

## RESEARCH ARTICLE

# Ancestral plastics exposure induces transgenerational disease-specific sperm epigenome-wide association biomarkers

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## Abstract

Plastic-derived compounds are one of the most frequent daily worldwide exposures. Previously a mixture of plastic-derived toxicants composed of bisphenol A, bis(2-ethylhexyl) phthalate, and dibutyl phthalate at low-dose exposures of a gestating female rats was found to promote the epigenetic transgenerational inheritance of disease to the offspring (F1 generation), grand-offspring (F2 generation), and great-grand-offspring (F3 generation). Epigenetic analysis of the male sperm was found to result in differential DNA methylation regions (DMRs) in the transgenerational F3 generation male sperm. The current study is distinct and was designed to use an epigenome-wide association study to identify potential sperm DNA methylation biomarkers for specific transgenerational diseases. Observations indicate disease-specific DMRs called epimutations in the transgenerational F3 generation great-grand-offspring of rats ancestrally exposed to plastics. The epigenetic DMR biomarkers were identified for testis disease, kidney disease, and multiple ( $\geq 2$ ) diseases. These disease sperm epimutation biomarkers were found to be predominantly disease-specific. The genomic locations and features of these DMRs were identified. Interestingly, the disease-specific DMR-associated genes were previously shown to be linked with each of the specific diseases. Therefore, the germline has ancestrally derived epimutations that potentially transmit transgenerational disease susceptibilities. Epigenetic biomarkers for specific diseases could be used as diagnostics to facilitate clinical management of disease and preventative medicine.

**Key words:** EWAS; BPA; phthalate; DEHP; DBP; transgenerational; sperm; testis; prostate; kidney

## Introduction

Bisphenol A (BPA), di-(2-ethylhexyl) phthalate (DEHP), and dibutyl phthalate (DBP) are some of the most prominent synthetic chemicals known to act as endocrine disruptors and result in detrimental alterations in development. These chemicals are

also considered ubiquitous in the environment, meaning it is effectively not possible to avoid exposure to these chemicals. Prenatal exposure is particularly concerning in its potential to affect lifetime trajectories through detrimental alterations in development. Maternal exposure to BPA results in postnatal alterations to the methylation of DNA and altered gene

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expression in offspring (1). These alterations in DNA methylation and histone modifications resulting from exposure to BPA are extensive and likely provide an important mechanistic component of the toxicity of endocrine-disrupting chemicals (EDC) (2). In fact, both BPA and phthalates have been described as 'epigenetically toxic' and several specific detrimental DNA methylation and histone protein alterations have been described (3). The detrimental effects of BPA and phthalates are seen among individuals directly exposed to these EDCs. In addition, if pregnant females are exposed then the F1 generation fetus and germline in F1 generation fetus that will generate the F2 generation are also directly exposed (i.e. a multigenerational exposure), and detrimental effects are seen in these F1 and F2 generations, as well as in the un-exposed F3 generation great-grand offspring termed transgenerational individuals (4).

The detrimental effects of direct exposure to toxicants can be greatly enhanced when that exposure occurs at an early developmental stage when epigenetic programming occurs. The Developmental Origins of Health and Disease (DOHaD) hypothesis was first developed 30 years ago and suggests that health outcomes later in life can be strongly influenced by early life experiences (5, 6). The original demonstration of this phenomenon involved a correlation between an increased risk of heart disease later in life and a low birth weight due to fetal undernutrition during gestation (7). EDCs are one of the most widely implicated environmental factors associated with later life pathology outcomes. Because the developing fetus is particularly vulnerable to EDC exposure, there is mounting evidence that the DOHaD can impact later-life health. There is also mounting evidence that epigenetic alterations resulting from EDC exposure provide a mechanistic link in this relationship (8). In particular, the developmental exposure to anti-androgenic and estrogenic EDCs is associated with reproductive dysfunction in adulthood. The direct mimicry of sex steroids can cause interference with epigenetic regulation during tissue differentiation to promote altered expression of genes important for development and reproductive function (9). Taken together, the DOHaD provides an explanation for many of the detrimental health outcomes associated with BPA and phthalate exposure, while epigenetic alterations provide the mechanistic link implicated in many of these health outcomes.

These epigenetic alterations provide an important explanation for those detrimental health outcomes seen among individuals exposed in a multigenerational or transgenerational manner (10). Direct exposure involves the individual directly exposed, the F0 generation, and the developing fetus (F1 generation) within that individual at the time of exposure. The germline of the directly exposed individual is also affected, therefore, any individuals derived from that germline are considered to have experienced multigenerational exposure (11). This can include the F1 and F2 generations of gestating female exposure and the F1 generation of preconception adult exposed individuals which is defined as a multigenerational or intergenerational generation(s). Transgenerational individuals involve any generation offspring that did not directly experience the exposure, nor do they derive directly from a germline that experienced the exposure. The F3 generation great-grand offspring of a gestating F0 generation female exposure and the F2 generation of a preconception F0 generation adult exposure is the transgenerational generation (12). Germline epigenetic alterations that are inherited carry the effects of direct exposure to subsequent generations (13). Various epigenetic effects resulting from endocrine disruptors have been observed (14). These alterations in the germline genome involve DNA methylation,

histone post-translational modifications, and non-coding RNAs and have all been shown to result from endocrine disruptor exposure. Numerous studies have demonstrated EDCs can alter the mouse, rat, and human epigenomes and these alterations result in variable tissue pathology susceptibilities, involving altered reproductive, metabolic, and neurological phenotypes being predominant (6).

The implications of EDC exposure on both male and female reproductive development have been well documented. Delayed puberty and liver enlargement were observed among mice treated with several different doses of phthalates (15). Plastic-derived EDCs have been shown to induce a variety of pathologies in both F1 generation offspring and transgenerational F3 generation (4). Phthalate exposure also results in impaired reproductive outcomes for F1 generation female mice, alterations in secondary sex characteristics, and fertility complications in F2 and F3 generation female mice, as well as cystic ovaries in F2 generation female mice (16). Altered estrous cyclicity, increased ovarian cysts, and hormone levels were observed in F1, F2, and F3 generation female mice derived from gestational exposure to phthalates (17). Ovarian function is disrupted by generational exposure to phthalates with effects appearing in multigenerational and transgenerational generations (18).

Phthalate exposure can also affect male reproductive development. Alterations in spermatogenesis, reduced germ cell differentiation, and increased testis weight were all observed among males receiving several different doses of phthalates during development (19). Developmental disruption was observed through the F4 generation of mice derived from F0 generation phthalate-exposed individuals, namely disrupted testicular germ cell differentiation and decreased sperm counts and motility (20). The combination of BPA and phthalates may even result in emergent detrimental effects on reproductive development (4). Permanent alterations in the F1 generation male reproductive health were different among those exposed to BPA or phthalates alone than those exposed to both (21). The effects of these exposures, particularly phthalates, on murine models are similar to those seen in human male reproductive tract disorders which include testicular dysgenesis syndrome (22). The presence of multiple exposures leads to greater negative health outcomes.

Reproductive dysfunctions are not the only negative effects seen in directly exposed multigenerational animals and in transgenerational individuals to BPA and phthalates. Maternal phthalate exposure may be linked to asthma and allergy phenotypes in humans (23). Exposure to plastics (BPA and phthalates) are linked with behavioral deficits, changes in DNA methylation, and changes in gene expression (24). There is a well-established link between metabolic disease and BPA exposure, including transmission of metabolic disorder through transgenerational epigenetic inheritance (25). Transgenerational epigenetic inheritance is the phenomenon where abnormalities resulting from exposure to a toxicant or other environmental perturbation are transmitted through the germline to future generations, despite the absence of continued exposure in those future generations (12, 26). The altered germline epigenetics upon fertilization and formation of the zygote can subsequently alter the epigenetics and transcriptome of the developing embryonic cells. Therefore, the embryonic stem cells developed have altered epigenetics and transcription that can impact the subsequent development of all derived somatic cell epigenomes and transcriptomes. Those somatic cell types later in development that are sensitive to these alterations will have an increased susceptibility to develop pathologies. The germline

epigenetic alterations developed then transmit this to the subsequent generation to promote the epigenetic transgenerational inheritance phenomenon (10–12).

Transgenerational epigenetic inheritance requires an epigenetic alteration in the germline (10). This phenomenon has been shown to be induced after exposure to a wide variety of endocrine disruptors, including DDT (27), vinclozolin (28), glyphosate (29), atrazine (30), dioxin (31), N,N-diethyl-metoluamide (DEET) and permethrin (32), methoxychlor (33), hydrocarbons (34), and plastics-derived compounds (4). The connection between environmental toxicants, altered epigenetics, and altered health outcomes has been well established (11). There is also a potential for the use of epigenetic biomarkers in the identification of exposure risks. Epigenetic biomarkers for the disease have been identified in exposure lineages from DDT (27), vinclozolin (28), and atrazine (30).

A previous study demonstrated sperm epigenetic alterations following exposure to a combination of the plastic-derived compounds BPA and phthalates, as well as transgenerational inheritance of disease (4). The population examined was exposed to doses of BPA and phthalates lower than those considered safe for consumption. The plastics induction of epigenetic transgenerational inheritance of increased incidence of disease in conjunction with sperm epimutations was previously reported in the F1 and F3 generations (4). The current study is distinct and was designed to analyze individuals with and without a specific disease to compare in an EWAS (epigenome-wide association study) to identify potential transgenerational disease-specific epimutation biomarkers.

## Results

### Animal Studies

As previously described (4), outbred Sprague–Dawley gestating female rats (F0 generation) received a developmental exposure at the onset of fetal gonadal sex determination. The U.S. Environmental Protection Agency (EPA) established a no observable adverse effect level (NOAEL) for long-term exposure to Bisphenol A as 50 mg/kg body weight per day (35). The NOAEL for exposure to DEHP phthalate is 4.8 mg/kg body weight per day (36), while the lowest observable adverse effect level (LOAEL) is 5 mg/kg body weight per day (37). An additional phthalate administered in the current study, dibutyl phthalate (DBP) has a NOAEL of 50 mg/kg BW/day (38). On embryonic days 8–14 (E8–E14) of fetal gonadal sex determination, gestating females were administered intraperitoneal injections of the plastic-derived compound mixture (BPA 25 mg/kg BW/day, DEHP 375 mg/kg BW/day, and DBP 33 mg/kg BW/day). Therefore, the BPA and DBP were below the NOAEL and DEHP below the LOAEL. The F1 generation offspring were directly exposed as a fetus and F2 generation grand offspring exposed as the germline in the F1 generation. These were each bred at 90 days of age within the exposure lineage. The F3 generation great-grand offspring is required to establish the transgenerational inheritance generation after ancestral exposure. A control lineage was established that used F0 gestating rats exposed to the vehicle control dimethyl sulfoxide (DMSO). Disease pathology was evaluated in BPA and phthalates (hereafter plastics) exposure and control lineages at 1 year of age. The plastics exposure lineage transgenerational individuals with specific disease or pathology were grouped as representatives of the pathology exhibited. Only individuals with a specific disease and no other disease were used to minimize the confounder of comorbidities. The

remaining individuals with no disease were grouped as ‘no disease’. Comparisons between these two groups were made for analysis of sperm DNA methylation. The differential DNA methylation regions (DMRs) in sperm used an MeDIP-Seq analysis for an EWAS approach for the identification of specific disease-associated epigenetic biomarkers.

### Pathology Analysis

As described in the Methods section, a new pathology analysis was used with archived histology slides from the original study (4) to assess sections of testis, kidney, and prostate. The histological sections were captured and analyzed digitally for greater accuracy. The digital images were analyzed by two different observers blinded to the exposure. When discrepancies arose, a third counter blinded to the exposure analyzed the histological section. The raw count of abnormalities was obtained from each digital image. The pixel area was also determined from each image, allowing for a size correction between individual organ samples in the study. The final counts used for the analysis represent the number of abnormalities seen per mm<sup>2</sup> for each individual image. The pathology parameters identified are as previously described and imaged (4). In brief, each counter records the incidence of abnormalities in each tissue. In testis, atrophy of a seminiferous tubule, the arrest of maturation of sperm (indicated by sloughed cells in the center of the tubule), and the presence of vacuoles were indicated disease pathologies. The abnormalities counted in kidney include a reduction in size of glomeruli, a thickening of the Bowman’s capsule, and the presence of cysts. Prostate abnormalities counted included atrophy of the epithelial cells, hyperplasia in the epithelial layer, and the presence of vacuoles within the epithelial layer of the prostatic glands. Obese phenotypes were assigned following assessment of body and abdominal adiposity. The individual F3 generation animals are listed as (+) which indicates the presence of disease or (–) the absence of disease for the current F3 generation plastics lineage male pathology (Table 1). Only those individuals with a single disease for a specific pathology were used for that pathology molecular analysis, as indicated in Table 1. The number of total animals analyzed and individuals with a single disease are indicated in Table 1. Animals exhibiting more than one disease are all listed under the category ‘Multiple Disease’. Those animals used in the molecular analysis are indicated by gray highlights in Table 1. Due to the low prevalence of disease in the control animal groups (4), control lineage animals were not used in the identification of epigenetic biomarkers.

### Sperm DNA Methylation

The experimental design was focused on the identification of transgenerational disease DMR biomarkers in sperm. Sperm were collected from the plastic lineage F3 generation males for epigenetic analysis. DNA from the sperm was isolated and fragmented with sonication, as described in the Methods. The methylated DNA immunoprecipitation (MeDIP) using a methylcytosine antibody was used to identify alterations in DNA methylation. The methylated DNA fragments were then sequenced for an MeDIP-Seq analysis, as described in the Methods. The differential DNA methylation regions (DMRs) were identified between the disease versus non-disease within the plastics lineage animals (Fig. 1). The transgenerational sperm DMR numbers are presented in Fig. 1 for different edgeR statistical p-value cutoff thresholds, and  $P < 1e-04$  (diseased versus non-

**Table 1** Plastics lineage F3 generation pathology

Molecular ID	Puberty	Testis disease	Prostate disease	Kidney disease	Obesity	Tumor	Multiple disease	Total disease
LP1	-	+	-	-	+	-	+	2
LP2	-	+	+	-	-	-	+	2
LP3	-	+	-	-	-	+	+	2
LP4	-	+	-	-	-	-	-	1
LP5	-	-	-	-	-	-	-	0
LP6	-	+	-	-	-	-	-	1
LP7	-	-	-	+	-	-	-	1
LP8	-	-	-	-	-	-	-	0
LP9	-	-	-	-	-	-	-	0
LP10	-	-	-	-	-	-	-	0
LP11	-	-	-	-	-	-	-	0
LP12	-	+	-	+	-	-	+	2
LP13	-	-	-	-	-	-	-	0
LP14	-	-	-	-	-	-	-	0
LP15	-	-	-	-	-	-	-	0
LP16	-	-	-	+	-	-	-	1
LP19	-	-	-	-	-	-	-	0
LP20	-	-	-	+	-	-	-	1
LP21	-	-	-	-	-	-	-	0
LP17	-	+	-	-	-	-	-	1
LP18	NA	-	-	-	+	-	-	1
LP22	-	-	-	NA	-	-	-	NA
LP23	-	-	-	-	-	-	-	0
LP24	-	-	-	+	-	-	-	1
LP25	-	-	-	+	-	-	-	1
LP26	-	+	-	-	-	-	-	1
LP27	-	+	-	+	-	-	+	2
LP28	-	+	-	+	-	+	+	3
LP29	-	+	+	-	-	-	+	2
LP30	-	+	-	-	-	-	-	1
LP31	-	-	-	-	-	-	-	0
LP32	-	-	-	-	-	-	-	0
LP33	-	-	-	-	-	-	-	0
LP34	-	-	+	-	-	-	-	1
LP35	-	-	+	-	-	-	-	1
LP36	-	-	-	-	-	-	-	0
LP37	-	+	+	-	-	-	+	2
LP38	-	-	-	-	-	-	-	0
LP40	-	-	-	+	-	-	-	1
LP39	-	+	-	-	-	-	-	1
LP41	-	-	-	-	+	-	-	1
LP42	-	-	-	-	-	-	-	0
LP43	-	-	-	-	-	-	-	0
LP44	-	+	-	-	-	-	-	1
LP45	-	-	-	-	-	-	-	0
LP46	-	+	-	-	-	-	-	1
LP47	-	+	-	-	-	-	-	1
LP48	-	+	+	-	-	-	+	2
LP49	-	-	-	-	-	-	-	0
LP50	-	-	-	-	+	-	-	1
LP51	-	-	-	-	-	-	-	0
LP52	-	-	-	-	-	-	-	0
Total	0/48 = 0%	18/52 = 35%	6/52 = 12%	9/51 = 18%	4/52 = 7%	2/52 = 4%	9/52 = 17%	

The individual animals for the plastic lineage males are listed and a (+) indicates presence of disease and (-) absence of disease. The shaded disease boxes identify those animals used for the disease molecular analysis. The number of animals with disease vs total number of animals and percentage is presented in the bottom row. The comparisons with no disease animals are indicated with a (0).

diseased within the plastics lineage) was selected as the threshold for subsequent analyses. Potential disease-specific DMRs were identified among the plastics lineage animals as shown in

**Table 1.** The plastics exposure lineage included animals with testis disease, kidney disease, and multiple diseases (Fig. 1A–C). Although several animals with prostate disease and obesity

## Specific Transgenerational Disease DMR Identification

**A Testis Disease DMR Biomarkers**

p-value	All Window	Multiple Window
0.001	360	1
<b>1e-04</b>	<b>39</b>	<b>0</b>
1e-05	2	0
1e-06	0	0
1e-07	0	0
Significant windows		1
Number of DMR		39

**B Kidney Disease DMR Biomarkers**

p-value	All Window	Multiple Window
0.001	2900	17
<b>1e-04</b>	<b>384</b>	<b>0</b>
1e-05	53	0
1e-06	8	0
1e-07	1	0
Significant windows		1
Number of DMR		384

**C Multiple Disease DMR Biomarkers**

p-value	All Window	Multiple Window
0.001	1308	11
<b>1e-04</b>	<b>192</b>	<b>0</b>
1e-05	28	0
1e-06	6	0
1e-07	1	0
Significant windows		1
Number of DMR		192

**Figure 1** DMR identification and numbers. The number of DMRs found using different P-value cutoff thresholds. The All Window column shows all DMRs. The Multiple Window column shows the number of DMRs containing at least two significant windows (1000bp each). The number of DMRs with the number of significant windows (1000bp per window) at a P-value threshold of  $P < 1e-04$  for DMR is presented. (A) Testis disease DMRs; (B) kidney disease DMRs; and (C) multiple disease DMRs

were observed, insufficient numbers were present for the analysis, Table 1. All windows column in Fig. 1 represent all DMR windows, and multiple window column includes those with neighboring 1 kb sites. Only kidney disease and multiple disease DMRs had multiple adjacent sites. In the current analysis, 1000 bp windows were used in the identification of DMRs. The genomic locations and characteristics are presented in Supplementary Tables S1–S3. The alterations in DNA methylation are presented as a maximum log-fold change (maxLFC) and indicates that ~30–43% of the different disease-specific DMRs have an increase in DNA methylation, while the remaining have a decrease in DNA methylation.

Chromosomal locations of the disease-associated DMRs are shown in Fig. 2. DMRs are represented as arrowheads and DMR clusters are represented by black boxes. DMRs are present for each individual disease on all chromosomes, except the Y chromosome and mitochondrial DNA (MT). These results support the idea that the epigenetic effects of transgenerational pesticide exposure are genome-wide. Genomic features are shown in Supplementary Fig. S1. The CpG density of DMRs is low, 1–3

CpG/100 bp, and referred to as CpG deserts. DMR length for most disease associations is between 1 and 3 kilobases. The principal component analyses (PCA) of the RPKM adjusted read depths at differential DMR sites for each sample are shown in Supplementary Fig. S2. The PCA plots show how the DMR samples cluster according to disease compared to non-disease when DMR sites are considered. No outliers were observed within each of the DMR data sets. Therefore, the DMR principal components analysis clustered the DMRs according to disease or non-disease for each pathology (Supplementary Fig. S2).

The potential overlap between the disease-specific DMR biomarkers was investigated. All the DMRs associated with a specific disease at  $P < 1e-04$  are unique to each disease, with no overlap between disease-associated DMRs (Fig. 3A). An extended overlap of the disease-associated DMRs ( $P < 1e-04$ ), with a significance level of  $P < 0.05$  (Fig. 3B) shows relatively low overlap between specific disease-associated DMR. The highest overlaps include the multiple disease category, with 28% overlap with testis disease and 8% overlap with kidney disease. The multiple disease category includes individuals with several of the individual diseases. A final overlap analysis used all overlaps at  $P < 0.05$  (i.e. 426; Supplemental Fig.S3) and checked if any were common to the overlaps at  $P < 1e-04$  (Fig. 3C). None overlapped between all the disease and only a few between diseases. Observations indicate the DMRs are predominantly disease-specific with no common set of DMRs.

**DMR Gene Associations**

The transgenerational disease-specific DMR epimutation gene associations were identified and presented in Supplementary Tables S1–S3. Approximately 50–55% of the testis, kidney, and multiple disease DMRs had gene associations. Therefore, ~50% of the DMR are intergenic and the rest within 10 kb of a gene to include both the proximal and distal promoters. The gene associations were sorted with functional gene categories and presented in Fig. 4A. The metabolism, transcription, and signaling categories were predominant. The DMR-associated gene pathways were identified through a KEGG pathway analysis, as described in the Methods section (Fig. 4B). The testis disease-specific DMRs had few multiple DMR-associated genes in specific pathways. The kidney and multiple disease DMR-associated genes had higher numbers of pathways with multiple genes and the pathways in cancer and MAPK pathway with multiple genes and the pathways were common between the two. Because the signaling components in the cancer and MAPK pathways are common to most cellular processes, the correlation is expected.

The final analysis correlated the disease-specific DMR-associated genes with previously identified disease-linked genes. A Pathway Studio gene network was used as described in the Methods section. The testis disease and kidney disease DMR-associated gene correlations are shown in Fig. 5. Testis disease DMR had links to infertility and testis organ associated genes. The kidney disease DMR-associated genes included a significant number with links to kidney physiology and kidney pathologies (Fig. 5B). Multiple kidney abnormalities are associated with the kidney disease DMR-associated genes identified in this study. The multiple disease DMR-associated genes had links with cancer, kidney disease, infertility, and kidney and testis physiology (Fig. 6). The gene shapes are correlated with various gene functions and are presented in Fig. 6. Observations identified potential disease-specific DMR-associated genes correlated with the testis, kidney, and multiple diseases. This helps validate the

Transgenerational Disease DMR Chromosomal Locations

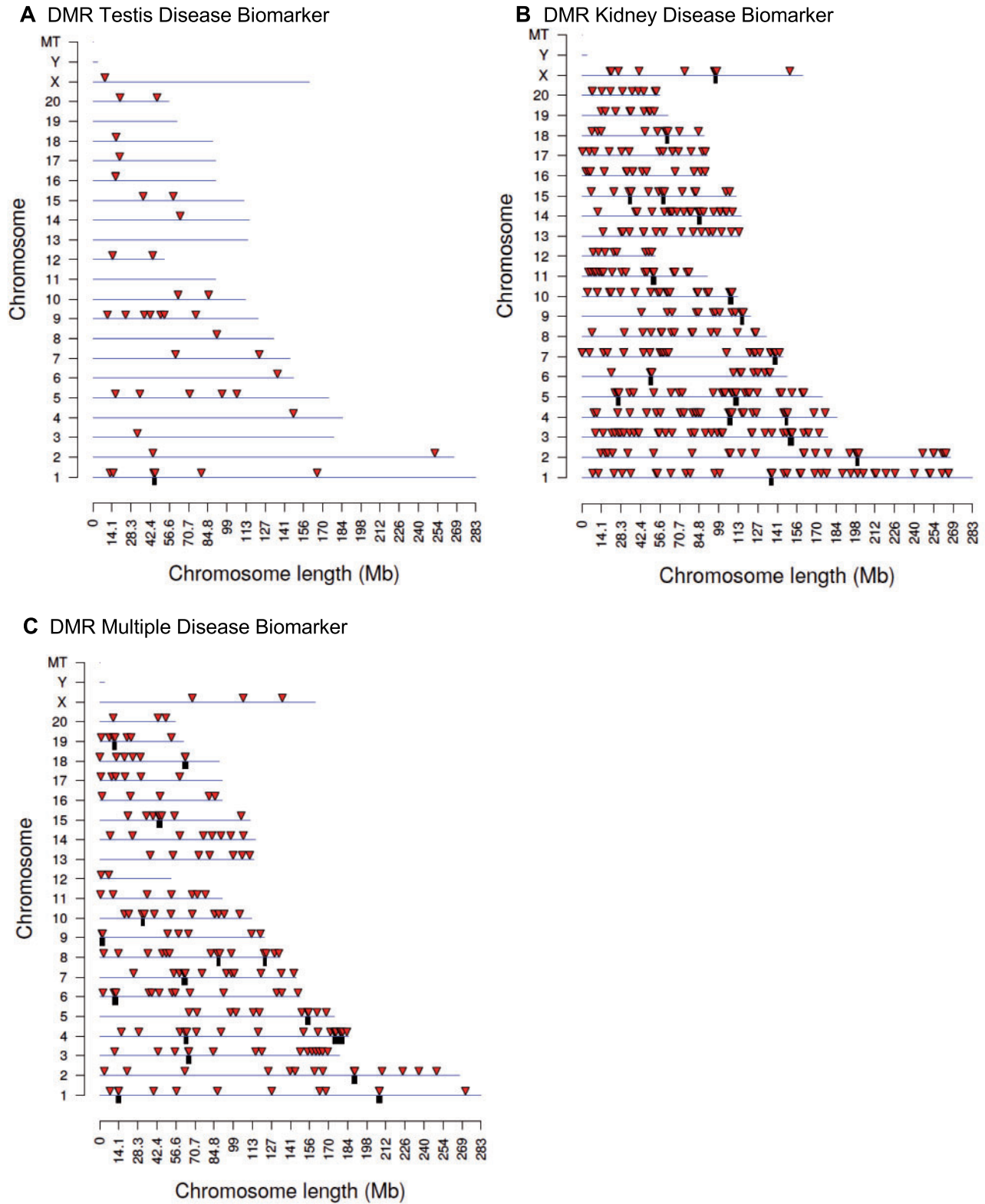
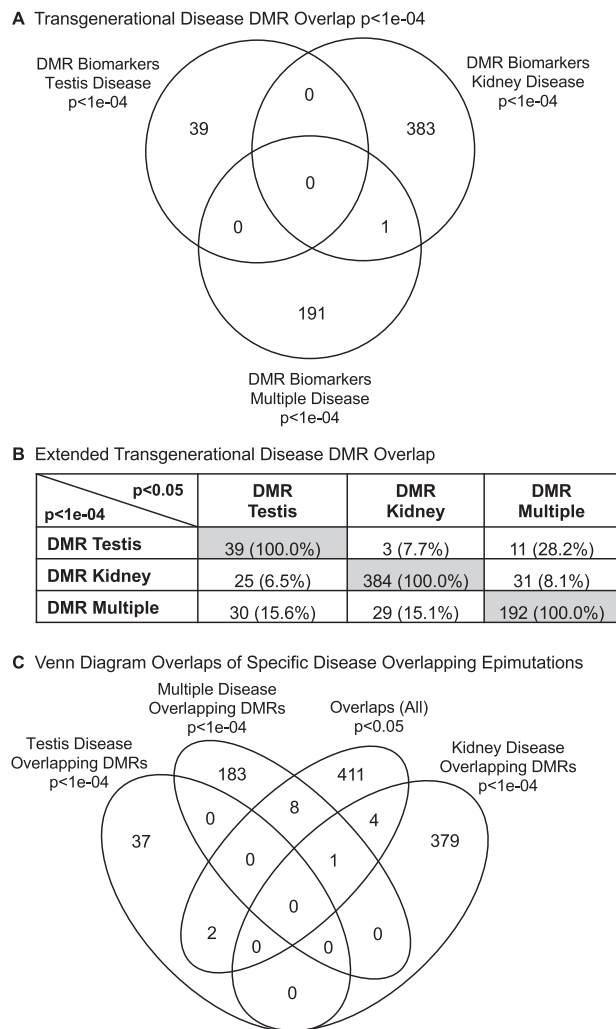


Figure 2 DMR chromosomal locations. The DMR locations on the individual chromosomes are represented with an arrowhead and a cluster of DMRs with a black box. The chromosome number is plotted against the size of the chromosome. All DMRs containing at least one significant window at a P-value threshold of  $1e-04$  for DMR are shown. (A) Testis disease DMRs; (B) kidney disease DMRs; and (C) multiple disease DMRs

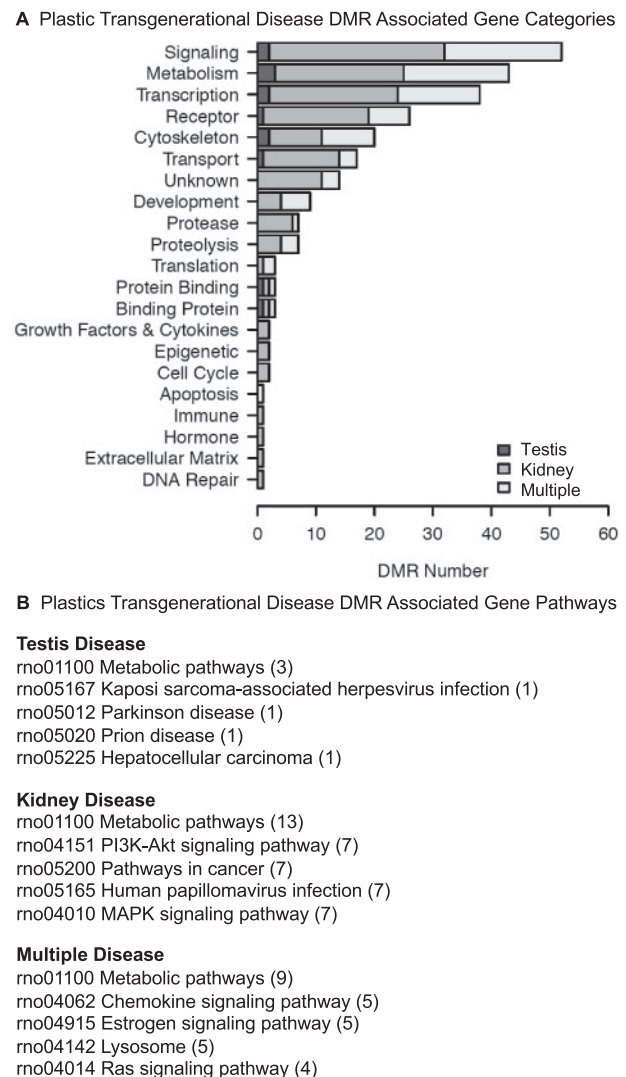


**Figure 3** Disease DMR overlap. (A) Venn diagram transgenerational disease DMR overlap at  $P < 1e-04$ . (B) Extended transgenerational disease DMR overlaps. The P-value data set at  $P < 1e-04$  is compared to the  $P < 0.05$  data to identify potential overlap between the different pathologies with DMR number and percentage of the total presented. The gray highlight is the expected 100% overlap. (C) Venn diagram overlaps of specific disease DMRs. The  $P < 1e-04$  disease DMRs are compared to the overlapping  $P < 0.05$  DMRs identified in [Supplementary Fig. S3](#)

DMRs identified and demonstrates the anticipated functional gene links.

## Discussion

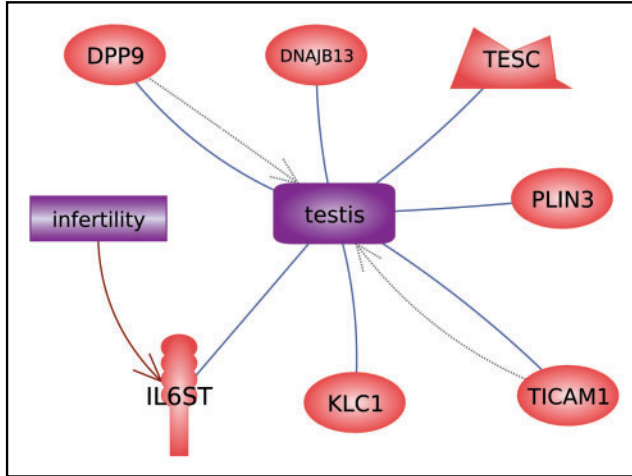
The epigenome is a very responsive and dynamic molecular system and therefore any epigenetic modifications which persist into later life can induce a variety of pathological states (39, 40). Various plastic compounds including BPA and a number of distinct phthalates can act as both endocrine disruptors and epigenetic effectors. Exposure to these chemicals can lead to epigenetic alterations and toxicity which promotes developmental, metabolic, and behavioral disorders (40). These epimutations can also be inherited through the germline, resulting in the transgenerational inheritance of altered phenotypes (10). Environmental toxicants, such as BPA and phthalates, are so widely used that they are considered ubiquitous and no population is exempt from exposure to them. The detrimental effects



**Figure 4** DMR-associated gene categories and pathways. (A) DMR-associated gene categories. The gene categories and number of associated genes are presented for each disease group. (B) DMR-associated gene pathways. The gene pathways with DMR-associated genes are listed from KEGG pathway analysis and the number in bracket presents the number of DMR-associated genes

of exposure to toxicants can be considered as embedded in the biology of human populations and will affect human health over a long period of time and across many generations (39, 41). Because many of these environmental toxicants result in epigenetic modifications among the exposed population, there is great potential to use the epigenetic profile in developmental epidemiology (42). Epigenetic modifications associated with exposure to environmental toxicants can be tracked and used in the projection of health and later life outcomes. In this scenario, any unique epigenetic mark and alteration (i.e. epimutation) associated with exposure to a particular toxicant can serve as a biomarker for that exposure and may be utilized to assist in the treatment and prevention of later-life disease. In this study, a rat model is used to demonstrate the effects of transgenerational (i.e. ancestral) exposure to environmental toxicants, a mixture of plastic-derived compounds, to identify potential epigenetic biomarkers associated with specific disease in the exposure lineage population.

**A Testis Disease DMR Associated Gene Correlations**



**B Kidney Disease DMR Associated Gene Correlations**

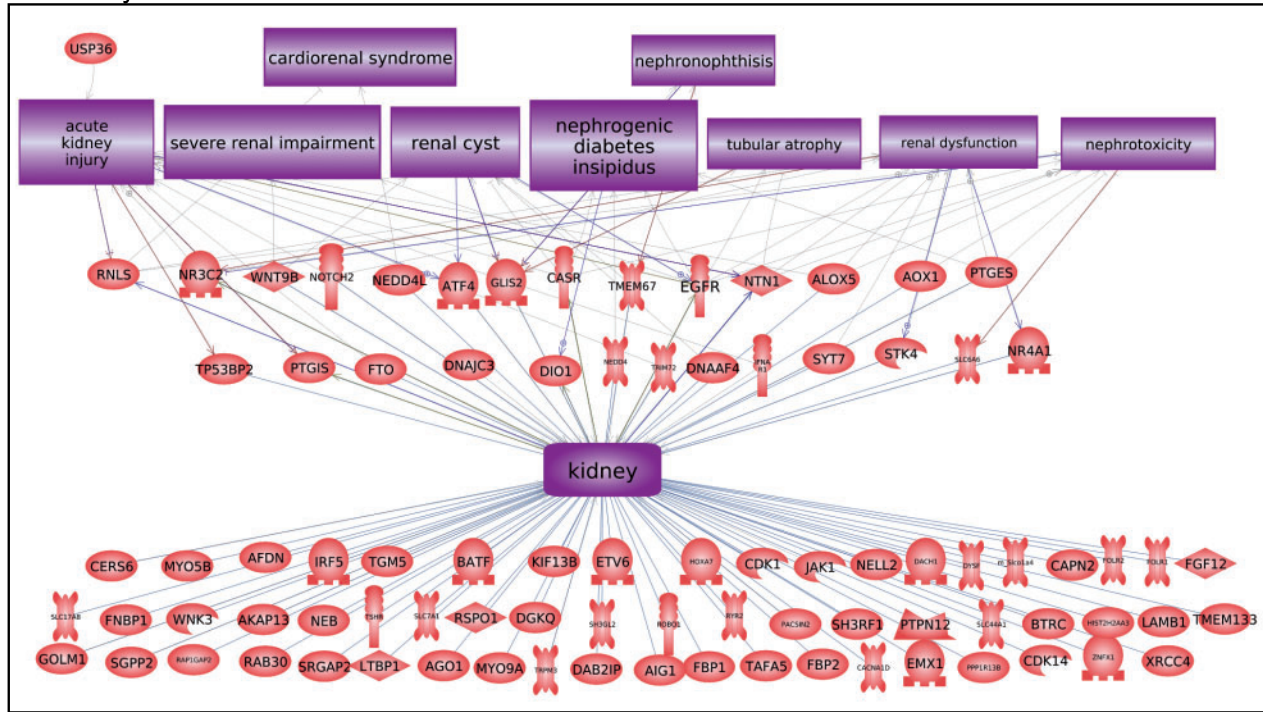


Figure 5 Specific disease DMR-associated gene correlation with previously identified disease genes. (A) Epimutation associated testis disease genes. (B) Epimutation associated kidney disease genes. The tissue physiology and related diseases are presented

Exposure to BPA and phthalates has been linked to an increased incidence of disease and transgenerational epigenetic alterations in the germline (4). BPA, in particular, has been linked with hypomethylation and a shift in the coat color distribution of yellow agouti mouse offspring (43) demonstrating a clear link between toxicant exposure, epigenetic modifications, and a shift in phenotype in a murine model. In rat models, exposure to a combination of BPA, DEHP, and DBP compounds resulted in transgenerational inheritance of increased incidence of reproductive disease, obesity, and epimutations (4). The current study utilizes the transgenerational F3 generation individuals ancestrally exposed to plastics in Manikkam et al. (4) to identify potential epigenetic biomarkers associated with a specific disease. The pathologies examined in the current study are relevant to human populations and include testis disease,

kidney disease, and multiple diseases. The exposure to a ubiquitous environmental toxicant is also relevant to the hypothesis of fetal origins of adult disease originally proposed by Barker (7). The analysis extends this hypothesis to numerous disease states, perhaps most relevant being the multiple disease state where a single individual experiences multiple disease as a result of ancestral exposure to environmental toxicants (39).

Epigenetic alterations in DNA methylation in sperm were examined in this study. Comparisons were made between diseased and non-diseased individuals whose great-grandparents were exposed to the plastic-derived compound mixture (Table 1). In these comparisons, the alterations in DNA methylation (i.e. DMRs) were identified and these serve as possible epigenetic biomarkers of disease resulting from ancestral exposure to plastics. Using an edgeR *P*-value of  $P < 1e-4$  as a statistical



Multiple ( $\geq 2$ ) Disease DMR Associated Gene Correlations

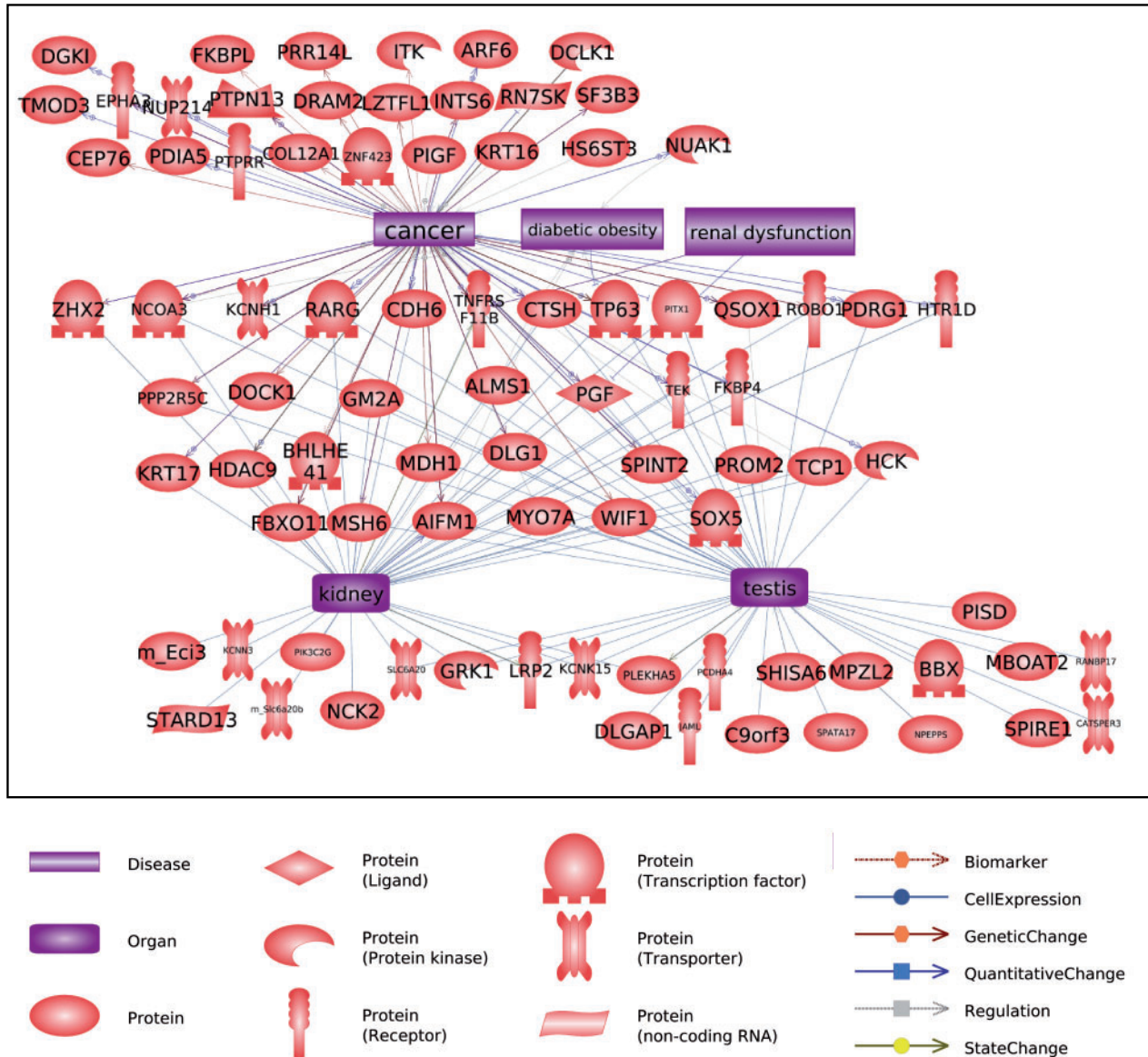


Figure 6 Multiple disease DMR-associated gene correlations with previously identified disease genes. The tissue physiology and related diseases are presented. Gene functional category shapes presented at the bottom with arrow function presented

threshold, several hundred DMRs are associated with each of the diseases examined (Fig. 1). The epimutations identified are widespread across the chromosomes of the study species (Fig. 2). Therefore, the transgenerational epigenetic effects of ancestral exposure are epigenome-wide. DMRs identified for each disease studied are unique with no overlap between diseases at a high statistical threshold (Fig. 3). An extended overlap of DMRs associated with disease also shows relatively little overlap, although individual disease overlaps with the multiple disease state are as high as 28% (Fig. 3). The relative lack of overlap of DMRs between diseases suggests that the epigenetic alterations associated with each disease are unique. This supports the potential use of these unique epigenetic marks for disease susceptibility diagnostics to assess development of later life health outcomes and facilitate preventative medicine. The results of the current study show that the ubiquitous exposure of the human population to plastic-derived compounds will not

only have detrimental effects on the current generation but also will possibly continue to plague future generations. The transgenerational epigenetic modification of the germline (sperm or egg) will impact the early embryo cell epigenome and transcriptome to then alter all derived somatic cell types' epigenomes and transcriptomes. Those somatic cells sensitive to these alterations will have an increase susceptibility to develop pathologies. These subsequent generations germlines will then transmit this to propagate the epigenetic transgenerational inheritance phenomenon (10-12).

Among the DMRs associated with each disease, 50-55% are associated with genes. The most common gene categories represented are metabolism, transcription, and signaling which are all common and critical molecular functions. Analysis of the DMR-associated genes with correlated tissue pathology and disease linked genes identified associations to related pathologies (Figs 5 and 6). These associated disease links establish the

relevance of the disease-specific biomarker DMRs identified. Observations help validate the experimental design and biomarker disease associations.

The number of animals with a specific individual disease was in some instances low. This is a limitation of the current study. Although an edgeR *P*-value was used to identify and analyze the disease-associated DMRs, further analyses adjusting for multiple testing using the false discovery rate (FDR) resulted in FDR *P* values for the disease epimutations of >0.1 in all comparisons. Potential higher variability in the data needs to be considered even though higher edgeR values were used.

The current study examined the phenotypic and epigenetic effects of ancestral exposure to a common mixture of plastic-derived chemicals at or near the no observable adverse effect level (NOAEL). The phenotypic effects examined were the incidence of disease in the ancestrally exposed transgenerational generation, and these included testis disease, kidney disease, and multiple diseases. The epigenetic effects were examined in the DNA methylation profiles of the sperm. Among the epigenetic alterations were DMRs uniquely associated with each of the diseases identified. These epigenetic differences could serve as important diagnostic marks for determining disease susceptibility and predicting health trajectories of individuals and populations. Due to the ubiquity of plastics exposure, and the embedded nature of these exposures and disease in the human population, epigenetic epidemiology utilizing biomarkers such as those identified herein could prove crucial to the efforts to mitigate negative health outcomes and improve the population's healthcare.

## Methods

### Animal Studies and Breeding

As previously described (4), female and male rats of an outbred strain Hsd:Sprague Dawley SD (Harlan) at 70–100 days of age were fed ad lib with a standard rat diet and ad lib tap water. Timed-pregnant females were mated and on embryonic days 8 through 14 (E8–E14) of gestation were administered daily intraperitoneal injections of the plastic compound mixture (BPA 25 mg/kg BW/day, DEHP 375 mg/kg BW/day, and DBP 33 mg/kg BW/day) or vehicle control dimethyl sulfoxide (DMSO).

The gestating female rats exposed were designated as the F0 generation. F1–F3 generation control and plastics lineages were housed in the same room and racks with lighting, food, and water. Non-littermate females and males aged 70–100 days from the F1 generation of pesticides or control lineages were bred within their treatment group to obtain F2 generation offspring. Unrelated F2 generation rats were bred to obtain F3 generation offspring. No sibling or cousin breeding was used to avoid any inbreeding artifacts. Only the F0 generation received treatments. All animals were aged to 1 year for pathology and epigenetic analysis. All experimental protocols for the procedures with rats were pre-approved by the Washington State University Animal Care and Use Committee (IACUC approval # 2568). All methods were performed in accordance with the relevant guidelines and regulations. The sperm samples and tissue sections from the previous study (4) were archived and used for the current study.

### Tissue Harvest and Histology Processing

As previously described (4), at 12 months of age, rats were euthanized by CO<sub>2</sub> inhalation and cervical dislocation for tissue

harvest. Testis, prostate, and kidney were fixed in Bouin's solution (Sigma) followed by 70% ethanol, then processed for paraffin embedding, and hematoxylin and eosin (H & E) staining by standard procedures for histopathological examination. Paraffin five-micron sections were processed and stained by Nationwide Histology, Spokane, WA.

### Histopathology Examination and Disease Classification

Archived histology slides or paraffin blocks from the previous study (4), were used for a new histology analysis for the current study. Stained testis, prostate, and kidney slides were imaged through a microscope using 4× objective lenses (testis and prostate) or 10× objective lenses (kidney). Tiled images were captured using a digital camera. Tiled images for each tissue were photo-merged into a single image using Adobe Photoshop (ver. 21.1.2, Adobe, Inc.). Images were evaluated and pathology features digitally marked using Photoshop software. The Washington Animal Disease Diagnostic Laboratory (WADDL) at the Washington State University College of Veterinary Medicine has board-certified veterinary pathologists and assisted in initially establishing the criteria for the pathology analyses and identifying parameters to assess. The tissue pathology evaluated used previously described histological criteria described in transgenerational models with representative images (4, 31–34). Histopathology readers were trained to recognize the specific abnormalities evaluated for this study in rat testis, ventral prostate, and kidney. Two individuals blinded to the exposure evaluated each tissue image for abnormalities. In the event of a disagreement about the disease status, a third individual blinded to the exposure evaluated the tissue. Sets of quality control (QC) slides were generated for each tissue and were read by each reader prior to evaluating any set of experimental slides. These QC slide results were monitored for reader accuracy and concordance.

Specific descriptions of histopathological analysis and example images were previously reported (4). Testis histopathology criteria included the presence of vacuoles in the seminiferous tubules, azoospermic atretic seminiferous tubules, and 'other' abnormalities including sloughed spermatogenic cells in center of the tubule and a lack of a tubule lumen. Prostate histopathology criteria included the presence of vacuoles in the glandular epithelium, atrophic glandular epithelium, and hyperplasia of prostatic gland epithelium. Kidney histopathology criteria included reduced size of glomerulus, thickened Bowman's capsule, and the presence of proteinaceous fluid-filled cysts >50 μm in diameter. A cutoff was established to declare a tissue 'diseased' based on the mean number of histopathological abnormalities plus two standard deviations from the mean of control group tissues, as assessed by each of the individual observers blinded to the treatment groups. This number (i.e. greater than two standard deviations) was used to classify rats into those with and without testis, prostate, or kidney disease in each lineage. A rat tissue section was finally declared 'diseased' only when at least two of the three observers marked the same tissue section 'diseased'. Onset of puberty was assessed in males starting at 35 days of age by the presence of balano-preputial separation. Obesity was assessed with an increase in body mass and a qualitative evaluation of abdominal adiposity. The statistical analyses for pathology results were expressed as the proportion of affected animals that exceeded a pre-determined threshold (testis, prostate or kidney disease frequency, tumor frequency, and obesity frequency). Groups were analyzed using Fisher's exact test.

### Epididymal Sperm Collection and DNA Isolation

The protocol used is as previously described (44). Briefly, the epididymis was dissected free of fat and connective tissue, then, after cutting open the cauda, placed into 6 ml of phosphate buffer saline (PBS) for 20 min at room temperature. Further incubation at 4°C will immobilize the sperm. The tissue was then minced, the released sperm pelleted at 4°C 3000 × *g* for 10 min, then resuspended in NIM buffer and stored at –80°C for further processing. An appropriate amount of rat sperm suspension was used for DNA extraction. Previous studies have shown mammalian sperm heads are resistant to sonication unlike somatic cells (45, 46). Somatic cells and debris were removed by brief sonication (Fisher Sonic Dismembrator, model 300, power 25), then centrifugation and washing 1–2 times in 1× PBS. The resulting pellet was resuspended in 820 μl DNA extraction buffer and 80 μl 0.1 M DTT added, then incubated at 65°C for 15 min. Proteinase K (80 μl of 20 mg/ml) was added and the sample was incubated at 55°C for 2–3 h under constant rotation. Protein was removed by addition of protein precipitation solution (300 μl, Promega A795A), incubation for 15 min on ice, then centrifugation at 13 500 rpm for 30 min at 4°C. One ml of the supernatant was precipitated with 2 μl of GlycoBlue (Invitrogen, AM9516) and 1 ml of cold 100% isopropanol. After incubation, the sample was spun at 13,500 × *g* for 30 min at 4°C, then washed with 70% cold ethanol. The pellet was air-dried for about 5 min then resuspended in 100 μl of nuclease-free water.

### Methylated DNA Immunoprecipitation

The archived sperm samples were prepared as previously described (44). Genomic DNA was sonicated and run on 1.5% agarose gel for fragment size verification. The sonicated DNA was then diluted with 1X TE buffer to 400 μl, then heat-denatured for 10 min at 95°C, and immediately cooled on ice for 10 min to create single-stranded DNA fragments. Then, 100 μl of 5× IP buffer and 5 μg of antibody (monoclonal mouse anti-5-methyl cytidine; Diagenode #C15200006) were added, and the mixture was incubated overnight on a rotator at 4°C. The following day magnetic beads (Dynabeads M280 Sheep anti-Mouse IgG; Life Technologies 11201D) were pre-washed per manufacturer's instructions, and 50 μl of beads were added to the 500 μl of DNA-antibody mixture from the overnight incubation, then incubated for 2 h on a rotator at 4°C. After this incubation, the samples were washed three times with 1× IP buffer using a magnetic rack. The washed samples were then resuspended in 250 μl digestion buffer (5 mM Tris PH 8, 10 mM EDTA, 0.5% SDS) with 3.5 μl Proteinase K (20 mg/ml), and incubated for 2–3 h on a rotator at 55°C. DNA clean-up was performed using a Phenol-Chloroform-Isoamyl-Alcohol extraction, and the supernatant precipitated with 2 μl of GlycoBlue (20 mg/ml), 20 μl of 5 M NaCl, and 500 μl ethanol in –20°C freezer for 1 h to several hours. The DNA precipitate was pelleted, washed with 70% ethanol, then dried and resuspended in 20 μl H<sub>2</sub>O or 1× TE. DNA concentration was measured in Qubit (Life Technologies) with the ssDNA kit (Molecular Probes Q10212).

### Methylated DNA Immunoprecipitation-Seq Analysis

Methylated DNA immunoprecipitation (MeDIP) DNA was used to create libraries for next-generation sequencing (NGS) using the NEBNext Ultra RNA Library Prep Kit for Illumina (San Diego, CA) starting at step 1.4 of the manufacturer's protocol to generate double-stranded DNA from the single-stranded DNA resulting from MeDIP. After this step, the manufacturer's protocol

was followed indexing each sample individually with NEBNext Multiplex Oligos for Illumina. The WSU Spokane Genomics Core sequenced the samples on the Illumina HiSeq 2500 at PE50, with a read size of ~50 bp and ~10–20 million reads per pool. Twelve libraries were run in one lane.

### Statistics and Bioinformatics

The DMR identification and annotation methods follow those presented in previously published papers (30, 44). Data quality was assessed using the FastQC program (<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The data were cleaned and filtered to remove adapters and low-quality bases using Trimmomatic (47). The reads for each MeDIP sample were mapped to the Rnor 6.0 rat genome using Bowtie2 (48) with default parameter options. The mapped read files were then converted to sorted BAM files using SAMtools (49). The MEDIPS R package (50) was used to calculate differential coverage between disease and non-disease sample groups. The edgeR P-value (51) was used to determine the relative difference between the two groups for each genomic window. Windows with an edgeR P-value less than an arbitrarily selected threshold were considered DMR. The site edges were extended until no genomic window with an edgeR P-value less than 0.1 remained within 1000 bp of the DMR. The edgeR P-value was used to assess the significance of the DMR identified. A false-discovery rate (FDR) analysis for each comparison was performed and provided a  $P > 0.1$  for all the comparisons. Due to the lower number of individuals with one specific disease type, an FDR analysis is generally not useful, nor was a permutation analysis, for the specific disease biomarkers (52–57). Differential epimutation sites were annotated using the biomaRt R package (58) to access the Ensembl database (59). The DMR-associated genes were then automatically sorted into functional groups using information provided by the DAVID (60) and Panther (61) databases incorporated into an internal curated database ([www.skinner.wsu.edu](http://www.skinner.wsu.edu) under genomic data). A Pathway Studio, Elsevier, database, and network tool was used to assess physiological and disease process gene correlations. All molecular data has been deposited into the public database at NCBI (GEO # GSE163412) and R code computational tools available at GitHub (<https://github.com/skinnerlab/MeDIP-seq>) and [www.skinner.wsu.edu](http://www.skinner.wsu.edu).

### Supplementary data

Supplementary data are available at *EnvEpig* online.

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publish, or preparation of the manuscript. **Conflict of interest statement.** None declared.

## Ethics

All experimental protocols for the procedures with rats were pre-approved by the Washington State University Animal Care and Use Committee (IACUC approval # 2568). All methods were performed in accordance with the relevant guidelines and regulations.

## Consent for Publication

Not applicable.

## Data Availability

All molecular data have been deposited into the public database at NCBI (GEO # GSE163412) and R code computational tools available at GitHub (<https://github.com/skinnerlab/MeDIP-seq>) and [www.skinner.wsu.edu](http://www.skinner.wsu.edu).

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