ENVIRONMENTAL EPIGENETICS

Environmental Epigenetics, 2018, 1–10

doi: 10.1093/eep/dvy017
Research article

RESEARCH ARTICLE

Longitudinal effects of developmental bisphenol A, variable diet, and physical activity on age-related methylation in blood

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Managing Editor: Susan Nagel

Abstract

Research indicates that environmental factors can alter DNA methylation, but the specific effects of environmental exposures on epigenetic aging remain unclear. Here, using a mouse model of human-relevant exposures, we tested the hypothesis that early-life exposure to bisphenol A (BPA), variable diet, and/or changes in physical activity would modify rates of age-related methylation at several target regions, as measured from longitudinal blood samples (2, 4, and 10 months old). DNA 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), Western high-fat diet (WHFD), or Western + BPA diets. In blood samples, *Esr1* DNA methylation increased significantly with age, but no other investigated loci showed significant age-related methylation. LINE-1 and IAP both showed significant negative environmental deflection by WHFD exposure ($P < 0.05$). *Esr1*also showed significant negative environmental deflection by WHFD exposure in female mice ($P = 0.02$), but not male mice. Physical activity had a non-significant positive effect on age-related *Esr1* methylation in female blood, suggesting that it may partially abrogate the effects of WHFD on the aging epigenome. These results suggest that developmental nutritional exposures can modify age-related DNA methylation patterns at a gene related to growth and development. As such, environmental deflection of the aging epigenome may help to explain the growing prevalence of chronic diseases in human populations.

Key words: epigenetics; DNA methylation; aging; bisphenol A; high-fat diet; physical activity

Introduction

The Developmental Origins of Health and Disease (DOHaD) hypothesis posits that exposure to nutritional and environmental factors during prenatal and early postnatal periods alters susceptibility to chronic diseases by influencing developmental plasticity [1, 2]. Based on their establishment during development and sensitivity to the environment, epigenetic marks—including chromatin modifications and DNA

Received 26 February 2018; **revised** 9 April 2018; **accepted** 9 May 2018

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methylation—have been identified as potential mechanisms driving the DOHaD hypothesis [3]. Despite growing evidence to support this idea [4, 5], however, the observed delay between developmental exposures and later-life disease remains difficult to explain using only cross-sectional measures of the epigenome.

Recent research shows that DNA methylation levels change as a function of age in both humans and animal models [6–10]. These age-related changes in methylation are gene- and tissue-specific, can be either random or predictable, and are thought to play a role in chronic disease development [7–9, 11]. Expanding on this idea, recent reports have shown nutrient- and toxicant-mediated shifts in the rate of age-related DNA methylation [12–14], a concept we have described as "environmental deflection" [11]. Given that environmental deflection of epigenetic mechanisms is expected to have long-term effects on transcriptional control, it is possible that this concept could play an important role in the delay between early-life exposures and later-life disease.

A number of environmental factors, including exposure to endocrine disrupting chemicals (EDCs), have shown associations with DNA methylation status. Bisphenol A (BPA) is an EDC used in the commercial production of polycarbonate and epoxy resins; it is found in a number of consumer products (e.g. plastic bottles and metal can linings) [15]. Previous research shows that BPA can bind growth-related nuclear receptors, activate specific transcription factors, and alter epigenome-wide DNA methylation [16–21]. In mouse models, developmental BPA exposure has been shown to alter both global and gene-specific methylation levels [19, 22, 23], suggesting that BPA exposure during development could lead to environmental deflection of age-related methylation patterns.

In addition to chemical exposures, developmental diet has also shown associations with DNA methylation [24, 25]. Previous animal model studies showed that modifications to maternal diet, including methyl donor content, can alter DNA methylation in offspring, indicating that altered nutrition may induce inter-generational changes in the epigenome [26–28]. Further supporting this idea, epigenome-wide studies have found nutrient-sensitive CpG sites throughout the epigenome, suggesting that dietary changes affect DNA methylation on a broad scale [29]. Expanding on this literature, we recently showed that developmental Western high-fat diet (WHFD) exposure can alter rates of age-related methylation at target genes in mouse tail tissue [14]. Based on these previous results, we hypothesized that maternal WHFD would also alter rates of age-related methylation in longitudinal mouse blood samples.

In addition to diet- and toxicant-induced changes to epigenetic marks, recent research has also shown associations between exercise/physical activity and gene promoter DNA methylation [30–32], suggesting that physical activity may play a role in regulating the epigenome. Further supporting this idea, a recent study in mice demonstrated that maternal exercise attenuated HFD-induced *Pgc-1α* promoter hypermethylation in skeletal muscle of offspring [33]. As such, physical activity is a lifestyle factor that may not only alter the epigenome, but specifically mitigate the negative epigenetic effects of WHFD exposure.

Building on these previous studies, we tested the hypothesis that developmental exposure to BPA, WHFD, and/or changes in physical activity would result in environmental deflection of age-related DNA methylation at five target loci—Long Interspersed Nuclear Element-1 (LINE-1), Intracisternal A-Particle (IAP), Insulin-like growth factor 2 (*Igf2*), *H19*, and Estrogen receptor α (*Esr1*). These target genes represent three categories—repetitive elements (LINE-1, IAP), imprinted genes (*Igf2*, *H19*), and a protein-coding gene (*Esr1*). Environmental deflection of longitudinal DNA methylation patterns at these target genes could alter growth and development, thereby contributing to later-life disease development.

Results

Litter Parameters

A subset of *a/a* non-agouti wild type mouse pups—1 male and 1 female per litter—from the four exposure groups were followed until 10 months of age. In total, longitudinal data was collected for 86 *a/a* pups—Control: $n = 22$ (10 female, 12 male), Control + BPA: $n = 19$ (9 female, 10 male), Western: $n = 22$ (11 female, 11 male), Western + BPA: $n = 23$ (12 female, 11 male). Across all mice, developmental BPA and/or WHFD exposure did not significantly alter litter size, sex ratio, or *a/a* to A^{vy}/a genotypic ratio (Supplementary Table S1). The number of surviving pups per litter was significantly lower in the Control + BPA exposed offspring compared to Control ($P = 0.008$), but was not significantly different in other comparisons.

Age-Related DNA Methylation

DNA methylation was quantified from matched 2, 4, and 10-month blood samples using bisulfite pyrosequencing. When adjusting for exposure group, sex, age: exposure, and physical activity, only the *Esr1* gene demonstrated significant changes with age ($\beta = 4.094$, $P = 3.64E\text{-}14$) (Table 1). Specifically, *Esr1* showed a significant increase in mean blood DNA methylation from 2 to 10 months of age. Furthermore, the included sex variable showed a significant relationship with mean *Esr1* DNA methylation ($\beta = 8.24$, $P < 2E\text{-}16$), indicating that sex may be modifying the association between age and blood DNA methylation.

Developmental Exposures Alter DNA Methylation

Within the linear mixed models, we also examined whether BPA and/or WHFD exposure had a significant effect on mean blood DNA methylation compared to Control. At the IAP, *Igf2*, *H19*, and *Esr1* loci, there were no significant effects of developmental exposures on mean DNA methylation (Table 2). At the LINE-1 repetitive element, WHFD exposed mice showed a significant increase in mean blood methylation compared to Control ($\beta = 0.762$, $P = 0.003$); no other exposure groups showed a significant change in mean blood methylation at this repetitive element (Table 2).

Environmental Deflection by WHFD Exposure

To further examine the potential effects of exposure on the rate of age-related methylation, age: exposure interaction terms were included in mixed effects models for mouse blood DNA methylation to test for environmental deflection of age-related methylation. There was significant WHFD-induced environmental deflection of age-related blood methylation at LINE-1 ($P = 0.01$), IAP ($P = 0.04$), and *Esr1* ($P = 0.02$) (Table 2). At all three of these gene regions, WHFD exposed mice had higher % methylation at 2 months and lower % methylation at 10 months compared to Control (Fig. 1). The *Igf2* and *H19* gene loci demonstrated no significant environmental deflection of age-related methylation by exposure.

To further examine the effects of sex on this relationship, sex-stratified models of age-related methylation were run for the *Esr1* gene region. These models showed that the effect of

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Table 1: age-related DNA methylation in mouse blood[a]

Gene	N (matched)	2M % methylation (SE)	4M % methylation (SE)	10M % methylation (SE)	Methylation by age - beta coefficient†	P-value
LINE-1	84	65.85 (0.10)	65.62 (0.10)	65.47 (0.10)	0.085	0.542
IAP	84	92.64 (0.09)	92.80 (0.10)	92.62 (0.13)	0.167	0.244
Igf2	82	46.45 (0.28)	47.07 (0.22)	47.05 (0.19)	0.218	0.469
H19	79	42.79 (0.19)	42.87 (0.23)	43.00 (0.28)	−0.049	0.867
Esr1	81	50.71 (0.73)	56.21 (0.62)	58.69 (0.40)	4.094	**3.64E-14**

[a]Linear mixed effect models were used to compare absolute methylation levels over time. Age, exposure group, sex, age: exposure, and log(AEE) variables were included as terms in all models. *N* (matched) refers to the number of mice with matched pyrosequencing data at each gene region across time. Linear mixed models for each gene also included a paired factor to account for matched, within-individual data. Separate models were run for each gene; beta coefficients and associated *P*-values for age predictor from each model are reported. Bold = $P < 0.05$; † = Beta coefficient for age predictor in linear mixed effects model.

Table 2: blood DNA Methylation by exposure group[a]

			DNA methylation			Relative methylation by exposure		Environmental deflection	
Gene	Exposure	N	2M % methylation (SE)	4M % methylation (SE)	10 month % methylation (SE)	Exposure group beta coefficient	P-value	Age: exposure interaction beta coefficient	P-value
LINE-1	Control	64	65.58 (0.24)	65.39 (0.20)	65.66 (0.17)	(Reference)	n/a	(Reference)	n/a
	BPA	57	65.89 (0.19)	65.44 (0.23)	65.52 (0.19)	0.365	0.172	−0.228	0.249
	Western	66	66.24 (0.18)	65.80 (0.17)	65.31 (0.26)	0.762	0.003*	−0.493	0.010*
	Western +BPA	65	65.69 (0.16)	65.84 (0.23)	65.38 (0.19)	0.347	0.173	−0.181	0.343
IAP	Control	64	92.44 (0.18)	92.69 (0.12)	92.78 (0.20)	(Reference)	n/a	(Reference)	n/a
	BPA	57	92.60 (0.19)	92.95 (0.15)	92.75 (0.32)	0.228	0.430	−0.099	0.626
	Western	66	92.71 (0.14)	92.84 (0.26)	92.27 (0.22)	0.367	0.307	−0.395	0.046*
	Western +BPA	65	92.77 (0.17)	92.73 (0.21)	92.71 (0.29)	0.299	0.185	−0.199	0.312
Igf2	Control	63	46.50 (0.35)	46.91 (0.39)	46.92 (0.41)	(Reference)	n/a	(Reference)	n/a
	BPA	55	46.43 (0.50)	47.10 (0.48)	48.17 (0.65)	−0.270	0.676	0.636	0.138
	Western	65	46.58 (0.33)	46.97 (0.47)	46.49 (0.51)	0.165	0.785	−0.323	0.429
	Western +BPA	62	46.29 (0.42)	47.32 (0.44)	46.69 (0.59)	−0.004	0.994	−0.033	0.937
H19	Control	60	43.16 (0.39)	42.96 (0.44)	43.06 (0.46)	(Reference)	n/a	(Reference)	n/a
	BPA	56	42.44 (0.36)	42.01 (0.51)	42.25 (0.72)	−0.739	0.256	−0.095	0.816
	Western	62	42.31 (0.27)	42.47 (0.36)	42.89 (0.56)	−0.747	0.235	0.261	0.513
	Western +BPA	59	43.32 (0.50)	43.93 (0.43)	43.76 (0.52)	0.307	0.631	0.264	0.518
Esr1	Control	61	50.62 (0.90)	56.36 (1.34)	59.18 (1.46)	(Reference)	n/a	(Reference)	n/a
	BPA	55	50.25 (0.84)	56.30 (1.35)	59.58 (1.34)	−0.327	0.732	0.503	0.480
	Western	63	51.48 (0.80)	56.21 (1.11)	56.64 (1.57)	1.417	0.114	−1.489	0.029*
	Western +BPA	65	50.41 (0.68)	56.00 (1.27)	59.47 (1.42)	0.139	0.876	0.389	0.565

[a]Average methylation by age group was compared across exposure groups using a linear mixed effects model. Age, exposure group, age: exposure, and log(AEE) were included as terms in all models. Linear mixed models included an ID random factor to account for intra-individual effects. Separate models were run for each gene; beta coefficients and associated *P*-values for the exposure categories from each model are reported. Western diet exposure led to a significant increase in mean LINE-1 methylation ($P = 0.003$). Environmental deflection of age-related methylation was tested across all exposure groups via inclusion of an age: exposure interaction term in the model. LINE-1, IAP, and *Esr1* all showed significant negative environmental deflection of age-related DNA methylation ($P < 0.05$). Control diet was used as the reference exposure in all comparisons. *$P < 0.05$.

age and WHFD exposure on *Esr1* promoter methylation in mouse blood varied by sex. Males showed a significant increase in *Esr1* methylation with age ($\beta = 4.22$; $P = 0.004$), but no significant effects of exposure. Meanwhile, females demonstrated both a significant increase in *Esr1* methylation with age ($\beta = 2.11$, $P = 0.001$) and a significant negative age: WHFD interaction term ($\beta = -1.67$, $P = 0.02$). Combined, these data show WHFD-mediated environmental deflection of age-related *Esr1* methylation in female mouse blood (Fig. 2).

Environmental Deflection by Physical Activity

From the CLAMS physical activity data, we used penalized spline regression models to calculate activity-related energy expenditure (AEE) values. An age: log(AEE) interaction term was then included in sex-stratified linear mixed models for the *Esr1* gene region, the only gene region in blood with significant age-related methylation. In the male and female models, the age: log(AEE) term was non-significant, but positive, showing opposite directionality to the significant negative age: WHFD coefficient in the female model (Supplementary Table S2). This indicates that AEE may partially offset the significant negative WHFD-mediated environmental deflection in female mice, a result that is apparent in a visualization of the female mixed model results (Fig. 3).

Discussion

BPA Exposure

We did not find any significant effects of developmental BPA exposure on either cross-sectional DNA methylation or age-related methylation at the five investigated genetic loci. These

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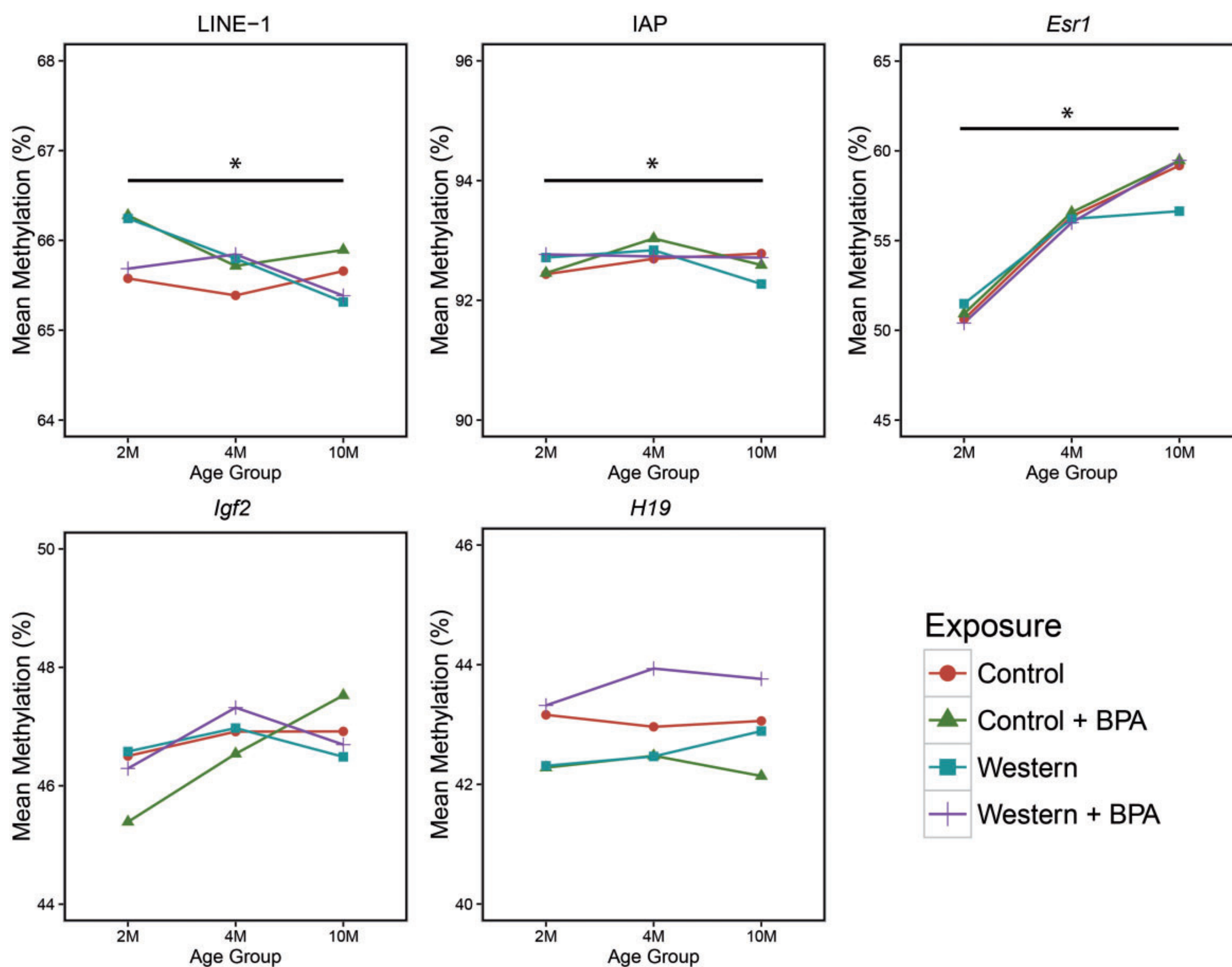

Figure 1: environmental deflection of age-related methylation by exposure group. This figure provides visualization of environmental deflection by exposure at five target loci. LINE-1, IAP, and *Esr1* demonstrated significant negative deflection of age-related methylation in Western-exposed mice compared to control. At the three significant genes, WHFD exposed mice had higher % methylation at 2 months and lower % methylation at 10 months compared to Control. *Igf2* and *H19* did not display environmental deflection by WHFD. * = age: exposure interaction term *P* value <0.05 for WHFD exposure group in linear mixed model.

results match our previous work, which showed that the same dose of BPA (50 μg/kg diet) did not significantly alter LINE-1 repetitive element methylation in liver [34] or *Esr1* methylation in tail and liver [14, 35]. However, contrary to our results, separate work in mice has shown that developmental BPA exposure (10 mg/kg bw/day) can alter DNA methylation at the *Igf2* DMR in embryonic tissue. Similarly, separate studies in our lab have shown that BPA exposure can alter genome-wide and global methylation levels in mouse liver and tail, respectively [22, 23]. Taken together, these contradictory results suggest that the epigenetic effects of BPA may be tissue-, dose-, and gene-specific. Therefore, the lack of significant BPA-related changes in DNA methylation in this study may simply reflect an absence of exposure effects in blood at the selected dose or investigated loci.

Age-Related Methylation

In longitudinal blood samples, the *Esr1* promoter region demonstrated significant age-related hypermethylation with age. This age-related increase in *Esr1* promoter methylation matches results from tail [14] and murine cortex [36], suggesting that the directionality of age-related methylation at the *Esr1* locus is consistent across tissues. Given that gene promoter methylation levels have been associated with altered transcription [37], age-related hypermethylation of the *Esr1* promoter could lead to long-term shifts in expression. Previous work has shown that *Esr1* expression affects a number of biological processes, including reproductive function, fat deposition, brain function, and bone density [38]. As a result, age-related methylation at the *Esr1* gene could modify risk for chronic disease development. This concept should be tested via follow-up measurement of longitudinal *Esr1* methylation levels in additional target tissues—e.g. liver, kidney, and adipose.

The effect of age on *Esr1* promoter methylation was sex-specific, with males showing a more pronounced effect of age compared to females. This may be a reflection of sex-specific estrogen receptor α (ERα) regulation during the aging process. Previous work in mouse cortex has shown that adult females have increased ERα protein levels, *Esr1* mRNA expression, and *Esr1* promoter methylation compared to males [36, 39], suggesting that females may show decreased effects of age due to a sex-specific requirement for *Esr1* expression into adulthood.

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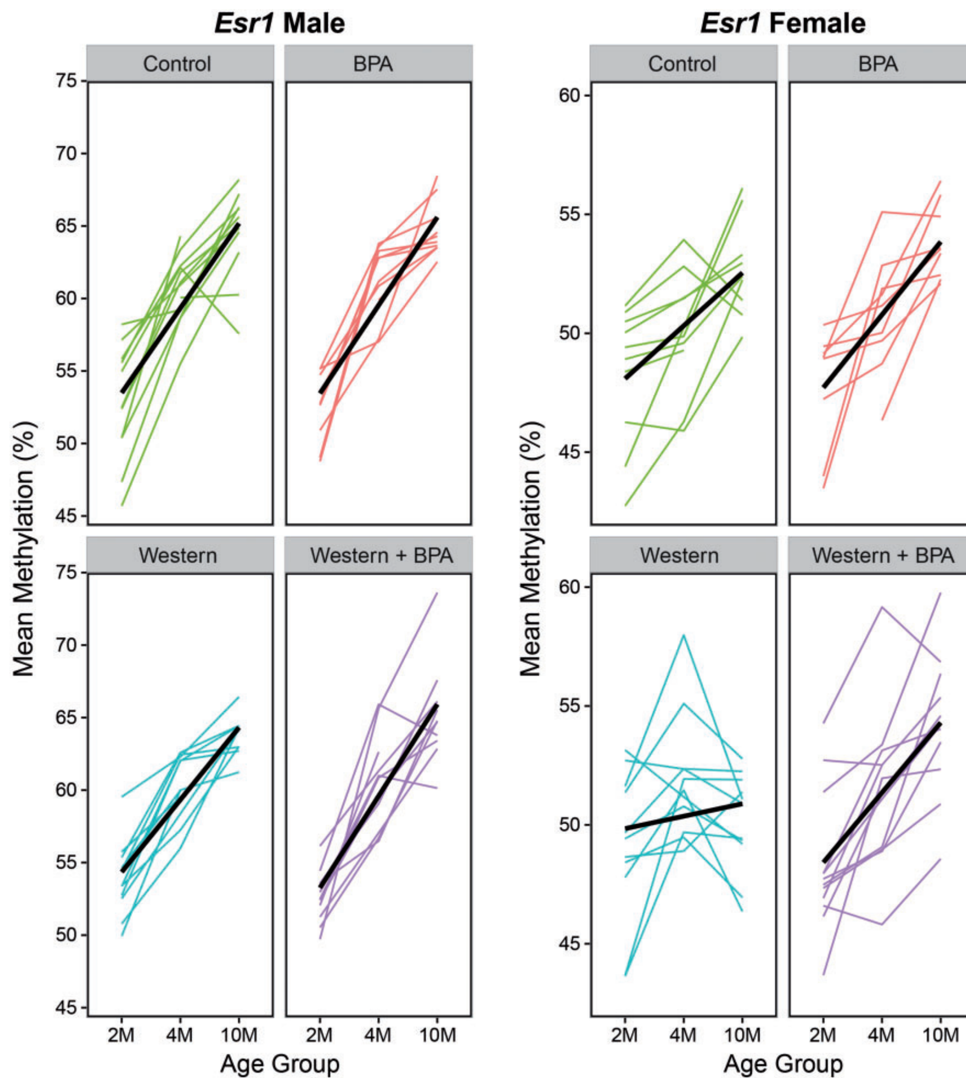

Figure 2: age-related *Esr1* DNA methylation split by exposure and sex. Spaghetti plot of age-related *Esr1* methylation as it varies by both exposure and sex. WHFD-exposed female mice had a significant interaction between exposure and age ($P = 0.026$), showing a decreased rate of age-related methylation in WHFD-exposed mice compared to Control. No other exposure group showed a significant interaction between effects of age and exposure on DNA methylation.

Environmental Deflection

Based on age: exposure interaction terms, we found significant environmental deflection by WHFD exposure at the *Esr1*, IAP, and LINE-1 loci. At all three of these genes, WHFD exposure had a significant negative effect on age-related methylation compared to Control, with WHFD-exposed mice showing higher mean methylation at 2 months old and lower mean methylation at 10 months old. This pattern suggests differential effects of developmental WHFD exposure at distinct life-stages—specifically, increased DNA methylation early in life followed by decreased DNA methylation in adulthood. Although the exact mechanism remains undefined, the apparent delay between WHFD exposure and decreased DNA methylation could be the result of a two-phase exposure effect: early-life epigenetic programming followed by compensatory changes in regulation of methylation maintenance machinery—e.g. Dnmt3a and Tet1 enzymes.

The two-phase effect of WHFD exposure on the murine epigenome may be the result of oxidative stress (OS) induction. Previous work has demonstrated that Western diet can induce OS in mice [40], and other work has shown that OS can alter DNA methylation [41]. For example, research indicates that OS can induce DNA hypermethylation via targeted acceleration of the reaction between cytosine molecules and S-adenyl-methionine (SAM), a methyl group donor [41]. Research has also shown that oxidative conditions can activate Tet enzymes [42–44], suggesting that OS may affect active DNA demethylation. These bi-directional effects of OS on the epigenome fit into the proposed two-phase model for the effects of WHFD on DNA methylation. In the first phase, WHFD-induced OS would increase DNA methylation on a short time scale through direct acceleration of the interaction between SAM and CpG sites across the genome. In the second phase, OS recruits Tet enzymes, leading to hypomethylation at specific promoter regions via targeted oxidation of 5-mC to 5-hydroxymethylcytosine (5-hmC). 5-hmC could then be lost throughout the life-course via active or passive demethylation pathways [45]. While this model fits well with our results, 5-hydroxymethylcytosine (5-hmC) is not distinguishable from 5-mC in bisulfite-pyrosequencing data [46]. As a result, there may be an early-life wave of OS-induced 5-mC

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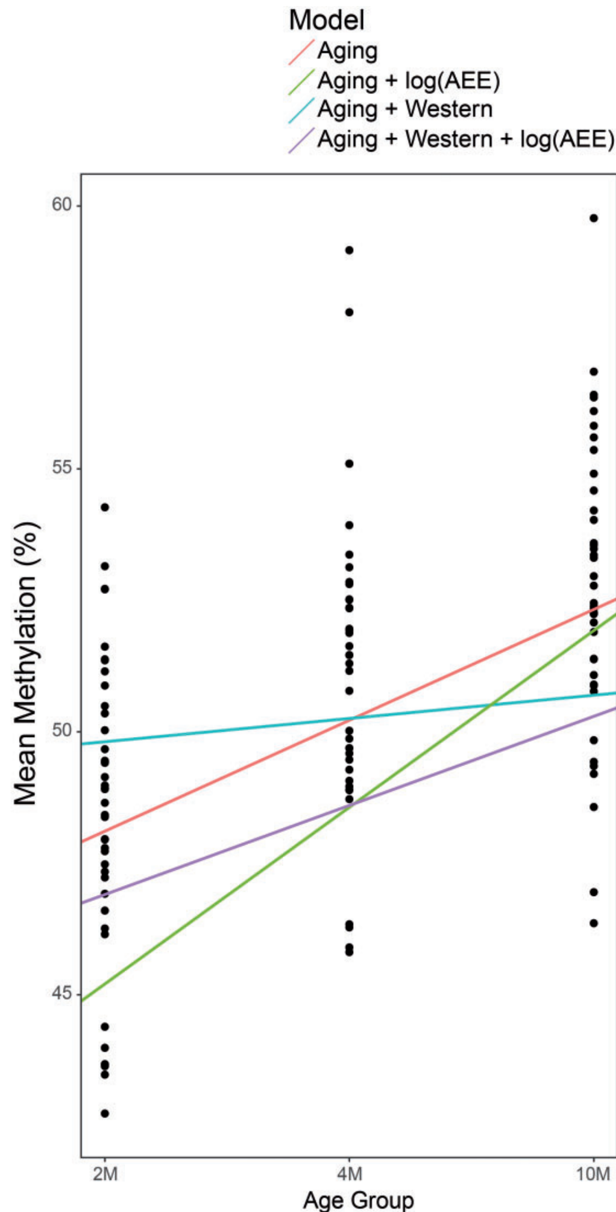

Figure 3: age-related *Esr1* methylation for female mice. Slope estimates of the relationship between age and *Esr1* methylation for female mice. Slope estimates are separated based on exposure to Western high-fat diet and/or AEE. Slope estimates are based on beta coefficient values from the *Esr1* linear mixed model for female mice. In the model legend, "aging" refers to the baseline rate of age-related methylation, which is conceptualized as the beta estimate for the age term in the model when effects of AEE and/or WHFD exposure are not considered. The other terms in the legend represent the effect of exposure and/or physical activity beta estimates on the baseline rate of age-related methylation. AEE and WHFD had opposing effects on the rate of age-related methylation at the *Esr1* locus in female mouse blood.

oxidation to 5-hmC that is not captured in our targeted pyrosequencing data. Additional work is needed to fully characterize the role of OS in WHFD-induced environmental deflection of epigenetic aging in mice.

Female mice showed opposing effects of WHFD and activity-related energy expenditure on longitudinal *Esr1* methylation, suggesting a mitigating effect of life-long physical activity in WHFD-exposed female mice. Supporting this idea, a recent study in mice showed that maternal exercise attenuated HFD-induced *Pgc-1α* promoter hypermethylation in skeletal muscle of offspring [33]. Combined with our results, these data suggest that physical activity—whether maternal or life-long—may offset HFD-related effects on the epigenome. In our study, the opposing effects of WHFD and physical activity on age-related methylation were less apparent in male mice. This may be a reflection of sex-specific regulation of the *Esr1* gene, which is more highly expressed in female mice [39]. Based on these data, sex should be considered in future studies investigating environmental deflection of age-related methylation.

Limitations and Future Directions

This study demonstrates measurable WHFD-based modifications to the rate of age-related methylation in murine blood, but the potential biological effects of this environmental deflection 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 phenotypic effects. As such, future studies investigating the effects of early-life toxicant exposure on epigenetic aging could expand the interpretability of their results by examining the effects of exposure and age on longitudinal gene expression.

DNA methylation levels vary by tissue [47], and a several comparative studies in humans have shown that the blood methylome can be quite distinct from other somatic tissues [48, 49]. However, little work has been done to investigate this question in mice, since blood is not often used as a target tissue in animal studies. As such, it is unclear whether the age-related methylation found in mouse blood would also be present in other tissues of interest, including liver, kidney, or adipose. To address this knowledge gap, the NIEHS TaRGET II consortium, which includes our lab, is investigating exposure-related differences in DNA methylation across multiple murine tissues [50]. Despite the unclear translatability of blood methylome data to other murine tissues, collection of longitudinal blood samples from the same mice allowed for direct measurement of intra-individual age-related methylation. This reduces the potential confounding introduced by inter-individual variability. Additionally, blood collection during adulthood was minimally invasive and did not negatively impact mouse health, meaning longitudinal changes in the methylome were not simply a reflection of decreased fitness. Finally, blood samples provide improved translatability to human aging studies, which typically rely on clinical blood draw samples. For these reasons, we used blood samples to investigate epigenetic aging in a mouse model; future studies should test whether our results are consistent across additional target tissues.

Conclusion

We measured longitudinal DNA methylation in matched blood samples collected from isogenic mice at 2, 4, and 10 months of age, then examined the effects of BPA, Western high-fat diet, and physical activity on age-related methylation patterns. The use of matched blood allows for greater translatability to bioavailable specimens in human populations. We found age-related hypermethylation at the *Esr1* locus, as well as diet- and sex-dependent alterations to this age-related methylation. Our results suggest that age-related methylation is a gene-specific biological mechanism that is sensitive to both early-life environmental exposures and life-course behaviors such as physical

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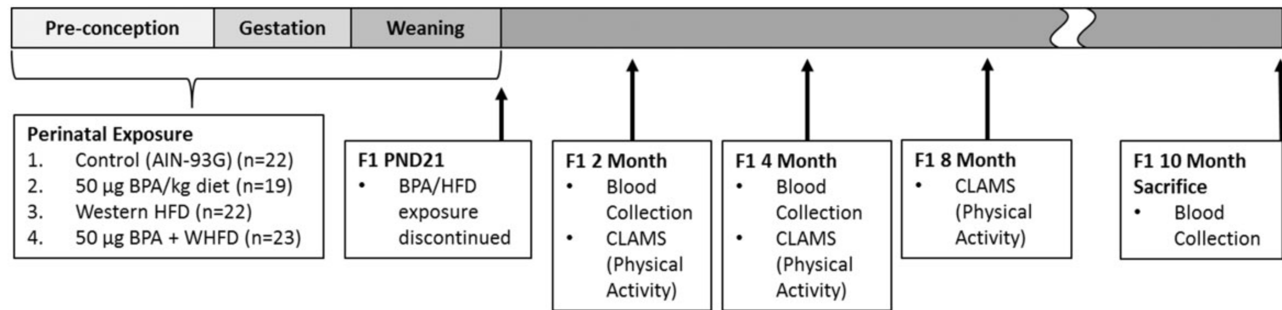

Figure 4: diagram of Exposure Timing. F0 dams were assigned to one of four dietary BPA/HFD exposure groups 2 weeks prior to mating. Exposure continued throughout conception, gestation, and lactation until weaning at post-natal day 21 (PND21). After weaning, F1 offspring were transferred to an ad libitum Control diet, which continued until sacrifice at 10 months of age. Matched blood samples were collected at 2, 4, and 10 months. CLAMS calorimetry data was collected at 2, 4, and 8 months.

activity. These results should be further investigated in mouse models, with a focus on additional tissues and phenotype characterization.

Methods

Offspring sourced from a genetically invariant A^{vy}/a mouse colony maintained in the Dolinoy Lab via sibling mating and forced heterozygosity for >220 generations [24]. Within the colony, the A^{vy} allele is maintained in the heterozygous male line (Supplementary Fig. S1), which has a genetically constant background 93% identical to C57BL/6J strain [24, 51]. The remaining 7% of the genome in this colony is from the C3H/HeJ strain [24, 51]. Two weeks prior to mate-pairing with A^{vy}/a males, 6- to 8-week-old wild-type *a/a* dams were placed on one of four experimental diet groups: (i) Control (custom AIN-93G), (ii) Control + 50 µg BPA/kg diet, (iii) Western HFD chow, and (iv) Western HFD + 50 µg BPA/kg diet (Fig. 4). As described previously, the Western HFD had altered nutrient levels and was designed based on the U.S. junk food diet [14]. Dietary exposure lasted through pregnancy and lactation, at which point pups in each treatment group were shifted over to a custom AIN-93G Control diet with 7% corn oil substituted for 7% soybean oil (Harlan Teklad). 50 µg/kg diet BPA was chosen as the treatment dose based on previous studies demonstrating epigenetic and phenotypic effects at 50 µg/kg BPA [22, 52]. To achieve the 50 µg/kg BPA concentration, BPA (0.01 g) was first mixed with sucrose (9.99 g) in glass containers, and then the resulting 0.1% BPA/sucrose mixture was included at 0.05 g/kg diet in custom Control/HFD diets (Harlan Teklad).

Tissue and Physical Activity Data Collection

For each exposure group, approximately 1 male and 1 female *a/a* wild-type pup per litter were maintained until 10 months of age—Control: $n = 22$ (10 female, 12 male), Control + BPA: $n = 19$ (9 female, 10 male), Western: $n = 22$ (11 female, 11 male), Western + BPA: $n = 23$ (12 female, 11 male) (Fig. 4). At 2, 4, and 8 months of age, offspring were relocated to a new cage outfitted with an integrated open-circuit calorimetry system—Comprehensive Lab Animal Monitoring System (CLAMS, Columbus Instruments); in this context, a host of phenotypic measures were taken, including body weight, oxygen consumption, food intake, and physical activity. After 3 days in the CLAMS cages, mice were returned to their original cages. During the 2 and 4 month cage transfers, tail vein blood samples were collected from all mice. At 10 months of age, mice were sacrificed, and cardiac puncture blood samples were collected. All blood samples were flash frozen in liquid nitrogen and stored at −80°C, until DNA was isolated. 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 [53]. The study protocol was approved by the University of Michigan Committee on Use and Care of Animals (UCUCA).

DNA Isolation

Genomic DNA was isolated from 2, 4, and 10 month frozen blood using the Qiagen Allprep DNA/RNA Mini Kit (Qiagen, Cat. #80204). 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, sodium bisulfite was added to 0.5 µg of genomic DNA to convert unmethylated cytosines to uracil. Bisulfite converted DNA was then amplified using polymerase chain reaction (PCR), causing uracils to be replaced with thymines. Remaining cytosines in the amplified PCR product represent a quantitative measure of DNA methylation [54]. 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 35 µl per sample, and gel electrophoresis was used to verify PCR product identity.

DNA Methylation Measurement

As previously described, pyrosequencing was used to measure DNA methylation levels in mouse tissue at five regions of interest (*Igf2, H19, Esr1*, IAP, and LINE-1) [14]. Briefly, PCR amplification was performed on bisulfite converted DNA using primers designed in the PyroMark Assay Design software 2.0 (assay conditions available in [14]). DNA methylation levels were then quantified using the PyroMark Q96 ID 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. 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.

Data Analysis

Matched blood samples were collected at 2, 4, and 10 months of age from a total of 86 *a/a* offspring across the four exposure

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groups—Control: $n = 22$ (10 female, 12 male), Control + BPA: $n = 19$ (9 female, 10 male), Western: $n = 22$ (11 female, 11 male), Western + BPA: $n = 23$ (12 female and 11 male). Cross-sectional DNA methylation for each target gene was compared between the Control, Western, Control + BPA, and Western + BPA exposure groups using ANOVA. For all ANOVAs, Tukey's post-hoc test was used to determine the significance of each group-to-group comparison. Physical activity (PA) data on multiple axes of movement—X ambulatory counts, X total counts, and Z total counts—were measured using CLAMS cages (Columbus Instruments). We summarized physical activity data, which was collected over the 3 days in CLAMS cages, in an activity-related energy expenditure (AEE) variable. AEE was calculated from total energy expenditure (TEE; kcal/kg/h) and resting metabolic rate (RMR; kcal/kg/h) using the following formula: $AEE = 0.9(TEE) - RMR$ [55–57]. The 0.9 multiplier was based on the thermic effect of food (TEF), which was assumed to be ~10% of TEE [56]. RMR was calculated from CLAMS data using a three step protocol. First, physical activity data was preprocessed using an optimized power function based on the CLAMS 20 min sampling interval [55]. Second, a penalized spline regression model was used to model the time-course relationship between Energy Expenditure and Physical Activity for each mouse. Third, intercepts from the TEE vs. PA spline models were used to represent the RMR for each mouse. The AEE variable was log-transformed prior to its addition to regression models.

Linear mixed effect models were used to compare mean 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 target region included a random factor to account for matched, within-individual data. Environmental deflection of age-related methylation was compared by exposure group via inclusion of an age*exposure interaction term in all mixed models. Age*sex and age*log(AEE) interaction terms were also considered in mixed models; these terms were only included in the final model for *Esr1*, where age demonstrated statistical significance.

Mixed models for blood tissue were fit using the following format: $Methylation \sim Age + Sex + Exposure + log(AEE) + Age{:}Exposure + Age{:}Sex + Age{:}log(AEE) + [1|ID]$. 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.r-project.org). Alpha significance levels were set at $P \leq 0.05$ for all statistical comparisons.

Supplementary data

Supplementary Data are available at *EnvEpig* online.

Acknowledgements

This work was supported by the University of Michigan (UM) NIEHS/EPA Children's Environmental Health and Disease Prevention Center P01 (ES022844/RD83543601), the Michigan Lifestage Environmental Exposures and Disease (M-LEEaD) NIEHS Core Center (P30 ES017885), The University of Michigan Nutrition Obesity Research Center (P30 DK089503), as well as the UM NIEHS Institutional Training Grants T32 ES007062 (J.J.K. and E.H.M.), T32 HD079342 (E.H.M.), and F31 ES025101 (E.H.M.).

Conflict of interest statement. None declared.

Statement of Data Availability

Data from this project are available upon request.

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