Title Page

Title: Perinatal Lead (Pb) Exposure Results in Sex and Tissue-Dependent Adult DNA Methylation Alterations in Murine IAP Transposons

Short Running Title: Perinatal Lead Exposure and Adult IAP DNA Methylation

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Abstract: (250 words ---currently at 250)

Epidemiological and animal data suggest that adult chronic disease is influenced by early-life exposure-induced changes to the epigenome. Previously, we observed that perinatal lead (Pb) exposure results in persistent murine metabolic- and activity-related effects. Using phylogenetic and DNA methylation analysis, we have also identified novel intracisternal A particle (IAP) retrotransposons exhibiting regions of variable methylation as candidate loci for environmental effects on the epigenome. Here, we now evaluate brain and kidney DNA methylation profiles of 4 representative IAPs in adult mice exposed to human physiologically-relevant levels of Pb two weeks prior to mating through lactation. When IAPs across the genome were evaluated globally, average (sd) methylation levels were 92.84% (3.74) differing by tissue ($p<0.001$), but not sex or dose. By contrast, the 4 individual IAPs displayed tissue-specific Pb and sex effects. Medium Pb-exposed mice had 3.86% less brain methylation at IAP 110 ($p<0.01$), while high Pb-exposed mice had 2.83% less brain methylation at IAP 236 ($p=0.01$) and 1.77% less at IAP 506 ($p=0.05$). Individual IAP DNA methylation differed by sex for IAP 110 in the brain and kidney, IAP 236 in the kidney, and IAP 1259 in the kidney. Using Tomtom, we identified 3 binding motifs that matched to each of our novel IAPs impacted by Pb, one of which (HMGA2) has been linked to metabolic-related conditions in both mice and humans. Thus, these recently identified IAPs display tissue-specific environmental lability as well as sex-specific differences supporting an epigenetic link between early exposure to Pb and later-in-life health outcomes.

Introduction

The heavy metal lead (Pb) is a toxicant of public health concern and poses a particularly dangerous threat to children's health. One of the most common routes of Pb exposure is through ingestion of Pb-based paint (CDC 2016). As such, children are at a greater risk for adverse health effects from ingesting contaminated paint chips or dust due to increased hand to mouth behavior and higher rates of Pb absorption relative to adults (Pichery et al. 2011). Currently, there is an additional growing concern that the aging water infrastructure in the US is contributing to an increased risk of perinatal and childhood exposure through placental transfer, breast milk, and direct ingestion of contaminated tap water. Recent cases of elevated blood Pb levels in children linked to major water infrastructure contamination events such as the Flint, Michigan Water Crisis (Hanna-Attisha et al. 2016) suggest these concerns are warranted. Thus, it is imperative to understand the long-term impact of developmental Pb exposure.

Human and rodent studies find that Pb exposure is especially toxic during early periods of rapid growth (Senut et al. 2012) and can affect nearly all organ systems (CDC 2016). However, the Agency for Toxic Substances and Disease Registry states that some organs such as the brain and kidneys are known targets for Pb-related effects in humans. In a mouse model, Sun and colleagues measured organ-specific Pb distribution after chronic venous exposure to either 0.1 or 0.2 mg/mL Pb nitrate solution at 0, 7, 11, or 15 days. Their results showed that the kidneys were subject to the highest concentrations of Pb while the brain displayed the lowest concentrations (Sun et al. 2009). Nevertheless it is clear from the literature that even low-level developmental Pb exposure is not inconsequential and can have a functional impact on long-term health including immunotoxicity (Dietert et al. 2004), metabolic dysfunction (Faulk et al. 2013b; Faulk et al. 2014a) and cognitive defects (Anderson et al. 2016). Therefore, it is necessary to identify plausible mechanisms that are both sensitive to toxicant exposure and susceptible to early perturbations.

The developmental origins of health and disease (DOHaD) hypothesis posits that early-life exposures can impact later-in-life outcomes (Barker 2000), and it has been proposed that epigenetic mechanisms may provide the conduit for perinatal exposure-related effects that persist into adulthood (Sharif et al. 2013). The "epi"-genome literally means above the genome and is a collection of modifiable chromatin alterations that coordinate when and where specific genes are expressed, without changes to the DNA sequence itself. The most well-understood epigenetic alteration is DNA methylation, which is the covalent addition of a methyl group to a cytosine residue of a cytosine-guanine (CpG) dinucleotide in vertebrates. Given that DNA methylation is not uniformly distributed across the genome (Maunakea et al. 2010), it is important to strategically focus on areas requiring epigenetic repression to determine if these patterns are environmentally labile.

Transposable elements (TE) are an appealing target to evaluate the toxicoepigenetic effects of Pb because they make up nearly half of the mammalian genome and harbor large numbers of CpGs. Although TEs have been historically classified as "junk DNA", they are now recognized as critical regulators of genetic information (Miousse et al. 2015) and their activity is tightly regulated by epigenetic control marks including DNA methylation (Rebollo et al. 2012; Rebollo and Mager 2016; Ross et al. 2010). The retrotransposon class of TEs can relocate via an RNA intermediate, and because these elements can function as alternative promoters, their transposition can elicit ectopic gene expression (Ostertag and Kazazian 2001). Importantly, some recent evidence suggests that a small percentage of retrotransposons may be variably methylated and that these methylation patterns are potentially linked to disease status (Bakshi et al. 2016). Although the latter study was conducted in humans, due to ethical constraints, an investigation of the toxicoepigenetic effects of developmental Pb exposure on retrotransposon methylation patterns is most effectively performed in a model organism.

Intracisternal A particles (IAP) are a class of murine retrotransposons that have been previously linked to toxicoepigenetic effects including shifts in methylation at an IAP insertion located in the *Agouti* (A^{vy}) gene following exposure to dietary supplementation (Waterland and Jirtle 2003). IAPs belong to the endogenous retrovirus family (ERV), a family known to be highly active in rodents (i.e. "copying and pasting" themselves), making heavy DNA methylation advantageous in these genomic regions (Zhang et al. 2008). Epigenetic suppression of the IAP elements is established early in the development of the mouse embryo and is faithfully transferred through cell divisions (Lees-Murdock and Walsh 2008). Moreover, IAPs are among a very select group of genomic elements that are at least partially excluded from genome-wide methylation erasure that occurs in the primordial germ cells, suggesting that epigenetic suppression of these elements is paramount for maintaining chromosomal stability during this critical developmental window (Hajkova et al. 2002; Lane et al. 2003).

As noted, the literature suggests select IAPs display environmental lability, however the impact of developmental Pb has not been established. Thus, it is the goal of our study to evaluate the lability of individual IAP methylation patterns and the methylation pattern of all IAPs globally following maternally transferred developmental exposure to Pb in a murine model. Previously we phylogenetically characterized 1,388 IAPLTR1_Mm elements (Faulk et al. 2013a) and quantified DNA methylation from a random subset of these IAPs (n= 7 per clade) measured in liver DNA from 21-day old mice (n=17). We selected 4 elements that displayed inter-individual methylation variability, and here we test whether DNA methylation status of these individual IAPs and a measure of global IAP methylation in the adult mouse (10 months of age) brain and kidney tissue is altered following perinatal exposure to 0 (control), 2.1 (low), 16 (medium), or 32 (high) ppm Pb-acetate water.

Materials and Methods

Mice were obtained from a colony maintained for over 220 generations with the A^{vy} 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). Post-pubertal virgin *a/a* females (6–8 wk old) were mated with A^{vy}/a males (7–10 wk of age), and randomly assigned to one of 4 Pb exposure groups, drinking water supplemented with Pb-acetate (1) 0 ppm, (2) 2.1 ppm, (3) 16 ppm, and (4) 32 ppm, to model human relevant perinatal exposure (Faulk et al. 2013b). Treatment water was made by dissolving Pb (II) acetate trihydrate (Sigma-Aldrich) in single batches of distilled water. Water Pb concentrations were verified using inductively coupled plasma mass spectrometry with a limit of detection of 1.0 µg/L (ICPMS; NSF International). Maternal blood lead levels (BLLs) were measured in a subset of dams (at least 7 dams per exposure group) at weaning through blood collected by cardiac puncture and measured via ICPMS at the Michigan Department of Community Health, with the control group being below the limit of detection (1.3 µg/dL) and the three exposure groups resulting in human physiologically relevant levels; 2.1 ppm, 16 ppm and 32 ppm exposure groups resulted in a BLL range (mean) of 2.0 to 5.88 µg/dL (4.1), 13 to 40 µg/dL (25.1), and 16 to 60 µg/dL (32.1), respectively (Faulk et al. 2013b). Throughout the duration of the experiment, animals were maintained on a phytoestrogen-free modified AIN-93G diet (TD.95092, 7% Corn Oil Diet, Harlan Teklad). Wild type *a/a* dams in each group were exposed to Pb-supplemented drinking water for two weeks prior to mating A^{vy}/a males. Exposure was continued during gestation and lactation. After weaning, the resulting pups were weighed and switched to untreated Pb-free drinking water (Figure 1). A subset of *a/a* pups from each exposure group, representing approximately 1–2 male and 1–2 female offspring per litter, was maintained to 10 months of age and serve as the study population. Organs collected at sacrifice were snap frozen in liquid nitrogen. 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 Committee on Use and Care of Animals.

DNA isolation and methylation analyses

Total genomic DNA was isolated from ~20mg of kidney and brain tissue for each animal using a Maxwell 16 Tissue DNA Purification Kit (Promega) in conjunction with the automated Maxwell 16 System (Promega). Genomic DNA was bisulfite converted using the EZ-96 DNA Methylation Kit (Zymo). Briefly, sodium bisulfite was added to approximately 500 ng of genomic DNA, converting unmethylated cytosines to uracil, which are replaced with thymine during PCR; methylated cytosines remain unchanged (Grunau et al. 2001). Each primer set was designed to contain a forward primer within the IAP sequence and a reverse primer within unique flanking sequence. The CpG sites measured were located within the IAP element but were not always homologous due to drift. PCR amplification of gene regions of interest was performed after bisulfite conversion using HotStarTaq master mix (Qiagen), forward primer (50 pmol), and reverse biotinylated primer (50 pmol) in a 30 µl PCR. PCR fragments were analyzed by gel electrophoresis or automated capillary electrophoresis using the Qiaxcel Advanced System (Qiagen). DNA methylation quantitation of CpG sites was performed using pyrosequencing on a PyroMark ID or MD instrument (Qiagen). To determine percent methylation, PyroMark software calculated the fraction of methylated cytosines (%mC) among the total sum of methylated and unmethylated cytosines. All primers are based on the mm9 chromosomal position. Table1 lists the IAP global and IAP-specific PCR conditions and primers that were used for pyrosequencing. For quality control, all pyrosequencing plates included 0%, 50% and 100% methylated bisulfite converted mouse control as well as at least one no DNA template control. A subset of the samples from every 96-well plate was run in technical duplicate to calculate a coefficient of variation (CV) and if the average %CV was greater than 5% the plate was repeated.

Binding motif search

MEME suite is an online collection of tools for the discovery and analysis of DNA sequence motifs. Using MEME suite, a 16 base pair (bp) region (7bp +/- the CpG) for each CpG for each individual IAP was entered into a binding motif comparison tool called Tomtom (Gupta et al. 2007). The reference database chosen for this search was HOCOMOCOv10 MOUSE. For each CpG motif that was queried, Tomtom produces a list of motifs that are statistically significantly similar.

Data analysis

A total of 314 mice were generated for a longitudinal epigenetic study of perinatal Pb exposure, of which 120 mice were followed to 10 months of age (Faulk et al. 2014b), and for the purpose of the currently described study, matched kidney and brain tissue were available for 93 mice. The influence of perinatal Pb exposure on sex ratio was determined by the Fisher's exact test comparing exposure groups to control. IAP identification numbers followed previous publication (Faulk et al. 2013a). CpG sites for both individual IAPs and global IAP were compared by t-test to determine if methylation values differed by tissue. For each individual IAP and global IAP, we evaluated the effect of assignment to perinatal Pb exposure group on IAP methylation at 10 months using a General Linear Model (GLM) where CpG sites were treated as repeated measures and an unstructured covariance matrix was utilized to explicitly model the variance and correlations across sites (Goodrich et al. 2015). The final GLM model controlled for sex and included litter as a random intercept.

Results

The distribution of males and females, as compared to control, did not differ significantly for any of the exposure groups for all born mice as reported previously (Faulk et al. 2013b) or the aged subset of mice used in this study (Table 2) ($p>0.05$). A subset of *a/a* adult animals (n = 93) with

matched kidney and brain tissue was analyzed for IAP DNA methylation levels at 10 months of age, resulting in at least 9 males and 9 females per exposure group (Table 2).

IAP Global Methylation in the Kidney and Brain

An IAP consensus sequence with three CpG sites was analyzed for both kidney and brain tissue for an estimate of global methylation. Site-specific and 3-site average methylation values are found in Table 3. Overall, global IAP average (sd) methylation levels were 92.84% (3.74) and ranged from 85.25 to 100%. The IAP global 3-site average value for brain was significantly lower compared to kidney ($p < 0.001$) with an absolute difference of 0.84%.

The Influence of Pb and Sex on Global IAP Methylation

When the global CpG sites were treated as repeated measures in the GLM, Pb did not influence global IAP methylation values for either brain ($p > 0.05$) or kidney ($p > 0.05$). Further, the GLM resulted in no sex-specific effects for either brain ($p > 0.05$) or kidney ($p > 0.05$).

Individual IAP Methylation in the Kidney and Brain

Amplicons for the 4 individual IAPs where interrogated for DNA methylation values at five CpG sites by pyrosequencing. However, because of reoccurring quality assurance failure at sites 4 and 5 for IAP 506 and 1259, only IAP 110 and 236 have data for all five sites. Site-specific and 3 or 5-site average methylation values are found in Table 3. Excluding IAP 236 site 5, the individual IAP methylation values ranged from 58.08 to 99.54%. Notably, the average methylation of IAP 236 site 5 was nearly half of the next lowest value at 32.40 and 36.15 for kidney and brain respectively. Individual IAP methylation values were averaged across CpG sites for all animals to compare DNA methylation between tissue types. The 5-site average of IAP 110 was higher in the brain compared to the kidney with an absolute difference of 3.60% ($p<0.001$). By contrast, brain DNA methylation was lower compare to kidney for the 5-site

average of IAP 236 (3.54% less methylation; p<0.001) and the 3-site average of IAP 1259 (1.17% less methylation; p<0.001). The 3-site average for IAP 506 was not different for brain and kidney (p>0.05).

The Influence of Pb and Sex on Individual IAP Methylation in the Kidney and Brain

When individual IAP sites were considered as repeated measures in a GLM model, significant reductions in DNA methylation values between medium or high Pb exposure groups and control were observed in the brain, but not in the kidney (see Table 4). In the brain, compared to control, the 16 ppm exposure group had 3.86% less methylation for IAP 110 (p=0.004), the 32 ppm exposure group had 2.83% less methylation for IAP 236 (p=0.008), the 32 ppm exposure group had 1.77% less methylation for IAP 506 (p=0.03). Similarly in the brain, the 16 ppm exposure group displayed 1.01% less methylation for IAP 1259 (p=0.04) when controlling for sex only, however when controlling for litter this relationship was no longer significant (p=0.10). When Pb exposure group was modeled as a continuous variable and the effect of Pb on each individual IAP was tested, a negative trend was observed for all 4 individual IAPs in the brain, and these linear relationships were significant for IAP 110, 236, and 506. The GLM analysis also revealed sex-related differences in methylation in both brain and kidney tissue. In Table 4, the estimated effect of sex is presented after accounting for variance and correlation across individual IAP CpG sites and the relationships of the three levels of Pb exposures compared to control. Females had significantly less IAP 110 methylation in the brain (1.65% female<male, p=0.006) and kidney (1.60% female<male, p=0.01), while females had significantly higher IAP 236 methylation (3.27% female>male, p<0.001) and IAP 1259 methylation (0.61% female>male, p=0.04) in the kidney.

Assessment of Binding Motifs Encompassing Individual CpGs

A 16 base pair (bp) region (7bp +/- the CpG) for each CpG for each individual IAP was used in a binding motif comparison tool called Tomtom (Gupta et al. 2007). This search yielded a total of 33 significant matches of which many uniquely matched a single CpG from an individual IAP. Table 5 contains a list of binding motifs that matched 2 or more CpG regions. Of note, at least one CpG site within every IAP was encompassed by a binding motif for the TEA Domain Transcription Factor 4 (TEAD4), High Mobility Group AT-Hook 2 (HMGA2) and Far Upstream Element Binding Protein 1 (FUBP1). In addition, the 16 bp region around IAP 236 site 5 significantly matched a total of eight binding motifs, which included FUBP1, NK3 Homebox 1 (NKX31), and six variations of the Forkhead Box (FOX) protein (FOXJ3, FOXD3, FOXA2, FOXF2, FOXO1, FOXO4).

Discussion

Here we analyzed DNA methylation patterns for 4 selected individual IAP elements in two previously identified Pb target tissues and observed perinatal exposure-dependent effects in the adult mouse. Moreover, the results from our final statistical model, which accounted for the influence of sex, litter, and for clustering of proximally located CpGs, which were treated as repeated measures rather than averaging, showed that Pb exposure was associated with decreased brain IAP DNA methylation in 3 elements. Although the test for linear trend was significant for these 3 elements in the brain, it is notable that the effect estimates suggest a non-linear dose response. This finding is not altogether surprising as we have shown previously that the A^{vy} allele, a well studied IAP element, also displays a non-monotonic response to neonatal Pb exposure (Faulk et al. 2013b). Sanchez-Martin et al. also observed non-monotonic Pb-induced changes in DNA methylation, which modestly correlated with mRNA expression, and these observations were similarly made in the mouse brain, specifically the hippocampus (Sanchez-Martin et al. 2015).

The brain-related findings in our developmental Pb mouse study have potential human relevance. In most mammals, there is a high degree of maternal-fetal transfer of Pb (Chen et al. 2014). Further, it is believed that an immature blood-brain barrier during early development allows for the passage of Pb into the fetal brain (Stein et al. 2002). The fetal brain, even more than the adult brain, is a primary target of low level Pb exposure with impacts on processes such as cell differentiation, synapse formation, and programed cell death (Mousa et al. 2015). As such, low-level developmental exposures to Pb have been linked to cognitive deficits in humans (Lanphear et al. 2000; Miranda et al. 2007) and rodents (Cohn et al. 1993).

Retrotransposons are expressed in the brain and their activity in this organ has been positively associated with aging and some neurodegenerative diseases though it is unclear if increased retrotransposition is a cause or a consequence of aging and/or disease progression (Reilly et al. 2013). Here we observed that IAP DNA methylation patterns are negatively associated with physiologically relevant levels of Pb long after cessation of the exposure. It is prudent to question the relevance and potential functional consequences of small magnitude epigenetic effect sizes like those measured in this study (Breton et al. 2017). Indeed, a number of studies have found that very small changes in CpG methylation are correlated with differential gene expression (Argos et al. 2015; Kile et al. 2013; Maccani et al. 2015; Murphy et al. 2012). For example, among 418 mother-infant pairs, Murphy et al. found that an smoking-related change in methylation status of about 1% in the differentially methylated region of insulin-like growth factor II (*IGF2*) correlated with substantial increases or decreases (with less or more DNA methylation, respectively) in transcription of *IGF2* measured by the Affymetrix microarray (Murphy et al. 2012). Given that IAP activity (i.e. retrotransposition as well as alternative promotion of neighboring genes) is tightly regulated by DNA methylation, it is possible that Pb-related reductions in methylation could lead to ectopic IAP activity. Thus, as an exploratory aim, we followed up our findings by investigating which transcriptional regulatory factors might be

affected by these methylation pattern changes. Such shifts in DNA methylation, even if small in absolute terms, can directly impact the interactions between binding factors and their binding site, and potentially affect gene transcription or chromatin conformation.

Using Tomtom, we identified a total of 33 binding motifs that matched the DNA sequence proximal to the IAP-related CpGs. Of these, 3 were consistently matched to each of our novel IAPs. The TEAD4 transcription factor has been linked to narcolepsy, a rare disorder involving the dysfunction of normal sleep patterns (Luca et al. 2013), as well as cancer cell development in animals (Cai et al. 2016) and humans (Suzuki et al. 2016). HMGA2 is linked to both obesity and type 2 diabetes mellitus in humans (Markowski et al. 2013), and in a murine knock out model HMGA2 was shown to be a necessary constituent of adipogenesis through its regulation of the relationship between CCAAT enhancer binding protein (C/EBP) and peroxisome-proliferator activated receptor-gamma (PPAR γ) (Xi et al. 2016). In the latter study Xi et al. observed that overexpression of HMGA2 resulted in a marked increase in the formation of mature adipocytes and suggested this supports a link between HMGA2 and diet-induced obesity. FUBP1 is a binding protein involved in the activation of c-myc, a proto-oncogene (Duan et al. 2017) and aberrant expression of FUBP1 is linked to a brain tumor called oligoastrocytoma (Cahill et al. 2015). Interestingly, we observed that methylation at IAP 236 site 5 was considerably lower than all other CpG sites and this was consistent for both brain and kidney. The motif enrichment search for the sequence flanking this specific site revealed significant DNA-binding similarities with members of the FOX protein family, an evolutionarily conserved superfamily of transcriptional regulators (Myatt and Lam 2007). In addition to the superfamily's role in cancer, data suggests the FOXO subgroup is important for regulating metabolism (Gross et al. 2008; Guo 2014).

It is notable that both HMGA2 and FOXO are transcription factors that have been linked to metabolic-related conditions. In a recent series of studies, which used the same longitudinal mouse cohort referenced in the current manuscript, we observed that perinatal Pb exposure leads to metabolic-related physiological effects including increased food intake, body weight, body fat, activity changes, and insulin resistance in adult mice (Faulk et al. 2013b; Faulk et al. 2014a; Wu et al. 2016). Although Pb toxicity research has traditionally focused on neurological-related outcomes, other studies investigating developmental Pb exposure have also observed persistent physiological changes including decreased fetal and childhood growth in humans and late onset obesity in rodents (Afeiche et al. 2011; Gonzalez-Cossio et al. 1997; Iavicoli et al. 2004; Leasure et al. 2008; Little et al. 2009). Thus, epigenetic activation of transcription factors such as HMGA2 and FOXO may be part of the overall mechanism linking Pb exposure to metabolic-related outcomes and warrants further investigation. We do highlight that exploratory findings such as these, while useful for generating hypotheses for further investigation into potential disease prevention and mitigation strategies, should be interpreted cautiously (Tran and Huang 2014).

In addition to exposure-specific effects we also observed significant differences in DNA methylation between adult male and female mice in both brain and kidney tissue. Sexually dimorphic characteristics underscore normal and pathologic function in the brain (McCarthy et al. 2012) and kidneys (Tomat and Salazar 2014). Sex-related differences in disease outcomes following exposure may reflect the timing of exposure relative to epigenetic programming events that occur during different developmental periods for males and females (Schneider et al. 2016). Furthermore, previous studies in humans have highlighted that variations in methylation, even in a small percentage of retrotransposon loci, can underlie major changes in gene expression and contribute to a disease phenotype (Bakshi et al. 2016)..

Sexually dimorphic phenotypes and related biomarkers (e.g. DNA methylation) are of increasing interest. For example, a cross-sectional study of 51 healthy boys and girls found that DNA methylation profiles measured in peripheral blood were predictive of pubertal development and reproductive hormone levels (Almstrup et al. 2016). The authors suggest the recent decline of pubertal age in Western society may be linked to epigenetic programming. While developmental exposures were not within the scope of this study by Almstrup et al., it is reasonable to hypothesize that the link between Western society and epigenetic programming could be related to early life exposure-induced epigenetic alterations in relevant labile regions. It follows then that sex-specific observations such as those found in our study may be informative when designing future studies aimed at evaluating the links between early exposures and sexually dimorphic disease susceptibility.

The current study provides new insights into potential epigenetic mechanisms underlying the developmental origins of health and disease; however, there are some limitations, which will need to be addressed by future studies. First, DNA in this study was extracted from tissues made up of a heterogeneous collection of cells. DNA methylation can vary by cell type and therefore must be acknowledged moving forward. Second, we only evaluated DNA methylation profiles in a small subset of the novel IAP elements we have identified and did not assess their level of activity (i.e. ability to modulate gene expression). Finally, pyrosequencing does not differentiate between 5-methylcytosine and 5-hydroxymethylcytosine. Therefore, because 5-hydroxymethylcytosine is enriched in the brain, dynamically regulated throughout the lifecourse, and associated with neurodegenerative disorders (Sherwani and Khan 2015), in the future, it will be important to determine if reductions in DNA methylation associated with Pb exposure are TET mediated via altered 5-hydroxymethylcytosine or a product of programmed hypomethylation from the time of early development.

Conclusion

Using the mouse as a model organism to investigate the developmental origins of health and disease, we show that exposure to human physiologically relevant levels of Pb pre-conceptionally through the first 21 days of life results in persistent reductions of DNA methylation in adult brain in specific genomic regions in which epigenetic repression is critical. Further, these exposure-related shifts in retrotransposon methylation profiles displayed sex-specific differences.

Author Contributions

DCD provided mentorship at all stages including design, study implementation, analysis, and revision of the manuscript. JF aided in DNA extraction, PCR reactions and pyrosequencing. CF designed and implemented the mouse study, designed IAP assays and revised the manuscript. LM performed DNA extraction, PCR and pyrosequencing as well as performed the analysis and drafted the manuscript.

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Table 1. PCR primers, conditions, and location of sequence to be amplified

IAP	PCR Conditions	Location	Forward Primer	Reverse Primer (biotinylated)	Sequencing Primer	Sequence to Analyze
110	1) 95° 15min 2) 95° 30sec 3) 56° 1min 4) 72° 1 min 5) 72° 5min 6) 4° hold Repeat steps 2-5 49 times	chr2:72112889-72114056	AGGGAGTATT ATTTTTTGATT GGTTGTAGT	CTTCTTTCCCC TAAAATTCAATA TTCTT	AGTGGTAGTAA ATATTTTTGGTT T	ATGYGTAGAT TATTTGTTTA TTAATTTAGA ATATAGGATG TTAGYGTTAT TTTGTGAYGG YGAATGTGGG GGYGGTTTTT TATAGTATTT GAGAATAT
236		ch4:155057154-155058318	GGGAAAGGT AGAGTATAAG TAGTTATAAA	TACACAATCTA TTCTCAACCCT CTAA	ATTATTTAGAAT ATAGGATGTTA G	YGTTATTTTG TGAYGGYGAA TGTGGGGGYG GTTTTTAATA AAATTGTATA YGTTTTTTTT TGGGGGAAAA ATAAGA
506		chr12:74416066-74417247	GGGGAAAGT AGAGTATAAG TAGT	ATAATAAAAACT AAACTTCCTTC CCATTAA	ATAAGTAGTTA TAAGATATTTTT GG	TATATGYGTA GATTATTTGT TTATTATTTA GAATATAGGA TGTTAGYGTT ATTTTGTAAY GGYGAATGTG GGGGYGGTTT TTAATAAAGA GTTATTTAAT
1259		chr8:8319882-8321071	GGGGAAAGT AGAGTATAAG TAGTTATAAG	CTTACTAAAAA ATTCAATACAC CAACTCT	AAGTAGTTATA AGATATTTTTG GT	ATATGYGTAG ATTATTTGTT TATTATTTAG AATATAGGAT GTTAGYGTTA TTTTGTAAYG GYGAATGTGG GGTATATYGA TAAGATATTT TGTAGAGTTG GTG
Global	1) 95° 15 min 2) 95° 30 sec 3) 56° 30 sec 4) 72° 1 min 5) 72° 5 min 6) 4° hold Repeat steps 2-5 44 times		GTGTTATTTT TTGATTGGTT GTAGTTT	ACCAAAAATAT CTTATAACTACT TATACT	ATTTTTTGATTG GTTGTAGTTTA	TYGGTYGAGT TGAYGTTAYG GGGAAAGTAG AGTATAAGTA GTTA

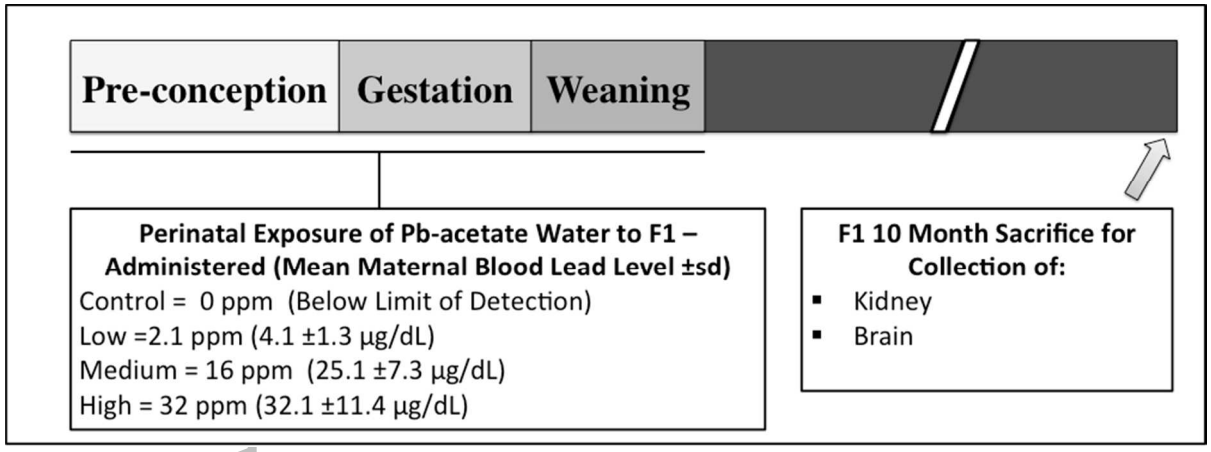

Figure 1. Exposure paradigm for perinatal lead exposure. Dams were exposed to either control water or Pb-acetate water starting two weeks prior to mating through gestation until postnatal day 21 (PND21). Following this perinatal exposure, all pups were transitioned to control water until 10 months of age at which time matched kidney and brain tissues were collected for DNA extraction.

Table 2. Distribution of mice by sex and exposure group

	Exposure Group			
	Control (0ppm)	Low (2.1 ppm)	Medium (16 ppm)	High (32 ppm)
Female	12	9	13	12
Male	10	10	13	14
Total	22	19	26	26

Table 3. Average IAP methylation for each CpG site stratified by tissue and naïve to exposure

		Brain				Kidney			
IAP	**CpG site**	**n**	**Avg Me%**	**Range**	**SD**	**n**	**Avg Me%**	**Range**	**SD**
110	1	70	88.94	76.94 - 100.00	5.24	64	84.61	77.87 - 100.00	4.18
110	2		90.43	71.69 - 100.00	6.59		87.79	76.65 - 100.00	7.21
110	3		68.79	53.78 - 90.94	7.07		62.70	55.21 - 72.00	3.68
110	4		62.27	37.8 - 73.35	5.47		58.08	46.91 - 80.01	4.62
110	5		89.59	74.00 - 100.00	7.88		89.30	70.58 - 100.00	8.79
110	5-site Avg		80.00 ***	68.03 - 85.71	3.58		76.40	68.56 - 85.55	3.21
236	1	76	83.12	69.24 - 100.00	4.68	67	86.69	77.18 - 100.00	6.09
236	2		78.84	66.64 - 100.00	5.43		81.33	70.63 - 100.00	5.05
236	3		78.60	66.97 - 85.78	4.10		85.66	72.07 - 100.00	59.32
236	4		80.65	50.55 - 100.00	7.00		89.85	81.07 - 100.00	7.07
236	5		36.15	26.95 - 47.77	4.56		32.40	24.46 - 41.27	3.85
236	5-site Avg		71.47	59.61 - 82.01	3.87		75.01 ***	69.87 - 86.99	3.73
506	1	66	95.31	74.89 - 100.00	7.03	87	93.95	81.63 - 100.00	5.21
506	2		99.54	84.27 - 100.00	2.65		98.93	91.12 - 100.00	2.36
506	3		71.07	55.99 - 100.00	8.41		74.61	61.28 - 100.00	6.36
506	3-site Avg		88.64	80.98 - 100.00	3.91		89.16	83.36 - 100.00	2.48
1259	1	80	92.34	84.59 - 100.00	1.92	86	94.35	90.47 - 100.00	1.84
1259	2		95.81	88.37 - 100.00	3.80		96.13	91.11 - 100.00	2.99
1259	3		74.39	68.09 - 79.26	2.32		75.55	57.54 - 81.46	4.67
1259	3-site Avg		87.51	83.33 - 92.05	1.69		88.68 ***	82.94 - 93.04	1.62
Global	1	85	93.73	87.81 - 97.85	2.61	87	93.89	86.64 - 100.00	3.42
Global	2		94.27	89.48 - 98.97	2.2		94	86.15 - 98.66	2.81
Global	3		88.46	85.25 - 92.46	1.58		91.1	87.81 - 100.00	1.49
Global	3-site avg		92.16	89.53 - 94.66	1.2		93.00 ***	90.45 - 96.60	1.13

* $p<0.05$, ** $p<0.01$, *** $p<0.001$ (t-test between tissue difference of the 5- or 3-site average methylation values)

Table 4. Results from GLM model showing effect of lead and sex on individual and global IAP methylation in brain and kidney tissue

IAP	Tissue	n	Lead Effect		Linear Trend for Lead Effect	Sex Effect
			Exp Grp Compared to Control	Effect Estimate	Effect Estimate	Effect Estimate
110	**Brain**	70	1	-1.24	-0.92 *	F<M 1.65% **
			2	-3.86 **		
			3	-2.29		
236	**Brain**	76	1	-0.70	-0.90 **	F<M 0.76%
			2	-1.23		
			3	-2.83 **		
506	**Brain**	66	1	-0.74	-0.52 *	F>M 0.10%
			2	-0.41		
			3	-1.77 *		
1259	**Brain**	80	1	-0.18	-0.19	F<M 0.19%
			2	-0.94		
			3	-0.36		
Global	**Brain**	85	1	0.17	0.06	F>M 0.26%
			2	0.00		
			3	0.26		
110	**Kidney**	64	1	1.20	-0.26	F<M 1.60% **
			2	0.51		
			3	-0.87		
236	**Kidney**	67	1	-0.25	-0.18	F>M 3.27% ***
			2	-0.34		
			3	-0.59		
506	**Kidney**	87	1	0.55	0.08	F<M 0.69%
			2	0.38		
			3	0.26		
1259	**Kidney**	86	1	-0.16	-0.02	F>M 0.61% *
			2	0.20		
			3	-0.20		
Global	**Kidney**	87	1	-0.03	0.08	F>M 0.07%
			2	0.01		
			3	0.24		

* p<0.05, ** p<0.01, **** p<0.001 1= low exposure group, 2= medium exposure group, 3= high exposure group

Table 5. Truncated results from the TomTom binding motif search—showing binding sites found within 2 or more targeted CpG regions of the assayed IAPs

IAP	CpG	(+/- 7 bp)	PO6F1	ONEC2	TEAD4	HMGA2	FUBP1	E4F1	CREB1	STAT3	NFYA	PO2F2	OVOL1	INSM1
110	1	AGTGGTCGTAAATAT												
	2	GTTTATGCGTAGATTA	x											
	3	ATGTTAGCGTTATTTT		x	x	x	x							
	4	TTTGTGACGGCGAATG						x	x					
	5	GTGACGGCGAATGTGG							x	x	x			
236	1	ATGTTAGCGTTATTTT		x	x	x	x							
	2	TTTGTGACGGCGAATG						x	x					
	3	GTGACGGCGAATGTGG							x	x	x			
	4	GTGGGGGCGGTTTTTA			x									
	5	TTGTATACGTTTTTTT					x							
506	1	GTATATGCGTAGATTA										x		
	2	ATGTTAGCGTTATTTT			x	x	x							
	3	TTTGTAACGGCGAATG											x	x
1259	1	GTATATGCGTAGATTA	x									x		
	2	ATGTTAGCGTTATTTT		x	x	x	x							
	3	TTTGTAACGGCGAATG											x	x