Epigenetics

ISSN: 1559-2294 (Print) 1559-2308 (Online) Journal homepage: http://www.tandfonline.com/loi/kepi20

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To cite this article: Christopher Faulk, Kevin Liu, Amanda Barks, Jaclyn M Goodrich & Dana C Dolinoy (2014) Longitudinal epigenetic drift in mice perinatally exposed to lead, Epigenetics, 9:7, 934-941, DOI: 10.4161/epi.29024

To link to this article: http://dx.doi.org/10.4161/epi.29024

Published online: 01 May 2014.

Epigenetics 9:7, 934–941; July 2014; © 2014 Landes Bioscience

Longitudinal epigenetic drift in mice perinatally exposed to lead

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Keywords: epigenetics, development, environment, DNA methylation, plasticity, environmental epigenomics, drift

Abbreviations: IAP, intracisternal A-particle; DMR, differentially methylated regions; A^{vy}, Agouti viable yellow; BPA, bisphenol A; Pb, lead; BLL, blood lead level; LOD, limit of detection

An understanding of the natural change in DNA methylation over time, defined as "epigenetic drift," will inform the study of environmental effects on the epigenome. This study investigates epigenetic drift in isogenic mice exposed perinatally to lead (Pb) acetate at four concentrations, 0 ppm (control), 2.1 ppm (low), 16 ppm (medium), and 32 ppm (high) prior to conception through weaning, then followed until 10 months of age. Absolute values of DNA methylation in a transposon-associated metastable locus, Cdk5-activator binding protein (*Cabp*^{IAP}), and three imprinted loci (*Igf2, Igf2r,* and *H19*) were obtained from tail tissue in paired samples. DNA methylation levels in the controls increased over time at the imprinted *Igf2* and *Igf2r* loci (both $P = 0.0001$), but not at the imprinted *H19* locus or the *Cabp*^{IAP} metastable epiallele. Pb exposure was associated with accelerated DNA hypermethylation in *Cabp*^{IAP} ($P = 0.0209$) and moderated hypermethylation in *Igf2r* ($P = 0.0447$), and with marginally accelerated hypermethylation at *H19* ($P = 0.0847$). In summary, the presence and magnitude of epigenetic drift was locus-dependent, and enhancement of drift was mediated by perinatal Pb exposure, in some, but not all, loci.

Introduction

The epigenome refers to mitotically heritable alterations to chromatin including histone modifications and covalent additions to DNA bases such as DNA 5-methylcytosine. As individuals age, the epigenome undergoes "epigenetic drift," a phenomenon of increasingly divergent DNA methylation marks over the life course.[1] Studies of aging and genetic dysregulation (e.g., cancer) support a decrease in global genomic DNA methylation (hypomethylation) concurrent with locus-specific increases in DNA methylation (hypermethylation).[2,3] Consequently, epigenetic drift has emerged as a biomarker for aging, with increased DNA methylation targeting developmental genes.[1,4] The preponderance of evidence to date, however, has been measured in cross-sectional samples, thereby lacking a longitudinal component of evaluating DNA methylation changes in paired samples over time.[5,6] Further, active environmental perturbations of the rates of epigenetic drift have yet to be studied.

Environmental influences on the epigenome are increasingly understood as having a role in later life morbidities, and serve as a likely mechanism associated with the developmental origins of health and disease (DOHaD). As the establishment of DNA methylation profiles occurs in early development, early life exposures are likely to be especially important in setting the trajectory of epigenetic drift.[7,8] For example, maternal nutritional supplementation with folate in mice has been show to protect against global and gene-specific DNA hypomethylation associated with aging.[9] Similarly, toxicant exposure in rodents affects the establishment of DNA methylation profiles across multiple tissues including liver and brain.[10,11] Likewise, clinical disorders, such as myelodysplastic syndrome, have been associated with accelerations in epigenetic drift.[12]

Historically, the heavy metal lead (Pb) has been studied as a potent developmental toxicant, and recent studies implicate epigenetic modifications as drivers of Pb's developmental dysregulation.[13,14] Pb is a persistent environmental pollutant with uptake via air, soil, water, and old paint as avenues for ingestion.[15] Currently, the reference level at which the US. Centers for Disease Control and Prevention (CDC) recommend public health actions be initiated is 5 μg/dL.[16] However, in the late 1970s, median BLL across US preschool children was 15 μg/dL, and 18.5% of African American children had a BLL of over 30 μg/dL, indicating that high historic exposures are relevant to the later life health outcomes of the current adult US population.[17] In mice, we recently showed that perinatal exposure to Pb sufficient to raise maternal blood lead level near 5 μg/dL was associated with increased wean weight in male offspring as well as shifts in DNA methylation at the A^{vy} locus at weaning.[18] Here, we answer the question of whether these shifts in DNA methylation are

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Submitted: 04/01/2014; Revised: 04/22/2014; Accepted: 04/25/2014; Published Online: 05/01/2014
http://dx.doi.org/10.4161/epi.29024

stable over time and whether changes in DNA methylation over the life-course are influenced by perinatal Pb exposure.

Two potential epigenetic susceptibility targets for environmentally induced effects are metastable epialleles and cis-acting regulatory elements of imprinted genes. Transposon-associated metastable epialleles, such as agouti viable yellow (A^{vy}) and CDK5-activator binding protein ($Cabp^{IAP}$), contain cryptic promoters in nearby Intracisternal A Particle (IAP) transposons that are normally silenced by DNA methylation. Imprinted genes contain parent-of-origin epigenetic marks, including differentially methylated regions (DMRs), resulting in mono-allelic expression. These commonly used biomarkers host CpG islands that are variably methylated[19] or differentially methylated,[18,20] respectively, and environmental exposures have been shown to stably influence their epigenetic marks and subsequent gene expression.[21-24] Therefore, measuring the relative change in DNA methylation from early to adult life in epigenetically labile regions is crucial to determining whether early life Pb exposure can alter the magnitude or direction of epigenetic drift.

The present study investigates the longitudinal change in absolute mean DNA methylation at representative metastable and imprinted gene loci in paired tail tissue from mice at weaning (day 22) and again at 10 mo of age. These regions were selected based on frequent use as biomarkers in animal and/or human epigenetics studies. Tail tissue was utilized as it can be collected at both weaning and sacrifice time points from the same animals, thus eliminating confounding effects of interindividual differences by using paired samples from the same animal over time. We also assess whether perinatal environmental exposure to the representative metal, Pb, accelerates the rate of change in DNA methylation in 3 imprinted genes (*Igf2, Igf2r, H19*) and the transposon-containing metastable epiallele $Cabp^{IAP}$. We observe significant absolute increases in DNA methylation over time for several exposure levels at all four loci examined. Changes in relative methylation reveal a significant trend of accelerated hypermethylation with Pb exposure for $Cabp^{IAP}$ and *H19,* and an exposure dependent moderation of the hypermethylation drift for *Igf2r.* As previous work concludes that epigenetic drift is insufficient to explain differences in age-related DNA methylation,[25] environmentally-induced changes to DNA methylation over time, shown here, help explain shifts due to factors beyond chronological age.

Results

Litter parameters

As reported previously, maternal blood lead levels (BLLs) were measured in a sample of animals with the control group being below the limit of detection (1.3 μg/dL). Maternal BLLs for the three exposure groups resulted in human physiologically relevant levels.[16] The 2.1 ppm exposure group resulted in a BLL range of 2.0 to 5.88 μg/dL (mean = 4.1). The 16 ppm group ranged from 13 to 40 μg/dL (mean = 25.1), and the 32 ppm group ranged from 16 to 60 μg/dL (mean = 32.1).[18] Perinatal Pb exposure did not result in overt maternal or offspring toxicity and did not alter litter size, sex ratio, or genotype ratio of *a/a* to A^{vy}/a offspring (n = 314). The number of pups surviving to weaning in the 2.1 ppm group was significantly reduced ($P = 0.01$) as compared with control, and was the result of poor maternal care in two litters where the dam did not nurture the litter, allowing entire litters to perish. The control, 2.1 ppm, 16 ppm, and 32 ppm groups had survival rates of 96%, 82%, 96%, and 90%, respectively. A subset of *a/a* animals (n = 120) was selected for longitudinal analysis using paired tail tissue at day 22 and 10 mo of age for DNA methylation analysis and will be referred to throughout this remainder of the report.

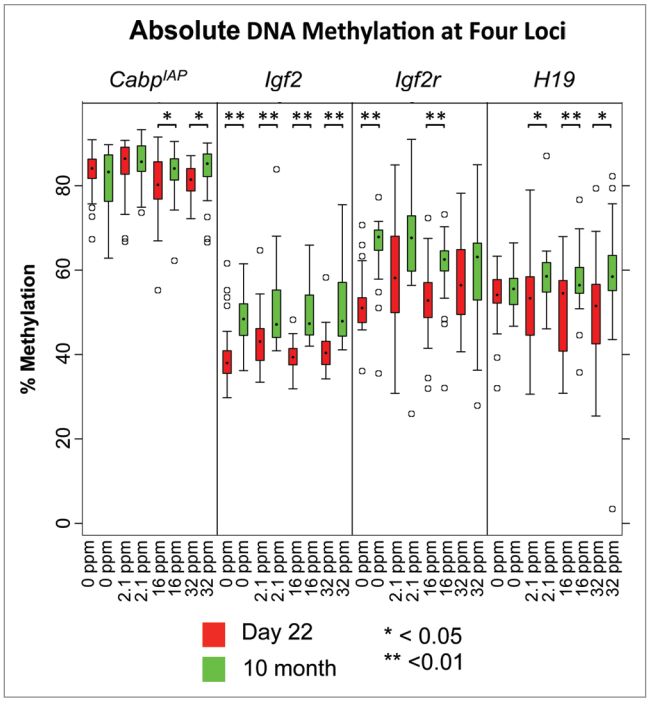

Figure 1. Absolute DNA methylation. DNA methylation percentage in four loci measured at two time points, day 22 (red) and 10 mo (green) in pairwise matched samples reveals epigenetic drift. Four lead exposure cohorts are indicated for each locus. Stars indicate significant changes in DNA methylation between time points.

Absolute levels of gene methylation

DNA methylation of the $Cabp^{IAP}$ metastable epiallele and the imprinted genes, *Igf2, Igf2r, H19* was measured in paired samples from day 22 and 10 mo tail tissue (**Table 1**). In the transposon associated $Cabp^{IAP}$ metastable epiallele, DNA methylation significantly increased in the two higher exposure groups, from 79.77% to 82.72% in the 16 ppm, medium group ($P = 0.0516$), and from 80.89% to 83.43% in the 32 ppm high group ($P = 0.0454$), but did not change over time in the 2.1 ppm low lead exposure group ($P = 0.3896$) or the 0 ppm control group ($P = 0.2796$) (**Fig. 1**). All three imprinted genes displayed an increase in DNA methylation over time with varying responses to perinatal lead exposure. DNA methylation of *Igf2* was significantly increased in all exposure groups from 40.13% to 48.40% in control ($P = 0.0001$), 43.51% to 50.75% in the low exposure group ($P = 0.0012$), 39.41% to 49.51% in the medium exposure group ($P = 0.0001$), and 40.75% to 51.00% in the high exposure group

Table 1. Absolute and relative DNA methylation

		Relative Change in Methylation Levels					Relative Change in Methylation Levels	
Gene	Exposure	N	Day 22 Methylation (SD)	10 Month Methylation (SD)	Change over Time *P* value		10mo - d22	P_{Trend}
Cabp^{IAP}	0 ppm	30	83.03 (5.26)	81.35 (6.97)	0.2796		-1.68	0.0209
	2.1 ppm	28	84.53 (6.47)	85.79 (4.63)	0.3896		1.27	
	16 ppm	29	79.77 (7.52)	82.72 (5.55)	0.0516*		2.95	
	32 ppm	28	80.89 (4.21)	83.43 (5.92)	0.0454*		2.54	
	Range		4.76	4.44		Avg	1.27	
Igf2	0 ppm	29	40.13 (7.32)	48.40 (6.12)	0.0001**		8.27	0.3050
	2.1 ppm	28	43.51 (6.59)	50.75 (9.73)	0.0012**		7.24	
	16 ppm	28	39.41 (3.44)	49.51 (6.53)	0.0001**		10.09	
	32 ppm	27	40.75 (4.91)	51.00 (9.28)	0.0001**		10.25	
	Range		4.10	2.60		Avg	8.96	
Igf2r	0 ppm	24	51.89 (7.38)	65.23 (8.45)	0.0001**		13.35	0.0447
	2.1 ppm	20	58.29 (14.27)	65.95 (12.62)	0.0708		7.66	
	16 ppm	22	52.05 (9.73)	60.44 (8.84)	0.0044**		8.39	
	32 ppm	27	57.23 (9.18)	60.70 (12.62)	0.2314		3.47	
	Range		6.40	5.51		Avg	8.22	
H19	0 ppm	23	53.44 (7.08)	55.43 (4.95)	0.2582		1.99	0.0847
	2.1 ppm	22	51.86 (11.23)	58.78 (7.90)	0.0224*		6.92	
	16 ppm	23	50.19 (9.95)	57.46 (8.16)	0.0042**		7.27	
	32 ppm	27	49.61 (11.77)	58.64 (14.10)	0.0120**		9.03	
	Range		3.83	3.35		Avg	6.30	

* < 0.05; * < 0.05.

(*P* = 0.0001). At the *Igf2r* locus, DNA methylation increased over time in the control group from 51.89% to 65.23% (*P* = 0.001) and in the 16 ppm exposure group, from 52.05% to 60.44% (*P* = 0.0044), while the lowest exposure group showed only a marginally significant increase in DNA methylation, with measurements of 58.29% to 65.95% (*P* = 0.0708), and the highest exposure group resulting in no significant change over time, shifting from 57.23% to 60.70% (*P* = 0.2314). *H19* responded to perinatal lead exposure with increasing DNA methylation over time in the exposed offspring but not in control. The 0 ppm group had 53.44% DNA methylation at day 22 and 55.43% at 10 mo (*P* = 0.2582). In contrast, the low exposure group increased from 51.86% to 58.78% (*P* = 0.0224), the medium exposure group increased from 50.19% to 57.46% (*P* = 0.0042), and the highest exposure group increased from 49.61% to 58.64% (*P* = 0.0120).

Relative levels of gene methylation

To determine whether perinatal lead exposure impacted the rate of change of DNA methylation longitudinally, we assessed relative changes from day 22 to 10 mo of age in the control and three exposure groups (**Table 1**). The increase in *Cabp*^{IAP} DNA methylation was significantly enhanced by perinatal lead exposure (linear trend *P* = 0.0209) (**Fig. 2**). On average, the magnitude of the increase in *Cabp*^{IAP} DNA methylation was 1.27% over time across the four exposure levels as shown by the significant increasing linear trend. Interestingly, the control group shows reduced methylation over time (–1.68%) in *Cabp*^{IAP}, while every exposure group increased in methylation (range 1.27% to 2.95%). While *Igf2* showed the greatest magnitude of change over time, averaging 8.96% across the four exposure groups, its increase was not correlated with lead exposure (linear trend *P* = 0.305). There was a slight but statistically insignificant increase in the relative methylation between the control group (8.27% increase) and the two higher lead exposed groups (both > 10%). In contrast, lead exposure moderated the age-dependent increase for *Igf2r*, which had a significant relative decrease concomitant with perinatal lead (linear trend *P* = 0.0447). *Igf2r* in control mice had the highest increase in methylation of any gene in any group, increasing by 13.35%, yet in the high exposure animals, this increase was only 3.47% (average 8.22% across all groups). In an opposite trend, *H19* showed only a marginally significant enhancement of DNA methylation (linear trend *P* = 0.0847) with an average gain of 6.30% across all groups. Importantly, however, the control animals showed no statistically significant increase in *H19* methylation over time (*P* = 0.2582), with a relative change of 1.99% methylation, yet al. lead exposed groups

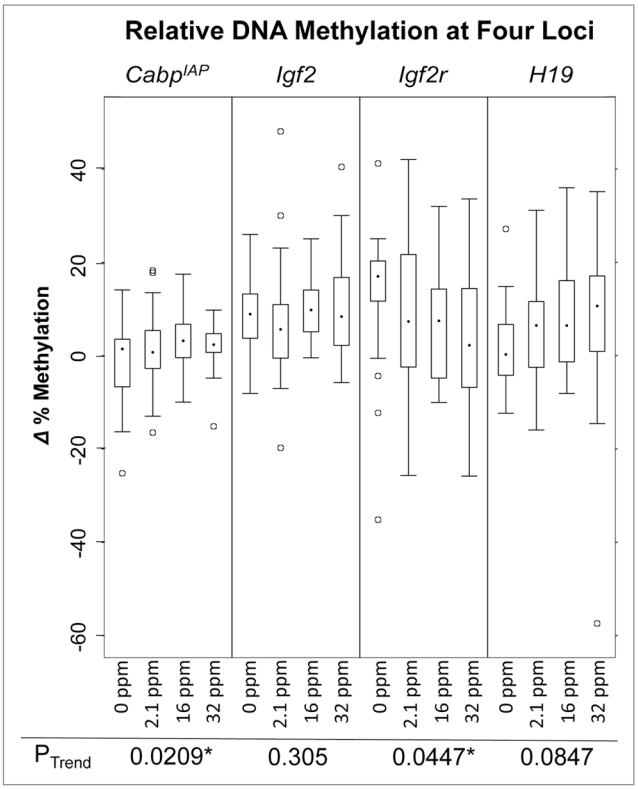

Figure 2. Relative DNA methylation. Changes in DNA methylation in four loci indicate change in DNA methylation over time relative to day 22. Each exposure group is delineated and linear trend p-values are shown below. Stars indicate significant linear trends < 0.05 in DNA methylation.

increased significantly in methylation, reaching a 9.03% increase in methylation in the 32 ppm exposure animals (P = 0.0120).

Discussion

The metastable and imprinted loci evaluated here are often used as biomarkers in both animal and human epigenetics studies, yet they are most often measured at one time point and are rarely measured longitudinally to account for epigenetic drift. Using an isogenic colony of mice and an established perinatal environmental exposure paradigm, we eliminate the impact of genetic variation and minimize the effects of nutritional and environmental variability, to isolate the impact of Pb exposure on epigenetic drift over the life course. We observed an increase in DNA methylation in four genes from weaning to 10 mo of age and, in some cases, alterations in the relative increase in DNA methylation were influenced by perinatal, yet transient, lead exposure. Recent research has focused on the rates of aging in somatic tissues as measured by DNA methylation differences, longitudinal changes over time, and shifts in late life DNA methylation values due to early life environmental exposure.[4,11,26] Here we combine measurement of longitudinal change with perinatal exposure to find whether shifts in early life DNA methylation at four loci are predictive of later-in-life DNA methylation values. As a direct relevance to human health, our lead exposure

Table 2. Litter parameters

Exposure	N (litter)	Male	Female	No. pups
Control	11	18	12	30
2.1 ppm	9	16	12	28
16 ppm	11	19	12	31
32 ppm	13	17	13	30
Total	44	70	49	119

paradigm resulted in human-relevant blood lead levels, with the low dose, 2.1 ppm, yielding a mean BLL of 4.1 μg/dL, just below the CDC action level of 5 μg/dL.[16]

Metastable epialleles are loci where the epigenetic state is stochastically established in development, and remains fixed thereafter. The metastable epiallele, CDK5-activator binding protein (*Cabp*^{IAP}) has been widely used as a biomarker of environmental exposure alteration to the epigenome.[27-30] Until now, the longitudinal changes in *Cabp*^{IAP} DNA methylation level had not been described. The *Cabp*^{IAP} locus has a high baseline level of methylation, explaining the small average increase across exposure groups (1.27%). This relative increase, however, was significantly higher in the high (2.54%) and medium exposure groups (2.95%), indicating perinatal exposure to Pb can influence the rate of change over time. Overall, the relative change in methylation level after 10 mo was accelerated with lead exposure as shown by the positive linear trend (P = 0.0209). Though the induced shift is small, multiple studies have shown correlations with small absolute methylation shifts in transposons are associated with human aging and exposure to air pollution.[20,31,32]

Imprinted genes are often used as candidate loci for environmentally induced epigenetic change, making a study of their natural change in DNA methylation over the life-course a valuable baseline measurement.[33,34] For example, human insulin-like growth factor 2 (*IGF2)* DNA methylation has been positively correlated with age in multiple cross-sectional studies.[33,35] Here *Igf2* showed a robust increase of 8 to 10% absolute DNA methylation over time in exposed and unexposed mice, respectively. Unlike the *Cabp*^{IAP} metastable epiallele, the relative rate of change of methylation was not influenced by perinatal lead exposure. Analysis of the Igf2 receptor, *Igf2r*, which is imprinted in mice but not humans,[36] showed a 13% increase in absolute DNA methylation over the 10-mo time course in control mice. Interestingly, lead exposure exhibited a moderating effect on the longitudinal *Igf2r* increase (P = 0.0447), with the 32 ppm exposed mice only increasing by DNA methylation by 3.5% over 10 mo. The DNA methylation of *Igf2* is of significant interest in evolutionary biology since, unlike *Igf2r*, it is imprinted in both humans and mice, and serves as a marker of historical exposure.[33,37]

Lastly, the *H19* imprinted gene behaved similarly to *Cabp*^{IAP} with a marginally significant relative acceleration in DNA methylation concomitant with perinatal lead exposure (linear trend P = 0.0847). As with *Cabp*^{IAP}, in control mice there was no statistically significant increase in DNA methylation from day 22 to 10 mo; however, in mice from every level of lead exposure, the absolute methylation level increased significantly (all P < 0.05).

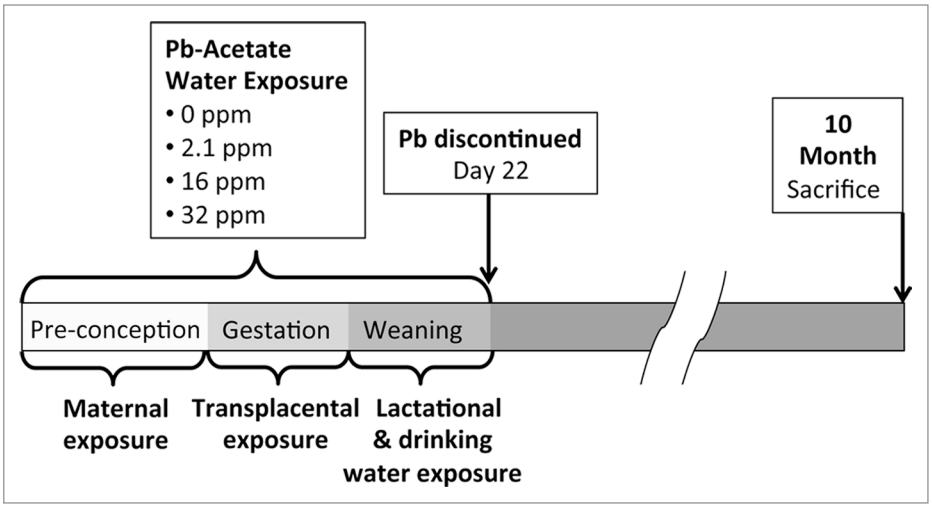

Figure 3. Exposure paradigm. Dams were exposed 2 wk prior to mating to one of four levels of lead (Pb)-acetate in drinking water. Offspring exposure continued through gestation and lactation until postnatal day 22, upon which offspring were transferred and maintained in a Pb-free environment thereafter, until 10 mo of age.

Thus, *H19* may serve as a sensitive biomarker of historic lead exposure and possibly other environmental contaminants. The *H19-IGF2* DMR is a hotspot of epimutations in humans, leading to Beckwith-Weidmann syndrome and Wilm's tumor, thus any changes to allelic contribution or dosage-compensation may have observable health effects.[38] Linking methylation to physiological change, Huang et al.[39] found an association with DNA methylation at the human *IGF2/H19* imprinting control region (ICR) and subcutaneous fat but not with BMI in adolescents. They hypothesize that peripheral fat deposits are influenced by *IGF2/H19* ICR methylation in early life obesogenic environments.

Our previous study found that Pb exposure did not alter DNA methylation at imprinted genes at weaning.[18] Here we find that Pb's effect on imprinted gene methylation was delayed, only becoming evident later-in-life. It is likely that the epigenetic developmental programming induced by Pb has not acted directly on these imprinted genes, since they are undifferentiated at weaning. Instead, it is more probable that the shift in methylation is a result from trans-acting factors manifesting from physiological or epigenetic effects magnifying over time as the animals age. Thus, small early life changes can potentially have outsize consequences on human health, even long after the cessation of an environmental insult. In the future, longitudinal human birth cohorts with well characterized early exposures and carefully collected expression and outcome data would be an ideal system to determine whether historical exposure can affect epigenetic drift.

Conclusion

We measured DNA methylation longitudinally in tail tissue of isogenic mice at postnatal day 22 and 10 mo of age to determine the magnitude of epigenetic drift at four loci commonly used as biomarkers in human and animal epigenetics studies. Assessing drift in isogenic mice eliminates confounding factors such as genetic heterogeneity and age cohort effects and allows for rigorous control of environmental and dietary conditions. The results of this study indicate that the DNA methylation of metastable epialleles and imprinted genes is not stable from childhood to adulthood. At the same time, perinatal lead exposure alters the rate and direction of change in DNA methylation that occurs during aging.

Materials and Methods

Animals and exposure

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.[40,41] 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 four Pb exposure groups to model human relevant perinatal exposure. Drinking water supplemented with Pb-acetate (1) 0 ppm (n = 11 litters), (2) 2.1 ppm (n = 9 litters), (3) 16 ppm (n = 11 litters), and (4) 32 ppm (n = 13 litters) (**Table 2**). Treatment water was made by dissolving Pb (II) acetate trihydrate (Sigma-Aldrich) in single batches of distilled water. Water lead concentrations were verified using inductively coupled plasma mass spectrometry with a limit of detection of 1.3 μg/dL (ICPMS; NSF International). Blood lead was measured in a subset of dams at weaning through blood collected by cardiac puncture and measured via ICPMS at the Michigan Department of Community Health, with limit of detection (LOD) of 1.3 μg/dL. 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

Table 3. PCR conditions

Primer/sequence to analyze	*Cabp*^{IAP} Assay	*Igf2* Assay	*Igf2r* Assay	*H19* Assay
Location	chr2:154179960–154180168 strand = reverse	chr7:149839707–149839926 strand = reverse	chr17:12935154–12935313 strand = reverse	chr7:149767589–149767843
Forward PCR primer	ATTATTTTTT GATTGGTTGT AGTTTATGG	TTTTTTAATA TGATATTTGG AGATAGTT	TGGTATAATT AGAATTATAG TTTAAT	GGGGGGTTAT AAATGTTATT AGGGGGGTAG G
Reverse PCR primer	biotin-CACCAACATA CAATTAACA	biotin-CCACATAATT TAATTCACTA ATAATTACTA	biotin-AAAAAACTCA AAAAATTCC	biotin-AACCCCTAAC CTCATAAAAC CCATAACTAT AAAATCA
Sequencing primer	TAGAATATAG GATGTTAG	AATATGATAT TTGGCGATAG TT	ATAATTAGAA TTATAGTTTA	GTGTAAAGAT TAGGGTTGT
Sequence to analyze	YGTTATTTTG TGAYGGYGAA TGTGGGGGYG GTT	YGYGGGAYGT TTGYGTAGAG GTTTGTTTGT TTTTTTGYGT GTTYGTYGGG GTYGT	ATYGGAATYG TATTAAAATT TTTYGAATTT TTGGGTAGYG	GYGGTYAGTG AAGTTTYGTA TATYG
Amplicon length	209	220	127	255
Temperature	47	56	52	55
Number of cycles	40	50	50	40

A^{vy}*/a* males. Exposure was continued during gestation and lactation. At 22 d of age, animals were tail tipped with tips stored frozen (–80 °C). After weaning, the resulting pups were weighed and switched to untreated Pb-free drinking water (**Fig. 3**). 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 mo of age: 0 ppm, n = 30; 2.1 ppm, n = 28; 16 ppm, n = 31; 32 ppm, n = 30, and serve as the study population evaluated for epigenetic drift here (**Table 1**). All animals had access to food and drinking water ad libitum throughout the experiment while housed in polycarbonate-free cages and were maintained in accordance with the Institute of Laboratory Animal Resources guidelines.[42] Animals were treated humanely and with regard for alleviation of suffering. 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 day 22 tail tissue (≤3 mm) and again at 10 mo of age (~3 mm), upon sacrifice, using a standard phenol-chloroform-isoamyl alcohol protocol.[43] Genomic tail DNA was bisulfite converted using the Zymo Research 96-well EZ-methylation kit or the Qiagen Epitect kit automated on the Qiagen QIAcube® purification system. Briefly, sodium bisulfite was added to approximately 1 μg of genomic DNA, converting unmethylated cytosines to uracil, which are replaced with thymine during PCR; methylated cytosines remain unchanged.[44] 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. All bands were resolved by gel electrophoresis.

DNA methylation quantitation of CpG sites was performed using pyrosequencing on a PyroMark MD instrument (Qiagen). To determine percent methylation, PyroMark software calculated the fraction of 5-methylated cytosines (%5mC) among the total sum of methylated and unmethylated cytosines. All samples were run in duplicate, and duplicates were averaged for statistical analysis. All primers are based on the mm9 chromosomal position. **Table 3** lists primer location, annealing temperature, and sequences to analyze including CpG sites for pyrosequencing runs. The four CpG sites measured in the *Cabp*^{IAP} allele can be found at nucleotide positions 44, 57, 60, and 72 of GenBank accession number BB842254 (mm9 genomic position: chr2:154179960–154180168, reverse strand).[45] The eight CpG sites measured in the *Igf2* allele can be found at nucleotide positions 1227, 1229, 1234, 1240, 1264, 1270, 1273, and 1279 of GenBank accession number AY849922 (mm9 genomic position: chr7:149839707–149839926, reverse strand).[23] The five CpG sites measured in the *Igf2r* allele can be found at nucleotide positions 1070, 1076, 1091, 1106, and 1108 of GenBank accession number L06446 (mm9 genomic position: chr17:12935154–12935313 reverse strand).[46] The four CpG sites measured in the *H19* allele can be found at nucleotide positions 1621, 1624, 1638, and 1645 of GenBank accession number U19619 (mm9 genomic position: chr7:149767589–149767843, forward strand).[47] For quality control, all pyrosequencing plates included 0% and 100% methylated bisulfite converted mouse control DNAs, and all plates were run in duplicate with average differences >1.5% between replicates discarded and samples re-run.

Data analysis

A total number of 314 mice were generated with a subset of 120 *a/a* offspring followed to 10 mo. In the full sample set, perinatal Pb exposure influence on sex ratio, and pup survival rate significance was determined by the Fishes exact test comparing exposure groups to control as previously reported,[18] and litter size variation across the exposure groups in this subset was tested via ANOVA with a Tukey HSD post-hoc adjustment to determine intergroup significance. Pb exposure was an ordered factor, therefore in the longitudinal subset, both paired comparisons and linear, quadratic, and cubic trends were fit to determine trends for gene methylation and exposure. In all models, methylation variables were the average percent methylation of

all CpG sites in a given PCR amplicon. Absolute methylation drift measurements consisted of the change over time from day 22 to 10 mo for each exposure group, with p-values calculated similarly to a paired *t* test by using a linear mixed model with a paired factor and a random factor accounting for within litter effects (e.g., methylation ~time&gene + [1|mouseID] + [1|litter]). Linear trends were calculated using the difference in day 22 and 10-mo methylation percentage as the dependent variable and the exposure group as the independent variable. Statistical analyses were performed with R 2.13.2 (http://www.r-project.org/) using the lme4 package for liner mixed models. *P* values for linear trend analyses were calculated using Markov chain Monte Carlo resampling (pvals.func). Results were considered significant at values of $P \leq 0.05$ and marginally significant at values of $P > 0.05$ and ≤ 0.10.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This work was supported by the University of Michigan (UM) NIEHS/EPA Children's Environmental Health Center P20 ES018171/RD834800 and P01 ES022844/RD83543601 as well as the UM NIDDK Nutrition and Obesity Research Center P30 DK089503, the UM NIEHS Core Center P30 ES017885, the UM NIEHS Institutional Training Grant T32 ES007062 (CF), NIH Grant K99 ES022221 (CF), and National Center for Advancing Translational Sciences Grant 2UL1TR000433 (JMG). The authors have no conflicts of interest and declare no competing financial interests.

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