of differences in liver was observed between DEHP and control group compared with the differences in blood.
development. In support of DEHP causing more heterogeneity with age, we did observe slightly higher variance levels in the DEHP groups compared with control groups at 5 months for age-related CpGs (data not shown, average percentage increase of standard deviation $\mu = 5.65\%$).

In particular, we identified many enriched pathways at 5 months only, in either blood or liver. For instance, cellular response to estrogen stimulus and regulation of intracellular transport were significant in female liver, consistent with DEHP’s effect on female hormones (75), while cholesterol homeostasis and channel activity were significant in male liver (Supplementary Fig. 4B), and many of the aforementioned pathways are relevant to the action of DEHP in liver. Organ growth, morphogenesis, and cell–cell adhesion were enriched in female blood, suggesting the epigenetic effect of DEHP has potential effects on organ development and epithelial-mesenchymal transition (EMT) (76).

These methylation changes, however, did not translate to differential expression at the 5-month time point, which is in line with the finding of a recent study on endocrine disrupting chemicals showing that reprogrammed genes remained transcriptionally silent without any impact on metabolism until a later life stage (77). It may be that additional aging or an external stimulus leads to further differential gene expression. We did not identify many affected GO terms at PND21, possibly due to the lower degrees of differential methylation. We identified two genes, Dnmt3a and Abca2 with DEHP hypomethylation and upregulation in male liver at PND21. Dnmt3a is an important epigenetic modifier in embryonic development and imprinting, and a few previous studies re-confirmed the DEHP exposure

Figure 6: differential gene expression analysis on the same cohort. (A) Bar plot showing the number of upregulated and downregulated genes in blood and liver separately at the two time points. (B) Scatterplot showing all the DMCs that overlapped with upregulated or downregulated genes. The top half represents genes that are significantly upregulated in DEHP exposure groups, and the right half represents hypermethylated DMCs in DEHP exposure groups. Sites that showed opposite direction of DNA methylation and gene expression are labeled with gene symbols.
results in increased expression (78, 79). Another study on low-dose pollutant mixture emphasized the association between DEHP exposure and decreased expression of Aboa2 (80), which is a PPAR target gene and a member of the superfamily of ATP-binding cassette (ABC) transporters.

Our study did have limitations that may have resulted in missing some important findings. The use of ERRBS resulted in the analyzed CpG sites being biased toward CpG islands and shores, and other areas of higher-than-average CpG density. The technique of ERRBS does not distinguish 5hmC (5-hydroxymethylcytosine) from 5mC. Since we only focused on 5mC in this study, Oxy-RRBS would have been a more accurate measurement option. As the next step, we plan to analyze the methylation profile in the same cohort more comprehensively using whole genome bisulfite sequencing (WGBS), which captures CpG sites of the entire genome at single-base resolution. Due to limited resources, we did not measure the blood cell count before isolating DNA and RNA in our experiments, which could have ruled out the potential effects of blood cell type differences in the peripheral blood leukocytes between DEHP exposure and control groups (81). As an alternative to using the blood cell counts, we performed cell type deconvolution with the RNA-seq data on all the blood samples, and the results showed no significant difference between DEHP exposure and control groups regarding all cell types tested (Supplementary Fig. S5). Although this could indicate that the blood cell counts did not play a major role in the methylation level difference among each sex-specific group, we cannot rule out a slight overall shift in the percent of leukocytes. Our relatively small sample sizes when stratified by sex may also have resulted in missing many of the more subtle DNA methylation and gene expression differences, with small effect sizes at the given DEHP dosage.

As part of the TaRGET II consortium, one of the objectives of this study was to determine the extent to which surrogate tissues (blood) reflect the target tissues (liver) in terms of DNA methylation, and whether methylation marks are consistent or different ages of animals (26, 82). Although hundreds to thousands of DMCs and DMRs were detected in both liver and in blood at both PND21 and 5 months, only a few DMCs overlapped among them. For example, the same promoter DMC near the Esng gene was detected in female blood and in liver at PND21, and Slc6a9 had the same hypomethylated mark between the two tissues in male at 5 months, whose upregulation was linked to DEHP exposure (83). Overlapped enriched pathways between liver and blood at 5 months included cell fate commitment, transcription factor activity, and cyclic nucleotide metabolic process. Due to the small number of consistent marks between tissues in both juvenile and adult stages, we conclude that in general blood does not serve as a good surrogate tissue for liver, in terms of the DNA methylation profile changes due to our dose of perinatal DEHP exposure. However, the blood-specific methylation changes may be used as biomarkers for perinatal DEHP exposure. Indeed, we identified a small number of individual CpG sites affected in both tissues by DEHP in a sex-specific manner, which can serve as a starting point for targeted studies with larger sample sizes.

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Data Availability

Raw ERRBS data and Bismark coverage files supporting the conclusions of this article have been deposited in NCBI’s Gene Expression Omnibus (GEO) and can be accessed with accession number GSE150670. Both raw and processed RNA-seq data are available on GEO with accession number GSE146508.

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Conflict of interest statement

None declared.

Supplementary data

Supplementary data are available at EnvEpig online.

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