Perinatal exposure to lead results in altered DNA methylation in adult mouse liver and blood:
Implications for target versus surrogate tissue use in environmental epigenetics

LK Svoboda¹, K Neier¹, R Cavalcante², Z Tsai^{1,3}, TR Jones¹, S Liu³, JM Goodrich¹, C
Lalancette², JA Colacino¹, MA Sartor^{3,4}, DC Dolinoy^{1,5}

¹Environmental Health Sciences, University of Michigan
School of Public Health
1415 Washington Heights
Ann Arbor, MI 48109, U.S.A.

²Epigenomics core, University of Michigan
Medical School
1150 W. Medical Center Dr.
Ann Arbor, MI 48109, USA

³Department of Computational Medicine and Bioinformatics, University of Michigan
Medical School
Palmer Commons, 100 Washtenaw Ave #2017
Ann Arbor, MI 48109, U.S.A.

⁴Department of Biostatistics, University of Michigan
School of Public Health
1415 Washington Heights
Ann Arbor, MI 48109, U.S.A.

⁵Nutritional Sciences, University of Michigan
School of Public Health
1415 Washington Heights
Ann Arbor, MI 48109, U.S.A.

Corresponding Author: Dana C. Dolinoy, ddolinoy@umich.edu
Telephone: (734) 647-3155
Environmental Health Sciences, University of Michigan
School of Public Health
1415 Washington Heights
Ann Arbor, MI 48109, U.S.A.

Running Title: Perinatal exposure to lead and altered liver and blood DNA methylation

Acknowledgements: This work was supported by the National Institute of Environmental Health Sciences (NIEHS) TaRGET II Consortium (ES026697), along with the University of Michigan NIEHS/EPA Children's Environmental Health and Disease Prevention Center P01 ES022844/RD83543601, the Michigan Lifestage Environmental Exposures and Disease (M-LEEaD) NIEHS Core Center (P30 ES017885), Institutional Training Grant T32 ES007062, and NIEHS grant R01 ES028802.

Declaration:

The authors declare no conflict of interest.

ABSTRACT

Background

DNA methylation is a critical epigenetic mechanism linking early developmental environment to long-term health. In humans, the extent to which toxicant-induced changes in DNA methylation in surrogate tissues, such as blood, mirror those in the target tissues is unclear. The Toxicant Exposures and Responses by Genomic and Epigenomic Regulators of Transcription (TaRGET II) consortium was established by the National Institute of Environmental Health Sciences to address the utility of surrogate tissues as proxies for toxicant-induced epigenetic changes in target tissues.

Objectives

The objective of this study was to investigate the effects of perinatal exposure to a human environmentally relevant level (32 ppm in maternal drinking water) of lead (Pb) on liver and blood DNA methylation in adult male and female mice. We hypothesized that developmental Pb exposure would lead to persistent changes in DNA methylation, and that a subset of differentially methylated loci would overlap between liver and blood.

Methods

Enhanced reduced-representation bisulfite sequencing was used to assess DNA methylation in 5 month old Pb-exposed and control mice. Sex-stratified modeling of differential methylation by Pb exposure was conducted using an established bioinformatics pipeline.

Results

Although Pb exposure ceased at 3 weeks of age, we observed thousands of stably modified, sex-specific differentially methylated regions in the blood and liver of Pb-exposed animals, including 44 genomically imprinted loci. In males, we discovered 5 sites that overlapped between blood and liver, and exhibited changes in DNA methylation in the same direction in both tissues.

Conclusions

These data demonstrate that perinatal exposure to Pb induces sex-specific changes in hepatic DNA methylation in adulthood, some of which are also present in blood. Ongoing studies will provide additional exposure-specific insights, and include other epigenetic marks that will enable further refinement of the design and analysis of human studies where target tissues are inaccessible.

83

84 INTRODUCTION

85 It is increasingly evident that environmental exposures and nutritional perturbations during
 86 critical windows of development can influence the long-term risk of disease. One important
 87 mechanism by which early-life environment can reprogram disease risk is *via* modification of the
 88 epigenome. Normal human development is governed by precise spatio-temporal regulation of
 89 gene expression, and this is driven in large part by epigenetic mechanisms. Methylation of DNA
 90 at the 5th-position of cytosines (5-methylcytosine, 5mC) occurring in cytosine-phosphate-
 91 guanine (CpG) dinucleotides is critical for establishment of tissue-specific gene expression
 92 patterns, regulation of imprinted genes, maintenance of genome stability, and silencing of
 93 transposable elements (Jones 2012). 5mC undergoes distinct waves of reprogramming during
 94 early embryonic development and in primordial germ cells, and is stable and mitotically heritable
 95 (Reik et al. 2001). Epigenetic reprogramming during these periods of rapid growth and
 96 differentiation can be altered by environmental or nutritional perturbation. 5mC can undergo
 97 further oxidation to 5-hydroxymethylcytosine (5hmC) (Lopez et al. 2017). Although 5hmC is an
 98 intermediate in the DNA demethylation pathway, recent studies suggest that the genomic
 99 distribution, downstream targets, and functional roles of 5hmC are distinct from that of 5mC
 100 (Lopez et al. 2017). Environmental exposures may also lead to reprogramming of 5hmC, and
 101 these changes are associated with altered transcription and adverse health outcomes (Huang et
 102 al. 2019; Kochmanski et al. 2018; Ringh et al. 2019).

103 Genomic imprinting, which results in mono-allelic, parent-of-origin expression of a subset of
 104 genes, is mediated in large part by DNA methylation, and establishment of 5mC at imprinted loci
 105 occurs during early embryonic development (Barlow and Bartolomei 2014; Okae et al. 2014).
 106 Importantly, we and others have reported that environmental exposures during this critical
 107 developmental window lead to altered 5mC and 5hmC at imprinted loci (Bansal et al. 2017;
 108 Faulk et al. 2014b; Kochmanski et al. 2018; Nye et al. 2016; Susiarjo et al. 2013) and alterations
 109 in imprinted gene expression (Kochmanski et al. 2018; Susiarjo et al. 2013). Abnormal DNA
 110 methylation and expression patterns of imprinted genes are associated with several diseases,
 111 including growth, metabolic and developmental disorders, cardiovascular diseases, psychiatric
 112 illnesses, and cancer (Skaar et al. 2012).

113 Exposure to heavy metals, including lead (Pb), remains a significant public health concern,
 114 particularly in poor urban communities due to various sources of contamination (e.g., leaded
 115 paint and pipes, mining, lead-glazed ceramics). Although the neurological effects of early-life Pb
 116 exposure have been well-documented, substantially less is known about how Pb exposure
 117 impacts the liver and other metabolic organs. Notably, recent human studies suggest that blood
 118 Pb levels are associated with increased risk of liver disease and diabetes (Bener et al. 2001;
 119 Cave et al. 2010; Zhai et al. 2017). Likewise, maternal blood Pb levels are associated with
 120 increased DNA methylation of the imprinted *MEG3* locus in offspring blood, concomitant with
 121 rapid gains in body fat (Nye et al. 2016). In animal models, we and others have recently
 122 reported that perinatal (Faulk et al. 2013; Faulk et al. 2014a; Leasure et al. 2008; Wu et al.
 123 2016) and post-natal (Sun et al. 2017; Xia et al. 2018a; Xia et al. 2018b) exposure to Pb leads
 124 to sex-specific changes in body weight, metabolic function, and gut microbiota. However,
 125 although *post-natal* Pb exposure leads to hypermethylation of genes associated with glucose
 126 and lipid metabolism in the rat liver (Sun et al. 2017), the effects of perinatal Pb exposure on

hepatic DNA methylation, and the implications this may have for long-term disease risk, are unclear.

In human population-based environmental epigenetics studies, easily obtainable sources of DNA (e.g. hair, blood, stool, saliva) are used as surrogates for the tissue(s) targeted by the environmental exposure. It is unknown, however, to what extent exposure-induced epigenetic changes in surrogate tissues reflect those occurring in target tissues. Likewise, whether toxicant-induced epigenomic changes persist in both target and surrogate tissues over time within individuals is unclear. To address these important questions, the National Institute of Environmental Health Sciences (NIEHS) established the Toxicant Exposures and Responses by Genomic and Epigenomic Regulators of Transcription (TaRGET II) consortium (Wang et al. 2018). Using mouse models of human-relevant environmental exposures, the objective of TaRGET II is to assess the conservation of environment-induced epigenomic perturbations between easily obtainable surrogate and disease-relevant, but inaccessible, target tissues. As part of this effort, we used an established mouse model of perinatal Pb exposure to determine the effects of Pb on genome-wide DNA methylation in the liver and blood of 5-month old, adult animals. We hypothesized that perinatal Pb exposure would lead to alterations in DNA methylation in liver and blood that persist into adulthood, and that a subset of these sites would overlap between the two tissues. Additionally, given work from our lab and others demonstrating that Pb exposure is associated with alterations in 5mC at imprinted loci (Faulk et al. 2014b; Nye et al. 2016), we hypothesized that these loci would be targets of Pb exposure in our study.

METHODS

Animal Exposure Paradigm

Mice utilized for breeding and exposure were obtained from a colony maintained for over 230 generations with the *A^y* allele passed through the male line, resulting in forced heterozygosity on a genetically invariant background with 93% identity to C57BL/6J (Waterland and Jirtle 2003; Weinhouse et al. 2014). Virgin *a/a* females (6–8 wks old) were mated with virgin *a/a* males (7–9 wks old), and randomly assigned to control or Pb exposure via drinking water. For Pb exposure, Pb-acetate was mixed with water to result in a Pb concentration of 32 ppm in drinking water, to model human relevant maternal exposure in the 16-60 µg/dL range (Faulk et al. 2013). Exposure water was made by dissolving Pb (II) acetate trihydrate (Sigma-Aldrich) in a single batch of distilled water, and Pb concentrations were verified using inductively coupled plasma mass spectrometry with a limit of detection of 1.0 µg/L (ICPMS; NSF International). Animals were maintained on a phytoestrogen-free modified AIN-93G diet (TD.95092, 7% Corn Oil Diet, Harlan Teklad). The *a/a* dams in the Pb group were exposed to Pb-supplemented drinking water for two weeks prior to mating *a/a* males. Exposure was continued during gestation and lactation. After weaning, the resulting pups were weighed and switched to Pb-free drinking water (**Figure 1**). A subset of pups from each exposure group, representing approximately 1–2 male and 1–2 female offspring per litter, was maintained to 5 months of age (n=6 males and 6 females per treatment). All animals had access to food and drinking water *ad libitum* throughout the experiment while housed in polycarbonate-free cages. The study protocol was approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC).

Body Composition and Tissue Collection

Body weights were measured for each mouse on a weekly basis (Mettler Toledo). Total body fat and lean mass were measured via EchoMRI (Houston, TX USA). Body fat percentage and lean mass percentage were calculated as a percent of total body weight. Upon euthanasia at 5 months of age, mesenteric fat, blood, and liver samples were collected in accordance with protocols established by the TaRGET II Consortium (**Figure 1** and (Wang et al. 2018)). Briefly, mice were fasted for 6 hours prior to euthanasia. Euthanasia was carried out via CO₂ asphyxiation and bilateral pneumothorax. Blood was collected by cardiac puncture, followed by whole body perfusion with cell culture grade 0.9% saline solution (Sigma Life Sciences). Next, liver and mesenteric fat were collected and weighed. Mesenteric fat was collected by dissecting white adipose tissue away from the stomach, spleen, pancreas, and intestines. Relative liver and mesenteric fat weights were expressed as a percent of total body weight. Liver was separated into individual lobes, and the left lobe was used for the analyses in this study. Liver and fat samples were immediately snap frozen in liquid nitrogen and stored at -80°C. Blood was collected with EDTA anticoagulant, centrifuged, and plasma was removed. Red blood cells were lysed using Erythrocyte Lysis Solution (Qiagen, 79217). The remaining white blood cell fraction was washed with PBS, pelleted by centrifugation, and resuspended in Buffer RLT (Qiagen, cat # 1053393). Crude cell lysate was stored at -80°C until DNA extraction.

DNA Extraction and Enhanced Reduced Representation Bisulfite Sequencing

The left lobe of the liver was cryo-pulverized and suspended in Buffer RLT (Qiagen, cat # 1053393). Blood and liver DNA extraction were performed using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen #80224). Enhanced reduced representation bisulfite (ERRBS) was performed at the University of Michigan Epigenomics Core as described previously (Akalın et al. 2012; Garrett-Bakelman et al. 2015). Briefly, 50 ng of genomic DNA was digested using MspI, a restriction enzyme that preferentially cuts CG-rich sites. 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. Both methylated and hydroxymethylated CpGs are protected from bisulfite conversion. Therefore, ERRBS detects a combination of 5mC and 5hmC. Final 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). Single-end, 50 bp reads were obtained for each library by sequencing on the HiSeq 4000 system (Illumina). ERRBS samples were multiplexed, with three samples per sequencing lane.

Bioinformatics pipeline, quality control, and differential methylation analysis

Snakemake (Koster and Rahmann 2012) was used to manage the bioinformatics workflow, and Anaconda (Anaconda Software Distribution) was used to manage software dependencies and create reproducible software environments. For quality control, FastQC (v0.11.3) was employed to assess the overall quality of each sequenced sample and identify specific reads and regions for trimming. TrimGalore (v0.4.5) was used to trim low-quality bases (quality score lower than 20), adapter sequences (required overlap of 6bp), and end-repair bases from the 3' end of reads. Reads shorter than 20bp after trimming were discarded. For alignment and methylation

calling we used Bismark (Krueger and Andrews 2011) (v0.19.0), an integrated alignment and methylation call program that performs unbiased alignment (by converting residual cytosines to thymines prior to alignment in both reads and reference). Within Bismark, reads were aligned to the reference genome (mm10) using Bowtie2 (Langmead and Salzberg 2012) (v2.3.4) with default parameter settings (multi-seed length of 20bp with 0 mismatches). Methylation calls were reported for all nucleotides with a read depth of at least 5. We used the methylSig R package (Park et al. 2014) (v0.5.0) to identify differentially methylated CpG positions and/or regions. Differential methylation for each comparison was tested using methylSigDSS(), which tests for differential methylation under general experimental design using a beta-binomial approach with the 'arcsine' link function (Park and Wu 2016). To control for batch effects, run was included as a covariate in the model. After obtaining p-values, we adjusted for multiple testing using the FDR approach. The results of the differential methylation tests were then annotated using the annotatr R Bioconductor package (Cavalcante and Sartor 2017) (v1.5.9). The annotate_regions function was used to generate genomic annotations, which include CpG annotations [CpG islands (CGI), shores, shelves, open sea (InterCGI)], genic annotations (exon, intron, promoter, 5'UTR, 3'UTR), and gene IDs. CpGs with read coverage > 1000 were removed because they were likely the result of PCR amplification, and CpGs with read coverage < 10 were removed due to decreased power to detect differential methylation. Opposite strand CpGs at the same position were combined via destrandng. We performed differential methylation testing on individual CpG sites (DMCs), requiring sufficient sequencing coverage for a minimum of 4 samples from the Pb group, and 4 samples from the control group for a site to be tested. Differentially methylated regions (DMRs) were identified in 50 bp tiles using the same requirements. For both DMCs and DMRs, sites with FDR <0.05 and an absolute difference in methylation of > 10% were considered significant.

Pathway analysis of DMRs

Poly-Enrich (Lee C.T. 2019) was used to assess the biological pathways enriched among the DMRs. Separate pathway analyses were conducted for each sex, tissue, and direction of differential methylation, and were restricted to proximal promoter regions (within 1 kb of transcription start sites). The following Gene Ontology gene sets used for this analysis: Biological Process, Cellular Component, and Molecular Function. Pathways with FDR <0.05 were considered statistically significant.

Analysis of overlap in differentially methylated sites between blood and liver

Annotated lists of DMCs and DMRs for males and females were compared between blood and liver to identify chromosomal locations that directly overlapped between the two tissues. Overlapping sites with changes in methylation in the same direction in both tissues were considered biologically relevant. In a further analysis, the full lists of DMR-associated genes in blood were compared to those in liver to identify a list of genes in common between the two tissues. Separate lists were generated for males and females. These gene lists were analyzed using DAVID version 6.8, Laboratory of Human Retrovirology and Immunoinformatics (LHRI), (<https://david.ncicrf.gov>) to identify pathways that were significantly enriched (FDR <0.05).

RESULTS

Litter parameters and phenotype

Perinatal lead exposure did not significantly alter the offspring sex ratio, litter size, or mortality rates ($p = 1.0, 0.9, \text{ and } 0.2$, respectively, Fisher's Exact Test) (**Table 1**). We have previously reported that perinatal Pb exposure leads to sex-specific changes in food intake, body weight, and body fat later in life ($n=12-18$ mice per sex) (Faulk et al. 2014a). However, given the smaller sample size in this study ($n=6$ mice per sex), it is not surprising that we observed no significant differences in body weight, total body fat, or body fat percentages in either males or females (**Supplemental Figure 1 A-F**). Nevertheless, consistent with our previous observations, we observed a trend toward increased total and relative mesenteric fat weights in males at 5 months, although this did not reach statistical significance ($p=0.06$, **Supplemental Figure 1 G-J**). Total and relative liver weights did not differ significantly between exposed and control groups in either males or females (**Supplemental Figure 1 K-N**).

Epigenome-wide differential DNA methylation with Pb exposure

To assess the effects of perinatal Pb exposure on DNA methylation across target and surrogate DNA, ERRBS was conducted on liver and blood samples from 5-month-old Pb and control-exposed offspring ($n=6$ males and 6 females per exposure group). Tables 2 and 3 illustrate the total number of regions tested and the number of differentially methylated cytosines (DMCs) and DMRs identified in each exposure group. Although Pb exposure ceased at 3 weeks of age, this analysis revealed thousands of sex-specific DMCs and DMRs in the adult liver and blood (**Tables 2-3**). The relative proportion of hypo- and hyper-methylated regions/cytosines was similar for each condition (**Tables 2-3**). Although observed methylation changes were between 10-20% at most sites with Pb exposure, numerous sites exhibited changes in methylation that were much greater, up to 70% in females and 74% in males (**Figure 2 A-D and Supplemental Figure 2**). We annotated the DMCs and DMRs to the mouse mm10 genome to determine the genomic locations of DMCs and DMRs relative to all sites tested, stratified by sex, tissue, and direction of differential methylation. Compared to all regions tested, DMCs/DMRs were significantly depleted from CpG islands, promoters, 5'UTRs, and exons, and were significantly enriched for regions greater than 4000 bp from a CpG island (interCGI) and intergenic regions (chi squared test, $p<.05$) (**Supplemental Figure 3 A-H**). This pattern was similar across sex and tissue (**Supplemental Figure 3 A-H**).

Pathway analysis of DMRs

Having established that *early life* Pb exposure leads to changes in DNA methylation in adulthood, we next determined the cellular pathways enriched among the DMRs (**Tables S1-S4**). We stratified our analyses by sex, tissue, and direction of differential methylation, and focused on proximal promoter regions (within 1 kb of transcription start sites). Pathways with a $FDR<0.05$ were considered significant. This analysis revealed that the pathways enriched among hypo-methylated sites were distinct from those enriched among hyper-methylated sites. In addition, the enriched pathways differed across sex and tissue. In spite of these differences, overall, we observed that Pb altered methylation of CpGs in key organ developmental pathways including mammary gland, bone, nervous system, and kidney development. Interestingly, we observed enrichment of pathways involved in regulation of neurotransmitter levels and activity in female and male liver.

Perinatal Pb exposure leads to changes in methylation at imprinted loci

Given previous findings from our lab and others that developmental exposure to BPA and Pb led to altered CpG methylation at imprinted loci (Faulk et al. 2014b; Kochmanski et al. 2018;

Nye et al. 2016), we investigated whether perinatal Pb exposure disrupted methylation at imprinted loci in liver and blood, and whether these changes overlapped between the two tissues. To do this, we compared the lists of Pb-induced DMCs/DMRs for each condition to a database of mouse imprinted genes (Williamson CM 2013). Of 139 imprinted genes interrogated, 112 were covered in this study using ERRBS (see Supplemental Excel file). We observed Pb-induced DMRs/DMCs at 44 imprinted genes combined across tissue and sex. Specifically, we identified 3 in female blood, 18 in female liver, 22 in male blood, and 21 in male liver. See **Tables 4-5** for DMCs mapping to imprinted genes in females and males, respectively, and **Supplemental Tables 5-8** for DMRs. In liver samples, we discovered 5 imprinted genes with DMCs in both males and females, including *Commd1*, *Gnas*, *Nespas*, *Pde10a*, and *Pde4d* (**Tables 4-5**). Methylation at CpGs in *Gnas/Nespas* was in the same direction in both males and females (**Figure 3A**). In blood, CpGs in *Meg3* were hypermethylated in both males and females (**Figure 3B**). We next compared DMC-associated imprinted genes between blood and liver within each sex. In females, no imprinted genes overlapped between blood and liver. In males, we observed DMCs at *Begain*, *Cdkn1c*, *Commd1*, *Peg12*, *Rasgrf1*, and *Snrpn* in both blood and liver, and found that CpGs in *Begain*, *Peg12*, *Rasgrf1*, and *Snrpn* exhibited changes in methylation in the same direction in both blood and liver (**Table 5 and Figure 3D-G**). Collectively, these data suggest that Pb exposure alters methylation at imprinted loci in a tissue and sex-dependent manner.

Overlap of DMRs and DMCs between liver and blood

An important objective of the TaRGET II Consortium is to identify signatures of environmental exposures in surrogate tissues that mirror those in target tissues. To this end, we first compared the chromosomal locations for DMCs and DMRs between liver and blood to determine whether there was overlap in differentially methylated sites between the two tissues. In females, 4 DMCs and 3 DMRs overlapped between blood and liver; however, the Pb-induced change in methylation at each of the overlapping sites was in the opposite direction for each tissue (**Table 6**). In males, 4 DMCs and 5 DMRs each overlapped between blood and liver (**Table 7**). In contrast with females, several sites in males exhibited changes in methylation that were in the same direction in both blood and liver. Among DMCs, these sites mapped to *Grifin*, *Elmsan1*, *Amn*, and *Tbc1d30*. Among DMRs, overlapping sites mapped to *Grifin* and *Plekhhg3*. An additional region was identified that did not map to a gene (**Table 7**). Next, we evaluated the overlap in DMC-associated genes between blood and liver in males and females. In males and females, we identified 325 genes in males and 188 DMC-associated genes in females that overlapped between blood and liver (**Figure 4 and Supplemental Tables 9-10**). Gene ontology analysis of the overlapping genes revealed enrichment for cell adhesion, protein binding, synapse, and neuronal process pathways (**Figure 4**). Overlapping genes in females were enriched for cell junction, membrane, and synapse pathways. We next compared the lists of DMC-associated genes with blood-liver overlap between males and females. A small number of genes (35 genes) were common between tissues and sexes (**Supplemental Table 11**). Together, these data suggest that there exist sex-specific changes in DNA methylation with perinatal Pb exposure that are consistent across blood and liver in adulthood, with few specific CpG sites overlapping directly between tissues.

DISCUSSION

In this work, we have investigated the effects of perinatal Pb exposure on DNA methylation in the liver and blood of adult mice. The findings from this study are novel for several reasons.

First, although increasing evidence links Pb exposure to adverse metabolic outcomes (Niessen et al. 2018), the mechanistic basis for these associations is unclear. Indeed, little is known about the effects of Pb exposure on the liver. To our knowledge, this is the first report demonstrating that Pb exposure during pregnancy and lactation leads to sex-specific alterations in hepatic DNA methylation in adulthood. Second, we discovered that perinatal Pb exposure leads to changes in methylation at several imprinted loci that are also reprogrammed by perinatal BPA exposure (Kochmanski et al. 2018). Finally, we mapped DNA methylation genome-wide in both blood and liver of the same mice after perinatal Pb exposure, and although few differentially methylated sites directly overlapped between blood and liver, we discovered many DMC-associated genes that overlap between tissues in both males and females. This finding has important implications for the design and interpretation of human population-based epigenomics studies.

Genome-wide changes in DNA methylation with Pb exposure

Although Pb exposure ceased at 3 weeks of age, we discovered thousands of DMCs/DMRs in both male and female mice at 5 months of age. In accordance with previous findings from our lab and others (Faulk et al. 2013; Faulk et al. 2014a; Sen et al. 2015b), the effects of Pb on DNA methylation were highly sex-specific. Indeed, when comparing males and females, pathway analyses revealed enrichment of distinct biological pathways. Likewise, the DMRs/DMCs were mostly tissue type-specific, and the specific sites that directly overlapped between liver and blood differed by sex. Among DMC-associated genes that overlapped between blood and liver, only a small percentage overlapped between males and females. These data suggest that sex will be a critical factor in the design, analysis, and interpretation of human- and animal-based environmental epigenetics studies. Although we investigated the effects of perinatal Pb exposure on DNA methylation in liver and blood, pathway analysis of DMRs in promoter regions revealed enrichment for pathways involved in neurological development and function in male and female liver. Moreover, among overlapping genes between blood and liver, gene pathways associated with synapse function were significantly enriched in both males and females. The nervous system is a well-known target of Pb, and although we did not directly measure nervous system tissues in this study, it is nonetheless striking that neurological pathways were enriched in genes annotated to DMCs/DMRs in both liver and blood. Analysis of other tissues, including brain, from Pb-exposed mice is currently underway, and will provide important insight into whether there is a common subset of tissue-independent, environmentally labile genes or CpG sites.

Although Pb exposure affected DNA methylation at thousands of loci, the mechanism underlying these changes is currently unknown. DNA methyltransferases (DNMTs) methylate the 5-position of cytosine bases in DNA using S-adenosylmethionine (SAM), which is generated via one-carbon metabolism (Ducker and Rabinowitz 2017). Pb may disrupt DNA methylation via alteration in DNMT expression/activity or through perturbation of SAM levels. Consistent with the first hypothesis, Pb exposure *in vitro* and *in vivo* leads to inhibition and altered expression of DNMTs (Sanchez et al. 2017). In support of the second possibility, administration of exogenous SAM mitigates the deleterious effects of Pb exposure, suggesting that Pb may deplete endogenous levels of SAM (Cao et al. 2008; Paredes et al. 1986). As ERRBS cannot discriminate between 5mC and 5hmC, a subset of changes in methylation observed in this study may actually be 5hmC, and altered 5hmC has indeed been reported with Pb exposure (Sen et al. 2015a). 5hmC is generated by the activity of TET methylcytosine dioxygenases

(TETs), using the cofactor alpha-ketoglutarate (α -KG), which is generated by the TCA cycle (Wong et al. 2017). Interestingly, Pb exposure has been shown to reduce activity of TCA cycle enzymes, including isocitrate dehydrogenase, which catalyzes the formation of α -KG (Basha et al. 2012; Seddik et al. 2011). Finally, it is possible that developmental Pb exposure leads to shifts in the cellular composition of blood and liver (Hung et al. 2019; Trevino and Katz 2018), and that alterations in DNA methylation are a reflection of these population shifts. The mechanisms underlying the observed Pb-induced changes in DNA methylation are currently under investigation.

Imprinted Genes

Genomic imprinting is critical for normal fetal development, and epigenetic programming of imprinted gene expression is established very early in development (Dolinoy et al. 2007). For these reasons, this process may be highly susceptible to perturbation by environmental exposures. Notably, we discovered that Pb exposure during gestation and lactation leads to changes in DNA methylation at 44 imprinted genes. This finding is consistent with accumulating evidence from our lab and others demonstrating that early life environmental exposures disrupt the epigenetic programming of imprinted loci (Bansal et al. 2017; Kochmanski et al. 2018; Nye et al. 2016; Susiarjo et al. 2013). Although most of the effects were sex-specific, we observed DMCs at *Commd1*, *Gnas*, *Nespas*, *Pde10a*, and *Pde4d* in both males and females, and methylation changes at CpGs in *Gnas*, *Nespas*, and *Pde10a* were in the same direction in both sexes. The *Gnas* locus encodes for three distinct protein products, and disruptions in the regulation of this locus have been implicated in a number of disorders, including early-onset obesity (Hanna et al. 2018). We recently reported that developmental BPA exposure leads to changes in DNA methylation and hydroxymethylation at the *Gnas* locus, concomitant with changes in *Gnas* expression (Kochmanski et al. 2018). Thus, *Gnas* may be an important target of multiple environmental exposures, in both males and females. When comparing male liver and blood, we observed DMCs at *Begain*, *Cdkn1c*, *Commd1*, *Peg12*, *Rasgrf1*, and *Snrpn* in both tissues, and found that CpGs in *Begain*, *Peg12*, *Rasgrf1*, and *Snrpn* exhibited changes in methylation in the same direction in both blood and liver. *Snrpn* encodes for an RNA binding protein that is critical for neurological function, and disruption of *Snrpn* expression is implicated in Prader-Willi syndrome, a neuroendocrine disorder associated with intellectual disability and obesity (Reed and Leff 1994). Work from our lab and others suggests that environmental exposures are associated with changes in methylation of the *Snrpn* locus (Faulk et al. 2015; Kochmanski et al. 2018; Soubry et al. 2017; Susiarjo et al. 2013). Given that the *Snrpn* locus is hypermethylated in both blood and liver with gestational Pb exposure, it is plausible that it may represent an important biomarker of early life Pb exposure. We recently reported that perinatal BPA exposure leads to reprogramming of DNA methylation and hydroxymethylation at 12 imprinted genes (Kochmanski et al. 2018). Interestingly, 9 of the imprinted loci altered by Pb exposure were among those reprogrammed by BPA exposure. These data suggest that a core set of imprinted genes may be vulnerable to epigenetic reprogramming by multiple environmental exposures. As both Pb and BPA exposure have been implicated in adverse metabolic health outcomes (Bansal et al. 2017; Nadal et al. 2017; Niessen et al. 2018), it is plausible that reprogramming of genomic imprinting may represent a common mechanism linking both toxicants to these outcomes. The mechanistic consequences of environment-mediated dysregulation of imprinted genes, and the implications this may have for human health, should be investigated in future studies.

Selection of surrogate tissues for environmental epigenomics studies

When comparing the chromosomal locations of DMCs/DMRs between blood and liver, only a small number of sites directly overlapped between the two tissues. Among DMRs/DMCs that overlapped between blood and liver, we identified several in males that exhibited changes in methylation in the same direction with Pb exposure in both blood and liver. These regions mapped to *Grifin*, *Elmsan1*, *Amn*, *Tbc1d30*, *Plekhhg3*. An additional region did not map to a gene. Although the precise roles for the identified genes in normal physiology and disease are not fully characterized, recent evidence suggests they may have critical roles in development and in several disease states. (Griswold et al. 2011; Huyghe et al. 2013; Kalantry et al. 2001; Luder et al. 2008; Piraino and Furney 2017; Prisco et al. 2014). Although few differentially methylated sites overlapped directly, when comparing the lists of DMC-associated genes between blood and liver, we observed many DMC-associated genes that overlapped between tissues. The CpGs and genes identified in this study may therefore represent candidates for analysis in human environmental epigenetics studies. Future studies are needed to validate the plausibility of these loci as potential biomarkers of Pb exposure, as well as to investigate the functional and health-related consequences of altered DNA methylation at these sites.

Limitations

This study has several important limitations. First, we utilized ERRBS, a method that, while providing resolution at the level of individual CpGs, does not cover the entire genome. The use of whole-genome bisulfite sequencing techniques may yield additional insight into the effects of Pb exposure on the liver, and identify additional Pb-induced signatures that overlap between blood and liver. Second, ERRBS does not differentiate between 5mC and 5hmC. Thus, the differentially methylated loci identified in this study are likely a combination of both 5mC and 5hmC. Third, changes of interest in DNA methylation may only occur in specific subpopulations of cells (Hui et al. 2018). Thus, our approach using unfractionated tissues may have missed other potential markers of interest for Pb exposure. Additionally, blood cells undergo rapid turnover, as well as exposure-, age-, and disease-dependent changes in cellular composition (Babio et al. 2013; Jaffe and Irizarry 2014; Su et al. 2016). Future studies using single-cell transcriptomics and epigenomics approaches will help to address issues of tissue-specific cellular heterogeneity and composition (Hui et al. 2018; Kelsey et al. 2017). Finally, it is important to note that DAVID gene ontology analysis does not consider the differing number of CpGs per gene, and therefore may exhibit bias.

Conclusions

In summary, we have investigated the effects of perinatal Pb exposure on DNA methylation in paired samples of mouse blood and liver. There are several key strengths of this study, including the side-by-side comparison of blood and liver to identify signatures of Pb exposure present in the blood that reflect changes in the liver. Future studies should investigate the reproducibility of these findings, the mechanistic and functional consequences of persistent, Pb-induced changes in hepatic methylation, and the implications these changes have for human health.

REFERENCES

483 Akalin A, Kormaksson M, Li S, Garrett-Bakelman FE, Figueroa ME, Melnick A, et al. 2012.
484 MethylKit: A comprehensive R package for the analysis of genome-wide DNA methylation
485 profiles. *Genome Biol* 13:R87.
486 Babio N, Ibarrola-Jurado N, Bullo M, Martinez-Gonzalez MA, Warnberg J, Salaverria I, et al.
487 2013. White blood cell counts as risk markers of developing metabolic syndrome and its
488 components in the predimed study. *PLoS One* 8:e58354.
489 Bansal A, Rashid C, Xin F, Li C, Polyak E, Duemler A, et al. 2017. Sex- and dose-specific
490 effects of maternal bisphenol a exposure on pancreatic islets of first- and second-generation
491 adult mice offspring. *Environ Health Perspect* 125:097022.
492 Barlow DP, Bartolomei MS. 2014. Genomic imprinting in mammals. *Cold Spring Harb Perspect*
493 *Biol* 6.
494 Basha DC, Basha SS, Reddy GR. 2012. Lead-induced cardiac and hematological alterations in
495 aging wistar male rats: Alleviating effects of nutrient metal mixture. *Biogerontology* 13:359-368.
496 Bener A, Obineche E, Gillett M, Pasha MA, Bishawi B. 2001. Association between blood levels
497 of lead, blood pressure and risk of diabetes and heart disease in workers. *Int Arch Occup*
498 *Environ Health* 74:375-378.
499 Cao XJ, Huang SH, Wang M, Chen JT, Ruan DY. 2008. S-adenosyl-L-methionine improves
500 impaired hippocampal long-term potentiation and water maze performance induced by
501 developmental lead exposure in rats. *Eur J Pharmacol* 595:30-34.
502 Cavalcante RG, Sartor MA. 2017. Annotatr: Genomic regions in context. *Bioinformatics*
503 33:2381-2383.
504 Cave M, Appana S, Patel M, Falkner KC, McClain CJ, Brock G. 2010. Polychlorinated
505 biphenyls, lead, and mercury are associated with liver disease in american adults: Nhanes
506 2003-2004. *Environ Health Perspect* 118:1735-1742.
507 Dolinoy DC, Weidman JR, Jirtle RL. 2007. Epigenetic gene regulation: Linking early
508 developmental environment to adult disease. *Reprod Toxicol* 23:297-307.
509 Ducker GS, Rabinowitz JD. 2017. One-carbon metabolism in health and disease. *Cell Metab*
510 25:27-42.
511 Faulk C, Barks A, Liu K, Goodrich JM, Dolinoy DC. 2013. Early-life lead exposure results in
512 dose- and sex-specific effects on weight and epigenetic gene regulation in weanling mice.
513 *Epigenomics* 5:487-500.
514 Faulk C, Barks A, Sanchez BN, Zhang Z, Anderson OS, Peterson KE, et al. 2014a. Perinatal
515 lead (pb) exposure results in sex-specific effects on food intake, fat, weight, and insulin
516 response across the murine life-course. *PLoS One* 9:e104273.
517 Faulk C, Liu K, Barks A, Goodrich JM, Dolinoy DC. 2014b. Longitudinal epigenetic drift in mice
518 perinatally exposed to lead. *Epigenetics* 9:934-941.
519 Faulk C, Kim JH, Jones TR, McEachin RC, Nahar MS, Dolinoy DC, et al. 2015. Bisphenol a-
520 associated alterations in genome-wide DNA methylation and gene expression patterns reveal
521 sequence-dependent and non-monotonic effects in human fetal liver. *Environ Epigenet* 1.
522 Garrett-Bakelman FE, Sheridan CK, Kacmarczyk TJ, Ishii J, Betel D, Alonso A, et al. 2015.
523 Enhanced reduced representation bisulfite sequencing for assessment of DNA methylation at
524 base pair resolution. *J Vis Exp*:e52246.
525 Griswold AJ, Ma D, Sacharow SJ, Robinson JL, Jaworski JM, Wright HH, et al. 2011. A de novo
526 1.5 mb microdeletion on chromosome 14q23.2-23.3 in a patient with autism and spherocytosis.
527 *Autism Res* 4:221-227.
528 Hanna P, Grybek V, Perez de Nanclares G, Tran LC, de Sanctis L, Elli F, et al. 2018. Genetic
529 and epigenetic defects at the gnas locus lead to distinct patterns of skeletal growth but similar
530 early-onset obesity. *J Bone Miner Res* 33:1480-1488.
531 Huang Y, Lin S, Jin L, Wang L, Ren A. 2019. Decreased global DNA hydroxymethylation in
532 neural tube defects: Association with polycyclic aromatic hydrocarbons. *Epigenetics*:1-11.

533 Hui T, Cao Q, Wegrzyn-Woltosz J, O'Neill K, Hammond CA, Knapp D, et al. 2018. High-
534 resolution single-cell DNA methylation measurements reveal epigenetically distinct
535 hematopoietic stem cell subpopulations. *Stem Cell Reports* 11:578-592.

536 Hung PH, Van Winkle LS, Williams CJ, Hunt PA, VandeVoort CA. 2019. Prenatal bisphenol a
537 exposure alters epithelial cell composition in the rhesus macaque fetal oviduct. *Toxicol Sci*
538 167:450-457.

539 Huyghe JR, Jackson AU, Fogarty MP, Buchkovich ML, Stancakova A, Stringham HM, et al.
540 2013. Exome array analysis identifies new loci and low-frequency variants influencing insulin
541 processing and secretion. *Nat Genet* 45:197-201.

542 Jaffe AE, Irizarry RA. 2014. Accounting for cellular heterogeneity is critical in epigenome-wide
543 association studies. *Genome Biol* 15:R31.

544 Jones PA. 2012. Functions of DNA methylation: Islands, start sites, gene bodies and beyond.
545 *Nat Rev Genet* 13:484-492.

546 Kalantry S, Manning S, Haub O, Tomihara-Newberger C, Lee HG, Fangman J, et al. 2001. The
547 amnionless gene, essential for mouse gastrulation, encodes a visceral-endoderm-specific
548 protein with an extracellular cysteine-rich domain. *Nat Genet* 27:412-416.

549 Kelsey G, Stegle O, Reik W. 2017. Single-cell epigenomics: Recording the past and predicting
550 the future. *Science* 358:69-75.

551 Kochmanski JJ, Marchlewicz EH, Cavalcante RG, Perera BPU, Sartor MA, Dolinoy DC. 2018.
552 Longitudinal effects of developmental bisphenol a exposure on epigenome-wide DNA
553 hydroxymethylation at imprinted loci in mouse blood. *Environ Health Perspect* 126:077006.

554 Koster J, Rahmann S. 2012. Snakemake--a scalable bioinformatics workflow engine.
555 *Bioinformatics* 28:2520-2522.

556 Krueger F, Andrews SR. 2011. Bismark: A flexible aligner and methylation caller for bisulfite-seq
557 applications. *Bioinformatics* 27:1571-1572.

558 Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with bowtie 2. *Nat Methods*
559 9:357-359.

560 Leasure JL, Giddabasappa A, Chaney S, Johnson JE, Jr., Pothakos K, Lau YS, et al. 2008.
561 Low-level human equivalent gestational lead exposure produces sex-specific motor and
562 coordination abnormalities and late-onset obesity in year-old mice. *Environ Health Perspect*
563 116:355-361.

564 Lee C.T. CRG, Lee C., Qin T., Patil S., Wang S., Tsai Z., Boyle A.P., Sartor M.A. 2019. Poly-
565 enrich: Count-based methods for gene set enrichment testing with genomic regions. *bioRxiv*.

566 Lopez V, Fernandez AF, Fraga MF. 2017. The role of 5-hydroxymethylcytosine in development,
567 aging and age-related diseases. *Ageing Res Rev* 37:28-38.

568 Luder AS, Tanner SM, de la Chapelle A, Walter JH. 2008. Amnionless (amn) mutations in
569 imerslund-grasbeck syndrome may be associated with disturbed vitamin b12 transport into the
570 cns. *J Inherit Metab Dis* 31 Suppl 3:493-496.

571 Nadal A, Quesada I, Tuduri E, Nogueiras R, Alonso-Magdalena P. 2017. Endocrine-disrupting
572 chemicals and the regulation of energy balance. *Nat Rev Endocrinol* 13:536-546.

573 Niessen LW, Mohan D, Akuoku JK, Mirelman AJ, Ahmed S, Koehlmoos TP, et al. 2018.
574 Tackling socioeconomic inequalities and non-communicable diseases in low-income and
575 middle-income countries under the sustainable development agenda. *Lancet* 391:2036-2046.

576 Nye MD, King KE, Darrah TH, Maguire R, Jima DD, Huang Z, et al. 2016. Maternal blood lead
577 concentrations, DNA methylation of meg3 dmr regulating the dlk1/meg3 imprinted domain and
578 early growth in a multiethnic cohort. *Environ Epigenet* 2.

579 Okae H, Chiba H, Hiura H, Hamada H, Sato A, Utsunomiya T, et al. 2014. Genome-wide
580 analysis of DNA methylation dynamics during early human development. *PLoS Genet*
581 10:e1004868.

582 Paredes SR, Fukuda H, Kozicki PA, Rossetti MV, Conti H, Batlle AM. 1986. S-adenosyl-l-
583 methionine and lead intoxication: Its therapeutic effect varying the route of administration.
584 *Ecotoxicol Environ Saf* 12:252-260.

585 Park Y, Figueroa ME, Rozek LS, Sartor MA. 2014. MethySig: A whole genome DNA
586 methylation analysis pipeline. *Bioinformatics* 30:2414-2422.

587 Park Y, Wu H. 2016. Differential methylation analysis for bs-seq data under general
588 experimental design. *Bioinformatics* 32:1446-1453.

589 Piraino SW, Furney SJ. 2017. Identification of coding and non-coding mutational hotspots in
590 cancer genomes. *BMC Genomics* 18:17.

591 Prisco SZ, Priestley JR, Weinberg BD, Prisco AR, Hoffman MJ, Jacob HJ, et al. 2014. Vascular
592 dysfunction precedes hypertension associated with a blood pressure locus on rat chromosome
593 12. *Am J Physiol Heart Circ Physiol* 307:H1103-1110.

594 Reed ML, Leff SE. 1994. Maternal imprinting of human snrpn, a gene deleted in prader-willi
595 syndrome. *Nat Genet* 6:163-167.

596 Reik W, Dean W, Walter J. 2001. Epigenetic reprogramming in mammalian development.
597 *Science* 293:1089-1093.

598 Ringh MV, Hagemann-Jensen M, Needhamsen M, Kular L, Breeze CE, Sjöholm LK, et al. 2019.
599 Tobacco smoking induces changes in true DNA methylation, hydroxymethylation and gene
600 expression in bronchoalveolar lavage cells. *EBioMedicine*.

601 Sanchez OF, Lee J, Yu King Hing N, Kim SE, Freeman JL, Yuan C. 2017. Lead (pb) exposure
602 reduces global DNA methylation level by non-competitive inhibition and alteration of dnmt
603 expression. *Metallomics* 9:149-160.

604 Seddik L, Bah TM, Aoues A, Slimani M, Benderdour M. 2011. Elucidation of mechanisms
605 underlying the protective effects of olive leaf extract against lead-induced neurotoxicity in wistar
606 rats. *J Toxicol Sci* 36:797-809.

607 Sen A, Cingolani P, Senut MC, Land S, Mercado-Garcia A, Tellez-Rojo MM, et al. 2015a. Lead
608 exposure induces changes in 5-hydroxymethylcytosine clusters in cpG islands in human
609 embryonic stem cells and umbilical cord blood. *Epigenetics* 10:607-621.

610 Sen A, Heredia N, Senut MC, Hess M, Land S, Qu W, et al. 2015b. Early life lead exposure
611 causes gender-specific changes in the DNA methylation profile of DNA extracted from dried
612 blood spots. *Epigenomics* 7:379-393.

613 Skaar DA, Li Y, Bernal AJ, Hoyo C, Murphy SK, Jirtle RL. 2012. The human imprintome:
614 Regulatory mechanisms, methods of ascertainment, and roles in disease susceptibility. *ILAR J*
615 53:341-358.

616 Soubry A, Hoyo C, Butt CM, Fieuws S, Price TM, Murphy SK, et al. 2017. Human exposure to
617 flame-retardants is associated with aberrant DNA methylation at imprinted genes in sperm.
618 *Environ Epigenet* 3:dvx003.

619 Su D, Wang X, Campbell MR, Porter DK, Pittman GS, Bennett BD, et al. 2016. Distinct
620 epigenetic effects of tobacco smoking in whole blood and among leukocyte subtypes. *PLoS*
621 *One* 11:e0166486.

622 Sun H, Wang N, Nie X, Zhao L, Li Q, Cang Z, et al. 2017. Lead exposure induces weight gain in
623 adult rats, accompanied by DNA hypermethylation. *PLoS One* 12:e0169958.

624 Susiarjo M, Sasson I, Mesaros C, Bartolomei MS. 2013. Bisphenol a exposure disrupts genomic
625 imprinting in the mouse. *PLoS Genet* 9:e1003401.

626 Trevino LS, Katz TA. 2018. Endocrine disruptors and developmental origins of nonalcoholic fatty
627 liver disease. *Endocrinology* 159:20-31.

628 Wang T, Pehrsson EC, Purushotham D, Li D, Zhuo X, Zhang B, et al. 2018. The niehs target ii
629 consortium and environmental epigenomics. *Nat Biotechnol* 36:225-227.

630 Waterland RA, Jirtle RL. 2003. Transposable elements: Targets for early nutritional effects on
631 epigenetic gene regulation. *Mol Cell Biol* 23:5293-5300.

Weinhouse C, Anderson OS, Bergin IL, Vandenberg DJ, Gyekis JP, Dingman MA, et al. 2014. Dose-dependent incidence of hepatic tumors in adult mice following perinatal exposure to bisphenol a. *Environ Health Perspect* 122:485-491.

Williamson CM BA, Thomas S, Beechey CV, Hancock J, Cattnach BM, and Peters J 2013. Mrc harwell, oxfordshire. World wide web site - mouse imprinting data and references - http://www.Har.Mrc.Ac.Uk/research/genomic_imprinting/

Wong CC, Qian Y, Yu J. 2017. Interplay between epigenetics and metabolism in oncogenesis: Mechanisms and therapeutic approaches. *Oncogene* 36:3359-3374.

Wu J, Wen XW, Faulk C, Boehnke K, Zhang H, Dolinoy DC, et al. 2016. Perinatal lead exposure alters gut microbiota composition and results in sex-specific bodyweight increases in adult mice. *Toxicol Sci* 151:324-333.

Xia J, Jin C, Pan Z, Sun L, Fu Z, Jin Y. 2018a. Chronic exposure to low concentrations of lead induces metabolic disorder and dysbiosis of the gut microbiota in mice. *Sci Total Environ* 631-632:439-448.

Xia J, Lu L, Jin C, Wang S, Zhou J, Ni Y, et al. 2018b. Effects of short term lead exposure on gut microbiota and hepatic metabolism in adult zebrafish. *Comp Biochem Physiol C Toxicol Pharmacol* 209:1-8.

Zhai H, Chen C, Wang N, Chen Y, Nie X, Han B, et al. 2017. Blood lead level is associated with non-alcoholic fatty liver disease in the yangtze river delta region of china in the context of rapid urbanization. *Environ Health* 16:93.

TABLES

Table 1: Litter Parameters

Exposure Group	# Litters	# Pups Born	# Pups Died	Mean Pups per Litter ($\pm$ SD) ^a	Pup Mortality Rate (%) ^b	% Females ^a
Control	6	53	3	7.57 $\pm$ 3.36	5.7	46%
Pb	7	52	7	7.43 $\pm$ 1.51	13.5	47%

Note: Statistical analyses (Pb vs. Control) were conducted using Fisher's Exact Test.

^ap = $\geq$ 0.9

^bp = 0.2

Table 2: Differentially methylated cytosines (DMCs) in female and male blood and liver identified using ERRBS.

Condition	Total	# Hypermethylated (% Total)	# Hypomethylated (% Total)	Total Tested
Female Blood	1,628	747 (45.9)	881 (54.1)	1,356,386
Female Liver	2,404	1,350 (56.2)	1,054 (43.8)	1,228,997
Male Blood	2,175	1,076 (49.5)	1,099 (50.5)	1,362,718
Male Liver	3,808	1,598 (42.0)	2,210 (58.0)	1,173,995

Note: Methylation changes are expressed as lead vs. control.

Table 3: Differentially methylated regions (DMRs) in female and male blood and liver identified using ERRBS.

Condition	Total	# Hypermethylated (% Total)	# Hypomethylated (% Total)	Total Tested
Female Blood	1,035	551 (53.2)	484 (46.8)	646,876
Female Liver	1,740	792 (45.5)	948 (54.5)	597,077
Male Blood	1,404	724 (51.6)	680 (48.4)	650,782
Male Liver	3,342	1,874 (56.1)	1,468 (43.9)	563,681

Note: Methylation changes are expressed as lead vs. control.

Table 4: Differentially methylated cytosines (DMCs) identified at imprinted loci in female blood and liver.

DMCs in Blood				
Gene	Position	Location	Change in methylation	FDR
<i>Meg3</i>	109547833	Intron/1 to 5 kb	36.92	0.002
<i>Slc22a3</i>	12454881	Intron	-14.18	6.62E-05
<i>Tnfrsf23</i>	143670844	Intron/Exon/3'UTR	-10.12	0.002
DMCs in Liver				
Gene	Position	Location	Change in methylation	FDR
<i>Art5</i>	102100155	Promoter/Intron/Exon/5'UTR	15.81	0.021
<i>Axl</i>	25764245	Promoter/Exon/Intron	-33.18	0.048
<i>Axl</i>	25781580	Intron	24.81	0.039
<i>Commd1</i>	22972256	Intron	-36.26	0.007
<i>Commd1</i>	22972731	Intron/Exon	16.37	0.002
<i>Fthl17e</i>	9028755	1 to 5 kb	-14.42	0.0009
<i>Gatm</i>	122595535	Exon	-23.74	0.014
<i>Gnas</i>	174285424	Exon	13.02	0.001
<i>Nespas</i>	174285424	Intron	13.02	0.001
<i>Gnas</i>	174285427	Exon	10.32	0.024
<i>Grb10</i>	11948460	Intron	-19.24	0.040
<i>H19</i>	142578975	Promoter/1 to 5 kb	-23.47	0.033
<i>Kcnq1</i>	143295410	Intron	-13.35	0.001
<i>Kcnq1</i>	143295427	Intron	-16.02	0.021
<i>Kcnq1ot1</i>	143295427	Exon	-16.02	0.021
<i>Kcnq1</i>	143295437	Intron	-14.64	0.0001
<i>Kcnq1ot1</i>	143295437	Exon	-14.64	0.0001
<i>Kcnq1</i>	143295442	Intron	-21.31	0.005
<i>Kcnq1ot1</i>	143295442	Exon	-21.31	0.005
<i>Kcnq1</i>	143395334	Intron	22.05	0.0003
<i>Magel2</i>	62378976	Exon	-20.11	0.023
<i>Pde4d</i>	109659183	Intron	-18.56	0.039
<i>Pde10a</i>	8754083	Intron	-14.17	0.003
<i>Zrsr1</i>	22972256	Promoter/Exon/5'UTR	-36.26	0.007
<i>Zrsr1</i>	22972731	Promoter/Exon/5'UTR	16.37	0.002

Note: Methylation changes are expressed as lead vs. control.

Table 5: Differentially methylated cytosines (DMCs) identified at imprinted loci in male blood and liver.

DMCs in Blood				
Gene	Position	Location	Change in methylation	FDR
<i>Airn</i>	12822718	Intron	-11.17	0.0007
<i>Art5</i>	102104325	1 to 5 kb	23.58	0.029
<i>Axl</i>	25774103	Intron/Exon	-10.66	0.012
<i>Begain</i>	109056064	Intron/1 to 5 kb	10.21	0.044
<i>Cdkn1c</i>	143461132	Promoter/1 to 5 kb	17.77	0.034
<i>Commd1</i>	22973934	Intron/Exon/1 to 5 kb	19.41	0.012
<i>Grb10</i>	12025803	Intron	-10.20	0.047
<i>Grb10</i>	12038672	1 to 5kb	13.65	0.026
<i>H13</i>	152707734	Exon/3'UTR	-10.54	0.0005
<i>Igf2r</i>	12726414	Intron	17.13	0.008
<i>Meg3</i>	109540296	Promoter/1 to 5 kb	38.50	0.0009
<i>Mkrn3</i>	62419838	Exon	27.44	0.030
<i>Peg12</i>	62463945	Exon	-24.76	0.042
<i>Plagl1</i>	13117338	Intron	-23.32	0.006
<i>Rasgrf1</i>	89958385	Intron	19.19	6.68E-05
<i>Snrpn</i>	60012762	Intron	21.67	0.0002
<i>Usp29</i>	6770496	Intron	-12.12	0.003
<i>Zim1</i>	6678078	Exon	-12.05	0.0004
<i>Zrsr1</i>	22973934	Exon	19.41	0.012
DMCs in Liver				
Gene	Position	Location	Change in methylation	FDR
<i>Begain</i>	109055956	Intron/1 to 5 kb	23.07	0.012
<i>Cdkn1c</i>	143459490	Promoter/Intron	-49.58	0.004
<i>Cdkn1c</i>	143460320	Promoter/1 to 5 kb/Exon	-13.94	0.0033
<i>Commd1</i>	22978331	Intron/Exon	-36.13	0.001
<i>DLK1</i>	109449906	1 to 5 kb	10.67	0.031
<i>Fbxo40</i>	36990334	Exon/5'UTR	-10.96	0.021
<i>Gnas</i>	174298185	Intron/Exon	15.82	0.048
<i>Nespas</i>	174298185	1 to 5 kb	15.82	0.048
<i>Gnas</i>	174299492	Intron/Exon	14.23	0.006
<i>Nespas</i>	174299492	1 to 5 kb	14.23	0.006
<i>Htr2a</i>	74706653	Exon/3'UTR	17.33	0.029
<i>Klf14</i>	30958628	Exon	-14.23	0.014
<i>Pde4d</i>	108848465	Intron	28.82	0.025
<i>Pde4d</i>	109936716	Exon	12.18	0.030
<i>Pde10a</i>	8547124	Intron	11.66	0.037
<i>Pde10a</i>	8927375	Intron	-12.70	0.004
<i>Peg12</i>	62463837	Exon	-20.88	0.007
<i>Rasgrf1</i>	89953923	Exon	21.35	8.36E-36
<i>Rhox5</i>	37807531	Intron	22.33	0.006
<i>Sfmbt2</i>	10368329	1 to 5 kb	-28.39	0.004
<i>Snrpn</i>	60005199	Intron	11.12	0.040
<i>Snurf</i>	60005199	Promoter	11.12	0.040
<i>Th</i>	142895420	Intron/Exon/3'UTR	14.65	0.002

Note: Methylation changes are expressed as lead vs. control.

Table 6: Overlap between differentially methylated cytosines/regions (DMCs/DMRs) in female blood and liver.

Gene	Coordinate	Change in blood	FDR blood	Change in liver	FDR liver
<i>Ccin</i>	43985009	-11.08	0.00935	28.08	0.011
<i>Lsm14b</i>	180023832	-19.35	0.00035	16.92	0.017
<i>Cntnap2</i>	46349288	-17.51	0.00091	39.58	0.024
<i>Fam122c</i>	53314658	-18.76	0.00012	18.05	0.006
Gene	Coordinate	Change in blood	FDR blood	Change in liver	FDR Liver
<i>Glo1</i>	30597451	11.86	0.00092	-13.38	0.045
<i>Lsm14b</i>	180023801	-19.35	0.00026	16.92	0.012
<i>Cntnap2</i>	46349251	-17.51	0.00070	39.58	0.018

Note: Methylation changes are expressed as lead vs. control.

Table 7: Overlap between differentially methylated cytosines/regions (DMCs/DMRs) in male blood and liver.

DMCs						
Gene	Start	End	Change in blood	FDR blood	Change in liver	FDR liver
<i>Grifin</i>	140567250	140567250	-12.83	0.025	-25.08	0.03932
<i>Elmsan1</i>	84151896	84151896	15.08	0.0005	11.74	0.00020
<i>Amn</i>	111270178	111270178	19.13	0.033	11.97	0.00060
<i>Tbc1d30</i>	121294168	121294168	22.54	0.047	27.76	0.01649
DMRs						
Gene	Start	End	Change in blood	FDR blood	Change in liver	FDR liver
<i>Grifin</i>	140567201	140567250	-12.83	0.020	-25.08	0.026
<i>Mob2</i>	142016401	142016450	-23.64	0.038	21.0	0.021
<i>Dnm3</i>	162453101	162453150	12.63	0.001	-23.95	0.049
<i>Plekhg3</i>	76535801	76535850	17.04	0.017	17.14	0.047
<i>N/A</i>	114931401	114931450	21.20	0.022	13.43	0.039

Note: Methylation changes are expressed as lead vs. control.

FIGURE LEGENDS

Figure 1: Mouse treatment paradigm showing timing of Pb exposure and animal sacrifice/tissue collection. Dams were exposed to normal chow and either control or Pb-acetate water, starting two weeks prior to mating, through gestation, until offspring were weaned on postnatal day 21. At this point, Pb exposure ceased and offspring were administered control water and chow. At 5 months of age, matched liver and blood tissues were collected for DNA methylation analysis. Pb doses and average blood lead levels are shown.

Figure 2: Volcano plots showing differentially methylated regions (DMRs) for lead vs. control in female blood (A), female liver (B), male blood (C), and male liver (D). Red: regions significantly hypermethylated with Pb exposure. Green: regions significantly hypomethylated with Pb exposure.

Figure 3: Lollipop diagrams showing differentially methylated cytosines (DMCs) with Pb exposure in: (A) *Gnas* in male and female liver, (B) *Meg3* in male and female blood, (C) *Begain* in male blood and liver, (D) *Peg12* in male blood and liver, (E) *Rasgrf1* in male blood and liver, and (F) *Snrpn* in male blood and liver.

Figure 4: Analysis of overlapping DMC-associated genes in females (A) and males (B). Numbers in circles indicate the total number of DMC-associated genes in each tissue. In the tables, # Genes indicates the number of genes in the specified GO pathway that appear in the experimental dataset. Pathway analysis was conducted using DAVID, version 6.8 (Laboratory of Human Retrovirology and Immunoinformatics (LHRI)).

Figure 1

A

Females

188 genes

GO Term	# Genes	P-value	FDR
GO0030054: cell junction	22	7.33E-7	9.55E-4
GO0030054: membrane	87	1.29E-5	0.02
GO0045202: synapse	16	2.59E-5	0.03

B

Males

325 genes

GO Term	# Genes	P-value	FDR
GO0007156: homophilic cell adhesion	26	5.82E-18	9.76E-15
GO0005509: calcium ion binding	44	1.71E-14	2.40E-11
GO0005515: protein binding	111	7.30E-10	1.03E-6
GO0016020: membrane	148	3.13E-7	4.17E-4
GO0050885: neuromuscular process controlling balance	9	3.09E-6	0.01
GO0045202: synapse	23	8.56E-6	0.01
GO0045202: neuron projection	20	2.14E-5	0.03

A**DMCs in Female Blood** **B****DMCs in Female Liver** **C****DMCs in Male Blood** **D****DMCs in Male Liver**