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Longitudinal Effects of Developmental Bisphenol A and Variable Diet Exposures on Epigenetic Drift in Mice

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Abstract

Environmental factors, including exogenous exposures and nutritional status, can affect DNA methylation across the epigenome, but effects of exposures on age-dependent epigenetic drift remain unclear. Here, we tested the hypothesis that early-life exposure to bisphenol A (BPA) and/or variable diet results in altered epigenetic drift, as measured longitudinally via target loci methylation in paired mouse tail tissue (3 wks/10 mos old). Methylation was quantified at two repetitive elements (LINE-1, IAP), two imprinted genes (*Igf2, H19*), and one non-imprinted gene (*Esr1*) in isogenic mice developmentally exposed to Control, Control+BPA (50 μg/kg diet), Mediterranean, Western, Mediterranean+BPA, or Western+BPA diets. Across age, methylation levels significantly ($p<0.050$) decreased at LINE-1, IAP, and *H19*, and increased at *Esr1. Igf2* demonstrated Western-specific changes in early-life methylation ($p=0.027$), and IAP showed marginal negative modification of drift in Western ($p=0.058$) and Western+BPA ($p=0.051$). Thus, DNA methylation drifts across age, and developmental nutritional exposures can alter age-related methylation patterns.

Keywords

Epigenetics; DNA Methylation; Drift; Bisphenol A; High Fat Diet; Developmental Origins of Health and Disease

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest.

1.1 INTRODUCTION

The epigenome is a dynamic regulatory framework that utilizes epigenetic information to govern the response of cells, tissues, and entire organisms to environmental stressors. Epigenetic control mechanisms operate at several levels, including alterations to DNA itself (e.g. DNA methylation), chromatin remodeling (e.g. histone modifications), and non-coding RNA interactions [1,2]. DNA methylation, which is perhaps the best-studied epigenetic mark, is defined by the addition of a methyl group to the 5′-carbon of cytosine in a cytosine-phospho-guanine (CpG) dinucleotide. Recent evidence indicates that DNA methylation status changes as a function of age in both humans and animal models, and that this change is often gene- or tissue-specific [3–6]. This process of altered DNA methylation across time is termed “epigenetic drift,” and has important implications for gene expression and disease onset throughout the life course [6]. While this process of drift occurs across all individuals, twin studies have shown that genetically identical individuals can have vast divergence in their epigenetic marks as they age [7]. These results suggest that unique environmental exposures throughout life, rather than any inherent genetic predisposition, may lead to a modulation in the rate of age-related drift.

Mounting evidence indicates that exposure to environmental factors during key developmental windows may alter gene regulation and phenotype through changes in epigenetic marks [8]. As such, the epigenome represents a possible mechanism underlying the Developmental Origins of Health and Disease (DOHaD) hypothesis, which states that exposure to nutritional and environmental factors during prenatal and early postnatal periods alters susceptibility to chronic diseases by influencing developmental plasticity [9]. In general, methylation of DNA at specific promoter/enhancer sites is associated with decreased transcription factor binding, as well as decreased transcription [10]. However, gene-specific methyl marks do not accurately predict global methylcytosine levels, which are driven by CpG methylation of non-coding, repetitive DNA elements, including transposons, retrotransposons, and endogenous retroviruses [11,12]. In contrast to the transcriptional effects seen at gene promoters/enhancers, altered methylation of repetitive elements has the potential to affect genetic stability through increased movement of repetitive elements around the genome [13–15]. Based on the differential regulatory effects of site-specific and global methylation levels, it is important to measure both when investigating the biological effects of epigenetic drift. Recent data also indicate that early life exposure to environmental toxicants has the potential to alter age-related global and gene-specific methylation [16,17]. Supporting this idea, we recently demonstrated that developmental lead (Pb) exposure in congenic mice altered DNA methylation levels at imprinted genes, and that exposure was associated with alterations in the rate of epigenetic drift throughout the life-course [18].

Endocrine disrupting chemicals (EDCs) are an important class of environmental factors that have been linked to the developmental origins of adult disease [19]. One such chemical, bisphenol A (BPA), is a commercial monomer that makes up polycarbonate plastic and epoxy resins. BPA is found in a variety of consumer products (e.g. metal can linings, receipt paper, etc.), and has near ubiquitous and continuous human exposure across the world [20]. BPA can directly bind estrogen receptor α, has been shown to activate a variety of growth-

related transcription factors, and can also bind effectively to several nuclear receptors involved in cell maturation [21–24]. BPA exposure has also been shown to affect DNA methylation levels across the epigenome [24–27]. *In utero* doses of BPA in mouse models affect both global and gene promoter-specific methylation, indicating that BPA exposure could alter gene expression during development [24,28,29]. The effects of BPA exposure on epigenetic drift in matched samples have not been previously studied, but based on BPA's ability to alter the developing epigenome, BPA exposure has the potential to alter drift rates over time.

In addition to chemical exposure, maternal diet can also affect offspring DNA methylation levels [30,31]. The modern "Western High-Fat Diet" (WHFD) is characterized by high saturated and omega-6 polyunsaturated fatty acids, reduced omega-3 fatty acid intake, and increased salt and refined sugar intake [32]. Studies in animal models have shown that alterations in maternal diet, specifically levels of methyl donors, can alter gene-specific and global methylation in offspring, indicating that diet can induce long-lasting, inter-generational changes in methylation [33–35]. Genome-wide studies of the methylome have also noted nutrient-sensitive CpG sites throughout the epigenome, indicating that alterations in diet can affect DNA methylation at specific genomic sites [36]. Based on these results, maternal diet represents an important mediator of the epigenome that has the potential to affect offspring methylation throughout the life course.

To investigate the potential combined effects of diet and BPA exposures on the epigenome, DNA methylation was measured in murine target loci regions -- Long Interspersed Nuclear Element-1 (LINE-1) repeats, Intracisternal A-Particle (IAP) repeats, Insulin-like growth factor 2 (*Igf2*) differentially methylated region (DMR) 2 [18,37], *H19* DMR [18,38], and the promoter region of Estrogen receptor α (*Esr1*) [39]. These candidate regions fall into three classes – repetitive elements (LINE-1, IAP), imprinted genes (*Igf2, H19*), and a non-imprinted protein-coding gene (*Esr1*). Target region classes were chosen based on their potential to reflect global methylation levels, their use as frequent biomarkers in environmental epigenetic studies, and their involvement in growth and metabolism, respectively.

Long interspersed nuclear element-1 (LINE-1) is the most common transposable element in the mouse genome, representing more than 20% of the murine sequence [40]. LINE-1 elements are ancient retrotransposons that replicated in the genome over evolutionary time. Although most LINE-1 elements are no longer active, they have widespread distribution across the genome, making LINE-1 methylation a useful approximation of "global" methylation levels [12]. Intracisternal A-Particle (IAP) retrotransposons are murine, long terminal repeat (LTR)-type genetic elements that also utilize RNA intermediates to retrotranspose around the genome [41]. With the exception of metastable epialleles like the well-studied viable yellow (A^{vy}) IAP element [34], most IAPs are tightly regulated, and have lost their ability to retrotranspose [41]. However, evidence indicates that aging can cause demethylation of IAP promoters, potentially reactivating their retrotransposition competency [41,42]. The IAP assay in this study utilizes a conserved IAP sequence to measure methylation across all IAP retrotransposons present in the murine genome, thereby providing a second, but more genetically "active" approximation of global methylation.

Along with repetitive elements, several imprinted and non-imprinted genes were also investigated. Imprinted genes display parent-of-origin differential methylation and mono-allelic expression [43]. The imprinted genes included in this study, *Igf2* and *H19*, contain differentially methylated regions (DMRs) that exhibit variability in methylation associated with exposure to diet and/or EDCs [37,44–48], making them valuable biomarkers of exposure-induced changes in methylation. In addition to the imprinted genes, we also examined methylation levels at a non-imprinted protein-coding gene – *Esr1.* Estrogen receptor α is a transcription factor activated by estrogenic ligands, and it mediates estrogen's involvement in the regulation of growth and development [49]. Evidence indicates that methylation of the *Esr1* exon 2 promoter is positively associated with age in unmatched samples of murine small intestine [39]. This fact, combined with *Esr1*'s biological importance throughout life, makes it an ideal non-imprinted candidate gene for assessing the effects of developmental exposure on epigenetic drift.

The present study examines longitudinal changes in absolute mean DNA methylation from paired early- (day 21) and late-life (10 months) mouse tail tissue. Matched tail tissue was used due to availability at both weaning and sacrifice, and to eliminate inter-individual confounding. This study investigates whether developmental exposure to BPA and/or altered diet affects the rate of epigenetic drift at these genetic loci. We found clear, gene-specific changes in absolute mean DNA methylation across time in all measured loci. Western diet exposure had a significant modifying effect on the rate of drift at the non-imprinted *Esr1* locus. Similarly, exposure to both the Western and Western + BPA diets had a marginally significant modifying effect on age-related methylation at IAP repeats. Effects of BPA exposure in diet did not have a significant effect on the rate of epigenetic drift at LINE-1, IAP, *H19,* or *Esr1,* but did have a marginally significant effect on age-related methylation at *Igf2*. This study demonstrates measurable, gene-specific epigenetic drift, as well as diet-dependent alterations in the rate of drift at a class of repetitive elements and a non-imprinted locus related to murine growth and development.

1.2 RESULTS

1.2.1 Litter parameters

Developmental BPA and/or diet exposure did not significantly alter litter size, sex ratio, or *a/a* to A^{vy}/a genotypic ratio (n=277). Percent survival was significantly lower in the Control +BPA exposed offspring (survival = 73%) compared to Control (survival = 91%, p=0.006) and Mediterranean+BPA (survival = 85%, p=0.007) exposure groups, but was not significantly different in other comparisons. A subset of *a/a* non-agouti wild type mouse pups (n=133) was selected for inclusion in longitudinal follow-up up to 10 months of age, which incorporated collection of matched tail tip samples (Table 1).

1.2.2 Exposure and Diet Dependent Changes in PND21 Weanling Mice

For all exposure groups, no significant changes in cross-sectional DNA methylation were found in the LINE-1, IAP, and *H19* loci at either time point. However, several significant differences in PND21 cross-sectional methylation were identified at the *Esr1* and *Igf2* loci (Figure 1). The *Esr1* locus demonstrated significant alterations to PND21 methylation when

comparing Control, Mediterranean, and Western diets (ANOVA, p = 0.002). Specifically, mice exposed to Western diet showed significantly decreased methylation compared to Control (Tukey's test, p= 0.027) and Mediterranean (Tukey's test, p = 0.002), and the Western exposure group demonstrated a significant decrease in methylation compared to the Western + BPA group (Student's t-test, p=0.005). The *Igf2* locus demonstrated significant alterations to PND21 methylation when comparing BPA, Mediterranean + BPA, and Western + BPA diets (ANOVA, p = 0.020). At PND21, *Igf2* methylation in the BPA group was significantly lower than methylation in the Western + BPA group (Tukey's test, p=0.027); a similar, marginally significant decrease in methylation was seen when comparing Mediterranean + BPA and Western + BPA (Tukey's test, p=0.073).

1.2.3 DNA Methylation Drifts Over Time

DNA methylation at the LINE-1 and IAP repetitive elements, as well as the *Igf2, H19,* and *Esr1* genes, was quantified from paired PND21 and 10 month tail samples (Table 2). When adjusting for exposure group, sex, and the interaction between age and exposure/sex, LINE-1, IAP, and the *H19* locus demonstrated significant decreases in methylation over time (−0.94%, p=0.035; −1.32%, p=2.2E-06; −10.44%, p=6.22E-08; respectively). In contrast, the *Igf2* and *Esr1* genes demonstrated increased methylation over time; however, only the increase in the *Esr1* gene was statistically significant (7.60% increase, p=1.44E-12) (Table 2).

1.2.4 Developmental Exposures Affect Drift Over Time

To examine the potential effects of exposure on methylation, we first examined whether each exposure group had a direct, significant effect on mean methylation compared to Control. LINE-1, IAP, and *H19* showed no significant effects of developmental exposures on mean methylation (Table 3). At the *Igf2* locus, BPA exposure had a marginally significant negative effect on mean methylation compared to Control (β= −3.81, p=0.066); no other exposure groups had a significant effect on methylation at this gene. In the *Esr1* gene, Western diet exposure had a significant negative effect on mean methylation compared to Control (β=1.93, p=0.013); this was the only significant exposure effect in the *Esr1* gene (Table 3).

To further examine the potential effects of exposure on the rate of epigenetic drift, an interaction term between age and categorical exposure was included in the linear mixed model for each gene (Table 3). The *Igf2* and *H19* genes showed no significant interaction between age and exposure group, indicating that the relationship between age and methylation was not affected by developmental exposures in those genes. On the other hand, at LINE-1 repetitive elements, developmental Western diet exposure had a marginally significant negative effect on the association between age and methylation compared to Control (β= −1.05, p=0.069). Similarly, for IAP, both the Western and Western+BPA diets had marginally significant negative effects on age-related methylation relative to Control (β= −0.645, p=0.058; β= −0.0649, p=0.051). In the *Esr1* gene, developmental Mediterranean +BPA diet exposure had a marginally significant positive effect on age-related methylation when compared to Control (β=1.85, p=0.064). Directionality of the interaction between age and exposure was specific to each gene (Table 3, Figure 1).

Given that gene regulation can vary by sex, two additional variables – sex and a sex:age interaction term – were included in the linear mixed model for each gene. Neither sex nor sex:age were significant terms in the mixed models for LINE-1 (p=0.395, p=0.644), IAP (p=0.476, 0.786), *Igf2* (p=0.868, p=0.686), and *H19* (p=0.294, p=0.639). However, at the *Esr1* gene, while the sex categorical variable did not demonstrate a significant effect on methylation, the sex:age interaction term was statistically significant (p=0.003), indicating effect modification of age-related methylation by sex (Table 3).

1.3 DISCUSSION

Age-associated changes in level of DNA methylation occurred in all measured genetic loci, with statistically significant changes present at *Esr1* and *H19* loci and in the LINE-1 and IAP repetitive elements. Consistent with other results in the literature, directionality of drift over time was specific to each gene [6,16]. The non-imprinted gene promoter, *Esr1*, demonstrated an increase in methylation with age, a result consistent with documented decreases in ERα expression during aging [50]. Meanwhile, repetitive element methylation decreased with age, and the investigated imprinted genes either increased or decreased with age depending upon the locus. These results are also consistent with previous reports [4,6,51], and indicate that epigenetic drift varies in a region-specific manner.

The documented region-specific directionality of drift fits a growing hypothesis in the field – that age-related changes in methylation facilitate development of chronic disease (e.g. cancer) via increased genomic instability and altered regulation of genes related to growth and development [6,52,53]. Decreased methylation of repetitive elements with age has the potential to increase genomic instability through increased transposition of repetitive elements around the genome and dysregulation of expression via cis-chromatin modifying effects [13–15]. Additionally, increased methylation of promoter regions in protein-coding genes is associated with dysregulated transcription [10,54]. Combined, these effects have the potential to produce an epigenetic environment that alters gene expression and may increase the risk of disease states commonly associated with aging.

Given that exposure to exogenous chemicals and altered diet can alter the epigenome [8,24–27,34], we tested the effects of developmental exposure to exogenous chemicals and/or variable diet on the rate of epigenetic drift using an age:exposure interaction term. Age:exposure demonstrated marginal significance at IAP repeats and the *Esr1* locus, but not at LINE-1*, Igf2,* or *H19,* indicating that exposure is a potential gene-specific effect modifier of epigenetic drift. For IAP repeats, developmental exposure to the Western and Western + BPA diets was associated with a marginally significant decrease in the rate of age-related methylation. At this global locus, the magnitude of this effect did not differ between the Western and Western+BPA diets, suggesting that exposure to Western diet was driving the age:exposure interaction effect. Given that age-related demethylation of IAP promoters has the potential to reactivate retrotransposition competency [41,42], Western HFD, by increasing the rate of age-related methylation loss at IAP elements, may also increase IAP retrotransposition events. This suggests a mechanism by which developmental WHFD influences genomic stability throughout an organism's life.

Although epigenetic drift at the IAP repetitive element demonstrated effect modification by Western diet exposure, this result was not seen in LINE-1. This suggests that separate classes of repetitive elements exhibit distinct epigenetic responses to environmental factors. Therefore, when studying the effects of the environment on global DNA methylation – both in cross-section and across the life course – multiple classes of repetitive elements should be included in analysis.

At the *Esr1* locus, Med+BPA exposure was associated with a marginally significant increase in the rate of age-related methylation. The Med+BPA exposure group had *Esr1* methylation levels below Control at PND21, and higher than Control at 10M. This marginal increase in the slope of epigenetic drift at the *Esr1* promoter may reflect tighter control of Estrogen Receptor α expression during aging. However, given the lack of significance in the age:exposure interaction terms for the BPA and Mediterranean groups at this gene, it is difficult to determine whether the Med+BPA exposure effect seen here is truly an effect of exposure. Additionally, a protective effect on PND21 survival was observed for the Med +BPA exposure group. Previous studies have observed that nutritional supplementation counteracts negative epigenetic effects on the epigenome [34,55]. Furthermore, in multiple longitudinal human birth cohort studies, maternal adherence to a Mediterranean diet was associated with reduced risk of intrauterine growth restriction, low birth weight and low placental weight [56,57]. Mothers consuming a Mediterranean diet also had higher circulating folate and vitamin B12 concentrations [57]. Folate and vitamin B12 are critical nutrients in the regeneration of *S*-adenosyl methionine, a major participant in DNA methylation maintenance; this suggests adherence to the Mediterranean diet may impact fetal epigenetic reprogramming. Therefore, it is possible that the developmental Mediterranean diet is providing a protective effect on survival by offsetting BPA-induced changes to epigenetic marks and gene regulation. Future studies should investigate this toxicant-diet interaction more fully.

Sex did not significantly modify drift direction at LINE-1, IAP, *Igf2,* or *H19.* However, sex was a significant effect modifier of the relationship between age and methylation at the *Esr1* locus. Specifically, as age increased from PND21 to 10 months, there was a significant increase in the effect of sex on methylation at the *Esr1* gene. Across all exposures except Med+BPA, male mice demonstrated lower average *Esr1* methylation at PND21, but higher average *Esr1* methylation at 10 months. This trend, combined with the significant age:sex interaction term, suggests a sex-specific change in regulation of the *Esr1* gene during aging. This corroborates the fact that the sexes utilize estrogen for very different processes during reproduction and growth, with females of reproductive age demonstrating higher average serum estrogen than males [49]. Therefore, as the animals reach reproductive age, sex-specific effect modification of age-related *Esr1* methylation may occur as a regulatory response to sexually dimorphic estrogen activity.

Given the effect modification of epigenetic drift by exposure group, we also tested the effects of developmental exposure on cross-sectional methylation at PND21 and 10 months. PND21 DNA methylation showed significant changes by exposure group at two candidate regions – *Igf2* and *Esr1. Igf2* encodes the Insulin-like growth factor 2 protein, an important regulator of cellular glucose transport during development [58,59]. The cross-sectional

WHFD-mediated increase in early-life *Igf2* methylation may be a biological response to an increased simple sugar load, indicating that developmental exposure to WHFD can affect early-life establishment of epigenetic drift at a gene related to metabolism and growth. Similarly, at the *Esr1* gene promoter, WHFD had a significant effect on mean methylation, with a decrease in methylation compared to Control at PND21. Given the directionality of this exposure effect, developmental WHFD exposure may increase transcription of the Estrogen Receptor α (ERα) early in life. Previous studies have shown that ERα is involved in control of lipid metabolism [60], with ERα knockout mice demonstrating increased adipose tissue deposition with aging [61,62]. Developmental exposure to WHFD, which is high in fat and has been linked to obesity, was significantly associated with a decrease in the rate of age-related *Esr1* methylation. This suggests that developmental exposure to Western diet alters the epigenetic profile of the *Esr1* locus during development, predisposing animals to increased *Esr1* transcription in anticipation of the Western diet's altered nutritional profile. When a mismatch occurs between the developmental and postnatal environment, there is potential for improper regulation of epigenetic marks and disease development [8,9]. The significant effects of exposure on PND21 DNA methylation at the *Igf2* and *Esr1* loci support this idea, indicating that developmental exposure to environmental factors may not only alter the rate of drift, but also the cross-sectional establishment of DNA methylation at specific genetic loci during development.

At the *Igf2* locus, BPA exposure had a marginally significant effect on mean methylation, with a decrease in methylation compared to Control at both PND21 and 10 months. This result, which is not present in the Med+BPA or Western+BPA diets, indicates that BPA exposure alone may alter transcription of the *Igf2* gene compared to the Control diet group. A previous study demonstrated decreased *Igf2* methylation and increased *Igf2* expression in developing embryos as a result of early-life 10 mg/kg/day BPA exposure [46]. Given these past results, the marginally significant effects of 50 μg BPA/kg diet exposure on *Igf2* methylation presented in this report may reflect BPA exposure-mediated alterations in *Igf2* expression, but further investigation is required. The LINE-1, IAP, and *H19* loci did not demonstrate significant cross-sectional effects by exposure, suggesting resistance to exposure-mediated reprogramming effects at these three genetic regions.

This study demonstrates measurable exposure-based modification to the rate of epigenetic drift, but the potential biological effects of this modification remain unclear without concurrent, longitudinal measurements of gene expression. Longitudinal measures of gene expression would provide a validation of DNA methylation results, demonstrating whether age- and exposure-related alterations to the epigenome have measurable physiological effects. As such, future studies investigating the effects of early-life toxicant exposure on epigenetic drift could expand the interpretability of their results by examining the effects of exposure and age on longitudinal gene expression, and/or examining DNA methylation and expression levels in other biological tissues of interest including blood – which may be accessed at multiple time points – and target tissues such as liver and brain.

By using matched tail tissue in this longitudinal study, drift rates reflected defined changes within organisms in the study population rather than changes in time between two separate populations. This matched design, combined with the controlled developmental exposure,

isolates the effects of exposure for each organism in the study population, allowing for a direct test of the hypothesis that environmental factors can modify the rates of epigenetic drift. Despite the longitudinal design, an inherent limitation of this study is the inability of bisulfite sequencing to differentiate between 5-methylcyosine (5-mC) and 5-hydroxymethylcytosine (5-hmC). Although hydroxymethylation is not expected to be a major epigenetic mark in tail tissue, recent study showed that aging affects global hydroxymethylation in healthy hepatic tissue, with a general trend towards increasing 5-hmC levels in older mice [63]. This reported increase in global hepatic 5-hmC levels over time is at odds with the previously reported loss of global 5-mC in cancer cells [4,6,51], suggesting that these separate epigenetic marks can change in different ways during aging. As a result, future epigenetic drift studies must better characterize the effects of aging on 5-hmC levels at specific CpG sites in the genome, as well as across tissue types.

1.4 CONCLUSION

We measured longitudinal DNA methylation in tail tissue collected from isogenic mice at PND21 and again at 10 months of age, then quantified the magnitude of epigenetic drift from these samples at five genetic loci – two repetitive elements, two imprinted genes, and one non-imprinted gene. The use of matched tail tissue from an isogenic mouse colony allowed for strict control of genetic, environmental, and dietary measures, as well as removal of potential confounding. This study demonstrates clear, gene-specific directionality of epigenetic drift during aging, supporting the growing hypothesis that epigenetic drift plays an important role in the link between aging and cancer [6,52,53]. In addition, we showed several diet- and sex-dependent alterations to the rate of drift at both imprinted and non-imprinted genes. These alterations indicate that developmental exposure to altered diet or BPA can affect methylation changes during the life course. Diet-dependent changes in DNA methylation were also evident in two investigated loci at PND21, demonstrating the effect of developmental exposure on early-life establishment of epigenetic marks. To improve the generalizability of these results, the dynamics of epigenetic drift at the studied gene regions should be further evaluated in human cohorts.

1.5 MATERIALS AND METHODS

1.5.1 Mouse Colony

Mice included in longitudinal analysis were *a/a* offspring sourced from a genetically invariant A^{vy}/a mouse colony maintained by sibling mating and forced heterozygosity for more than 220 generations [64]. Within this colony, the A^{vy} allele is passed through the heterozygous male line, which has a genetically constant background 93% identical to C57BL/6J strain [64,65]. Two weeks prior to mate-pairing with A^{vy}/a males, six week old wild type *a/a* dams were placed on one of six experimental diet groups: (1) Control (modified AIN-93G), (2) Control + 50 μg BPA/kg diet, (3) Mediterranean HFD chow, (4) Mediterranean + 50 μg BPA/kg diet, (5) Western HFD chow, and (6) Western HFD + 50 μg BPA/kg diet (Figure 2). Dietary exposure was continued through pregnancy and lactation, at which point treatment group pups were shifted over to a modified AIN-93G Control diet containing 7% corn oil rather than 7% soybean oil (Harlan Teklad). The 50 ug/kg diet BPA

exposure level was chosen based on previous studies, which demonstrated both increased global methylation and sex-specific phenotypic effects at 50 μg/kg BPA [28,66]. BPA (0.01 g) was mixed with sucrose (9.99 g) in glass containers to achieve a 0.1% BPA mixture. To achieve the 50 μg/kg BPA concentration, 0.1% BPA/sucrose mixture was included at 0.05 g/kg diet in custom Control/HFD diets by the manufacturer (Harlan Teklad). Western HFD and Mediterranean HFD mixtures were designed based on the U.S. junk food diet and the human Cretan diet, respectively [67–71]. Protein was kept constant between the three base diets, but vitamin levels, lipid ratios, and carbohydrate types were altered to mimic human consumption (Table 4) [71].

1.5.2 Exposure and Tissue Collection

At postnatal day 21 (PND21), offspring were tail tipped, and collected tail tissue was frozen at −80°C. For each exposure group, a subset of PND 21 *a/a* wild-type pups were maintained until 10 months of age – Control: n = 22, Control+BPA: n=19, Mediterranean (Med): n=23, Mediterranean+BPA: n=24, Western: n=22, Western+BPA: n=23 (Table 1). At 10 months of age, remaining mice were sacrificed, and tail tissue was again collected. The offspring with tail tips collected at both PND21 and 10 months of age represent the population used to measure epigenetic drift in this paper. All animals in this study were stored in polycarbonate-free cages with *ad libitum* access to food and drinking water, and were maintained in accordance with Institute for Laboratory Animal Research (ILAR) guidelines [72]. The study protocol was approved by the University of Michigan Committee on Use and Care of Animals (UCUCA).

1.5.3 DNA isolation

Genomic DNA was isolated from PND21 tail tissue (≤3mm) using a phenol-chloroform-isoamyl alcohol protocol [73]. Genomic DNA was isolated from 10 month tail tissue (3 mm) using the Maxwell Mouse Tail DNA Purification Kit (Promega, Cat. #AS1120). Yield and purity of all DNA was measured using a NanoDrop spectrophotometer, and then genomic DNA was bisulfite converted using the Zymo Research 96-well EZ-methylation kit (Zymo Research, Cat. #D5004). Briefly, bisulfite conversion was accomplished through the addition of sodium bisulfite to 0.5–1 μg of genomic DNA, thereby converting unmethylated cytosines to uracil. During polymerase chain reaction (PCR) amplification, uracils are replaced with thymines, making any remaining cytosines a direct, quantitative measure of methylation [74]. PCR amplification was performed on bisulfite converted DNA using HotStarTaq master mix (Qiagen, Cat. #203443), RNAse-free water, forward primer (9 pmol), and biotinylated reverse primer (9 pmol). Total PCR volume was 30 μL per sample, and gel electrophoresis was used to verify PCR product identity.

1.5.4 DNA Methylation Measurement

Specific PCR amplification for regions of interest (*Igf2, H19, Esr1,* IAP*,* and LINE-1) was performed on bisulfite converted DNA with primers designed using the PyroMark Assay Design software 2.0 and mm9 mouse genome. DNA methylation levels were quantified using the PyroMark Q96 MD instrument (Qiagen). Pyrosequencing samples were run in duplicate, and the average of the duplicates provided the final methylation percentages. Sample duplicates with coefficient of variation (%CV) > 10% were discarded and re-run.

Pyrosequencing assay information, including primer sequences, chromosomal location, annealing temperature, and sequences to analyze are available in Table 5. In an effort to reduce plate-to-plate batch effects, matched samples were run on the same plate for all PCR amplification and pyrosequencing runs. All pyrosequencing plates included 0% and 100% bisulfite converted methylation controls, as well as a no template control, to ensure proper functioning of the instrument and to provide background standards of methylation for each gene.

1.5.5 Data Analysis

Matched tail tissue was collected at postnatal day 21 and 10 months of age from a total of 133 *a/a* offspring. The effect of developmental BPA/HFD exposure on sex ratio and litter survival rate was determined by Fisher's exact test, with Control as the reference group. Litter number, sex ratio, and litter survival rate were compared between exposure groups using a combined statistical approach involving both 3-way ANOVAs and Independent Student's T-tests. This same approach was used to compare cross-sectional PND21 or 10 month methylation data by exposure group. Separate 3-way ANOVAs were performed to compare Control vs. Mediterranean vs. Western and Control+BPA vs. Med+BPA vs. Western+BPA exposure groups. Separate Student's t-tests were performed to individually compare methylation between base diets and their associated BPA exposure diet (e.g. Control vs. Control + BPA). For all ANOVAs, Tukey's post-hoc test was used to determine the significance of each group-to-group comparison. Mixed effect linear models were used to compare absolute methylation levels over time by exposure group. Age, exposure group, and sex were included as explanatory variables in all models. Linear mixed models for each candidate region also included a paired factor to account for matched, within-individual data, as well as a random factor to account for within-litter effects. Homogeneity of relative age-related methylation was compared by exposure group via inclusion of an age:exposure interaction term in all mixed models. An interaction term between age and sex was also included in an effort to identify and/or control for potential modifying effects of sex on methylation levels.

Mixed models were fit using the following format: $Methylation \sim Age + Sex + Exposure + Age{:}Exposure + Age{:}Sex + [1|ID] + [1|Litter]$. For all models, the methylation outcome variable was defined as mean methylation across all amplicon CpG sites for two passing replicates. The *lme4* package within the statistical program R was used for all linear mixed models (R version 3.2.3, http://www.rproject.org). Alpha significance levels were set at $p \leq 0.05$ for all statistical comparisons.

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Highlights

- Age had a significant effect on DNA methylation at investigated candidate genes.
- Epigenetic drift directionality and magnitude was specific to each candidate gene.
- Developmental exposure to Western diet modified the rate of epigenetic drift.
- Western diet was associated with increased early-life DNA methylation at *Igf2*.

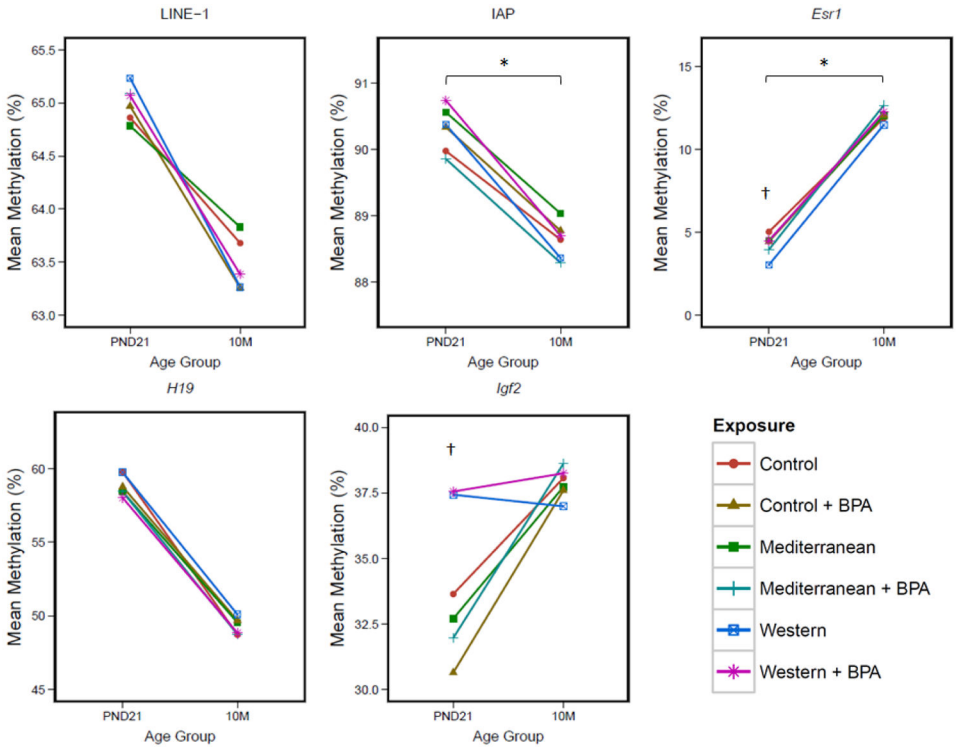

Figure 1. Methylation Drift by Exposure Group

Visualization of epigenetic drift over time at 5 genetic loci. LINE-1, *H19*, and IAP demonstrated a negative association between age and % methylation, while *Esr1* and *Igf2* demonstrated a positive association between age and % methylation. * = age:exposure interaction term p-value <0.10 for at least one exposure group in linear mixed model. † = ANOVA/t-test p-value <0.05 for cross-sectional comparison by exposure group.

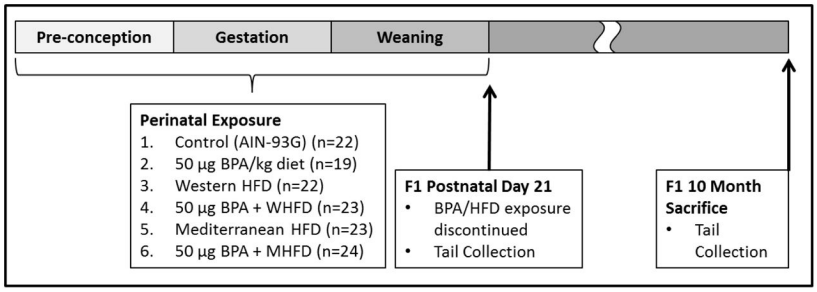

Figure 2. Diagram of Exposure Timing

F0 dams were assigned to one of six dietary BPA/HFD exposure groups two weeks prior to mating. Exposure continued throughout conception, gestation, and through lactation until weaning at post-natal day 21 (PND21). After weaning, offspring were transferred to an *ad libitum* Control diet, which continued until sacrifice at 10 months of age. Matched tail tips were collected at both PND21 and 10 months.

Table 1

Litter Parameters

A subset of n=133 mouse pups included in longitudinal follow-up. All pups in the longitudinal subset were maintained until sacrifice at 10 months of age.

Developmental Exposure Group	N (litter)	Female	Male	Pups (#)
Control	14	10	12	22
Control+BPA	25	9	10	19
Med HFD	20	11	12	23
Western HFD	21	11	11	22
Med+BPA	15	12	12	24
Western +BPA	23	12	11	23
Total	*118*	*65*	*68*	*133*

Table 2

Absolute Mean Methylation by Exposure Group

Linear mixed effect models were used to compare absolute methylation levels over time by exposure group. Age, exposure group, sex, age:exposure, and age:sex were included as terms in all models. Linear mixed models for each gene also included a paired factor to account for matched, within-individual data, as well as a random factor to account for within-litter effects. Separate models were run for each gene; beta coefficients and associated p-values for age predictor from each model are reported.

Absolute Change in Methylation Levels by Age					
Gene	**Paired Tail Samples (N)**	**PND21 % Methylation (SD)**	**10 Month % Methylation (SD)**	**Adjusted Methylation by Age - Beta coefficient†**	**p-value**
LINE-1	260	65.05 (1.35)	63.50 (1.22)	−0.943	**0.035**
IAP	222	90.31 (1.37)	88.62 (1.38)	−1.321	**2.20E-06**
Igf2	249	34.39 (8.69)	37.87 (2.87)	3.275	0.112
H19	257	58.87 (7.06)	49.31 (3.01)	−10.436	**6.22E-08**
Esr1	260	4.26 (1.86)	12.09 (3.00)	7.604	**1.44E-12**

BOLD = $p<0.05$

† Beta coefficient for Age predictor in Linear Mixed Model.

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Table 3

Relative Mean Methylation by Exposure Group

Average methylation by age group was compared across exposure groups using a linear mixed effects model. Age, exposure group, sex, age:exposure, and age:sex were included as terms in all models. Linear mixed models included a paired factor to account for matched, within-individual data, as well as a random factor to account for within-litter effects. Separate models were run for each gene; beta coefficients and associated p-values for the exposure categories from each model are reported. Slope of the age-methylation relationship was tested for heterogeneity across all exposure groups via inclusion of an age:exposure interaction term in the model. Control diet was used as the reference exposure in all comparisons.

Relative Methylation by Exposure								
			Methylation by Age		Methylation by Exposure		Slope Heterogeneity	
Gene	**Developmental Exposure Group**	**N**	**PND21 % Methylation (SD)**	**10 Month % Methylation (SD)**	**Methylation by Age Beta Coefficient†**	**p-value**	**Age : Exposure Interaction Beta Coefficient†**	**p-value**
LINE-1	Control	44	64.86 (1.14)	64.00 (1.40)	(Reference)	n/a	(Reference)	n/a
	Control+BPA	38	64.78 (2.18)	63.32 (1.32)	−0.079	0.855	−0.596	0.316
	Med	44	64.81 (1.53)	63.83 (1.43)	−0.040	0.925	−0.132	0.820
	Western	43	65.18 (1.03)	63.27 (1.13)	0.331	0.431	−1.052	**0.069**
	Med+BPA	47	65.08 (1.44)	63.41 (1.29)	0.232	0.568	−0.818	0.147
	Western+BPA	46	64.97 (1.25)	63.65 (1.09)	0.122	0.766	−0.450	0.426
IAP	Control	38	89.98 (1.37)	88.64 (1.60)	(Reference)	n/a	(Reference)	n/a
	Control+BPA	32	90.56 (1.02)	89.03 (1.19)	0.560	0.279	−0.358	0.299
	Med	38	89.86 (1.15)	88.29 (1.15)	0.783	0.169	−0.291	0.410
	Western	34	90.34 (1.37)	88.77 (1.37)	0.484	0.356	−0.645	**0.058**
	Med+BPA	42	90.74 (1.53)	88.70 (1.53)	0.206	0.689	−0.325	0.335
	Western+BPA	38	90.37 (1.38)	88.36 (1.38)	0.788	0.135	−0.649	**0.051**
Igf2	Control	43	33.64 (6.23)	38.07 (2.59)	(Reference)	n/a	(Reference)	n/a
	Control+BPA	37	30.65 (8.26)	37.59 (4.58)	−3.811	**0.066**	3.355	0.221
	Med	41	32.70 (10.69)	37.83 (2.05)	−0.513	0.804	0.338	0.900
	Western	41	37.43 (11.04)	36.99 (1.84)	2.846	0.163	−3.914	0.143
	Med+BPA	44	31.97 (8.02)	38.61 (1.81)	−2.506	0.207	2.965	0.259
	Western+BPA	45	37.55 (8.26)	38.25 (3.34)	3.162	0.108	−2.888	0.268
H19	Control	38	59.77 (7.22)	48.93 (2.86)	(Reference)	n/a	(Reference)	n/a

Relative Methylation by Exposure								
			Methylation by Age		Methylation by Exposure		Slope Heterogeneity	
Gene	**Developmental Exposure Group**	**N**	**PND21 % Methylation (SD)**	**10 Month % Methylation (SD)**	**Methylation by Age Beta Coefficient†**	**p-value**	**Age : Exposure Interaction Beta Coefficient†**	**p-value**
	Control+BPA	31	58.77 (6.43)	49.61 (2.29)	−0.830	0.650	1.588	0.521
	Med	42	58.43 (7.31)	49.51 (3.84)	−1.956	0.249	2.601	0.266
	Western	42	59.75 (8.28)	50.07 (3.23)	0.426	0.806	0.814	0.730
	Med+BPA	42	58.43 (6.25)	48.55 (2.67)	−1.047	0.532	0.688	0.766
	Western+BPA	37	58.02 (7.32)	49.28 (3.03)	−2.334	0.173	2.776	0.236
Esr1	Control	43	5.03 (2.25)	11.90 (1.93)	(Reference)	n/a	(Reference)	n/a
	Control+BPA	38	4.45 (1.56)	12.07 (3.00)	−0.587	0.458	0.758	0.468
	Med	45	4.53 (1.84)	12.07 (2.01)	−0.523	0.489	0.669	0.504
	Western	42	3.08 (1.12)	11.48 (1.65)	−1.933	**0.013***	1.620	0.112
	Med+BPA	46	3.96 (1.72)	12.67 (4.95)	−1.089	0.150	1.851	**0.064**
	Western+BPA	46	4.51 (1.97)	12.27 (3.14)	−0.604	0.424	0.988	0.321

† Beta coefficient for age predictor in Linear Mixed Model;

BOLD = p<0.10

* p<0.05

Table 4
Comparison of Nutrient Content by Diet

Three base diets were included as developmental exposures in this study – Control, Mediterranean HFD, and Western HFD. For all three base diets, protein was kept constant, but vitamin levels, lipid ratios, and carbohydrate types were altered to mimic human consumption. BPA was added to each diet to produce three additional developmental dietary exposure groups – Control+BPA, Med+BPA, and Western+BPA. Apart from BPA addition, nutrient content was not altered from the base diet levels in these three groups.

Nutrient Content in 3 Base Diets			
Diet Nutrients	**Control**	**Mediterranean**	**Western**
Kcal/g	3.98	4.53	4.72
%Calories from Fat	16%	42%	40%
PUFE : SFA : MUFA	1 : 0.2 : 0.5	1 : 1.3 : 5.6	1 : 1.9 : 1.6
Protein (casein)	20	19	19
Carb Content (g/100 g chow)			
Cornstarch	40	23	14
Sucrose	10	9.2	25.5
Cellulose	5	8	2
Vitamin A (IU)	4000	8000	4000
Vitamin C (mg)	0	500	0
Vitamin D (IU)	1000	1000	400
Vitamin E (IU)	75	75	25
Folic Acid (mg)	2	4	1
Sodium (mg)	1039	1039	7000
Potassium (mg)	3600	8000	3600
Magnesium (mg)	513	850	513

Table 5

PCR conditions

Information for each assay, including genomic location, primer sequences (5′-3′), sequence to analyze, amplicon length, annealing temperature, number of cycles, and number of CpG sites measured.

Primer/Sequence to Analyze	*Igf2*[1]	*H19*[1]	LINE-1	IAP	*Esr1*[2]
Location	chr7:149839707–149839926 strand = reverse	chr7:149767589–149767843	Repetitive Element	Repetitive Element	chr10:4712147–4712203
Forward PCR Primer	TTTTTTAATA TGATATTTGG AGATAGTT	GGGGGGTTAT AAATGTTATT AGGGGGGTAGG	AAGGGGTTTGTG TTTTAGATTAGG	GTGTTATTTTTTGA TTGGTTGTAGTTT	TTTGGAAGTTGT AGTTTTTGGTTA GT
Reverse PCR Primer	biotin-CCACATAATTTAAT TCACTAATAATT ACTA	biotin-AACCCCTAAC CTCATAAAAC CCATAACTAT AAAATCA	biotin-AACTCCCCACCA AATCCTAAAACC TCTA	biotin-ACCAAAAATATCT TATAACTACTTATA CT	biotin-ACAAAACACAA ATAACCCAACTC
Sequencing Primer	AATATGATATTT GGCGATAGTT	GTGTAAAGAT TAGGGTTGT	AGTTTGTTTTTTT ATGTATTATAGT	ATTTTTTGATTGGT TGTAGTTTA	GGAGAGGAGTA TGTAAAG
Sequence to Analyze	YGYGGGAYGT TTGYGTAGAG GTTTGTTTGT TTTTTTGYGT GTTYGTYGGG GTYGT	GYGGTYAGTG AAGTTTYGTA TATYG	TTTAGGTTTY GYGYGATTGG ATTGGGGTAG AYGTTGTGTT TTATTTATTA GAGGTTT	TYGGTYGAGT TGAYGTTAYG GGGAAAGTAG AGTATAAGTA GTTA	TTGGAGAATT YGGGAGYGTT TGGGTGYGTT TTTTGGAGTT GGGTTATTTG TGTTTT
Amplicon Length (bp)	220	255	132	87	131
Annealing Temperature (°C)	56	55	61	56	55
Number of Cycles	50	40	44	45	40
Number of CpG Sites	8	4	4	4	3

[1]Assays from Faulk et al. 2014[18];

[2]Assay adapted from Maegawa et al. 2010 [39].