Spring 2023 – Epigenetics and Systems Biology
Discussion Session (Epigenetics and Disease Etiology)
Michael K. Skinner – Biol 476/576
Week 14 (April 13)

Epigenetics and Disease Etiology

Primary Papers

1. Nilsson et al. (2018) Epigenetics. 13(8): 875-895. (PMID: 30207508)
2. King and Skinner (2020) Trends Endocrinol Metab. 31(7):478-494. (PMID: 32521235)
3. Beck et al. (2022) Sci Rep. 12(1):5452. (PMID: 35440735)

Discussion

Student 34 – Ref #1 above

- What environmental contaminants were examined?
- What is the transgenerational disease?
- How is the ovarian somatic cell epigenome modified to promote ovarian disease?

Student 35 – Ref #2 above

- What is the epigenetic transgenerational inheritance mechanism?
- Could the rise in obesity in the population today be in part due to transgenerational phenomenon from ancestral exposure?
- Do we have a responsibility to our future generations?

Student 36 – Ref #3 above

- What is the experimental design?
- What are the epigenetic and disease observations?
- How do the observations fit with classic genetic causes for disease?

EPIGENETICS
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RESEARCH PAPER

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Environmental toxicant induced epigenetic transgenerational inheritance of ovarian pathology and granulosa cell epigenome and transcriptome alterations: ancestral origins of polycystic ovarian syndrome and primary ovarian insufiency

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ABSTRACT

Two of the most prevalent ovarian diseases affecting women's fertility and health are Primary Ovarian Insufficiency (POI) and Polycystic Ovarian Syndrome (PCOS). Previous studies have shown that exposure to a number of environmental toxicants can promote the epigenetic transgenerational inheritance of ovarian disease. In the current study, transgenerational changes to the transcriptome and epigenome of ovarian granulosa cells are characterized in F3 generation rats after ancestral vinclozolin or DDT exposures. In purified granulosa cells from 20-day-old F3 generation females, 164 differentially methylated regions (DMRs) ($P < 1 \times 10^{-6}$) were found in the F3 generation vinclozolin lineage and 293 DMRs ($P < 1 \times 10^{-6}$) in the DDT lineage, compared to controls. Long noncoding RNAs (lncRNAs) and small noncoding RNAs (sncRNAs) were found to be differentially expressed in both the vinclozolin and DDT lineage granulosa cells. There were 492 sncRNAs ($P < 1 \times 10^{-4}$) in the vinclozolin lineage and 1,085 sncRNAs ($P < 1 \times 10^{-4}$) in the DDT lineage. There were 123 lncRNAs and 51 lncRNAs in the vinclozolin and DDT lineages, respectively ($P < 1 \times 10^{-4}$). Differentially expressed mRNAs were also found in the vinclozolin lineage (174 mRNAs at $P < 1 \times 10^{-4}$) and the DDT lineage (212 mRNAs at $P < 1 \times 10^{-4}$) granulosa cells. Comparisons with known ovarian disease associated genes were made. These transgenerational epigenetic changes appear to contribute to the dysregulation of the ovary and disease susceptibility that can occur in later life. Observations suggest that ancestral exposure to toxicants is a risk factor that must be considered in the molecular etiology of ovarian disease.

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Introduction

Two of the most prevalent ovarian diseases affecting women's fertility and health are Primary Ovarian Insufficiency (POI) and Polycystic Ovarian Syndrome (PCOS). POI is characterized by a marked reduction in the primordial follicle pool of oocytes and the induction of menopause prior to age 40 [1]. POI currently affects approximately 1% of female population [2]. While genetic causes can be ascribed to a minority of patients, around 90% of POI cases are considered idiopathic, with no apparent genetic link nor known cause [3].

PCOS is a multi-faceted disease that affects 6–18% of women [4,5]. It is characterized by infrequent ovulation or anovulation, high androgen levels in the blood, and the presence of multiple persistent ovarian cysts [6,7]. PCOS patients often show insulin resistance and a heightened risk for diabetes [8,9]. Both genetic and environmental factors have been linked to the development of PCOS, although these do not explain all cases [10,11]. For both PCOS and POI other underlying causes such as epigenetic transgenerational inheritance of disease susceptibility have seldom been considered.

Epigenetics refers to 'molecular factors and processes around the DNA that regulate genome activity independent of DNA sequence, and that are mitotically stable' [12]. Epigenetic factors include DNA methylation, histone modifications, expression of noncoding RNA (ncRNA), RNA methylation, and alterations in chromatin structure [13]. Epigenetic transgenerational inheritance is defined as 'the

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Supplemental data for this article can be accessed here.

germline transmission of epigenetic information and phenotypic change across generations in the absence of any continued direct environmental exposure or genetic manipulation' [12]. Epigenetic changes can be induced by environmental factors such as nutrition or toxicant exposure and are an important mechanism by which organisms alter gene expression in response to their environment. Although transgenerational epigenetic changes must be inherited via germ cells (i.e., sperm or eggs), these germ cell epigenetic changes subsequently promote in the early embryo and stem cells alterations in epigenetics and gene expression that impacts all somatic cells and organs of the individual. This can lead to increased disease susceptibility later in life. Therefore, disease development in organs such as ovaries can in part be due to ancestral exposures and epigenetic inheritance [14].

Previous studies have shown that exposure to a number of environmental toxicants can promote the epigenetic transgenerational inheritance of ovarian disease. Exposure of gestating female rats (F0 generation) to the agricultural fungicide vinclozolin resulted in a transgenerational increase in ovarian abnormalities in great-grand-offspring (F3 generation) [15]. These abnormalities included a decrease in the primordial follicle pool of oocytes that was similar to what is seen in POI, and an increase in ovarian cysts that was similar to what is seen in PCOS. In addition, the ovarian granulosa cells from the antral follicles of 6-month-old F3 generation vinclozolin lineage rats showed transgenerational changes in gene expression and alterations in the pattern of DNA methylation compared to F3 generation control lineage rats [15]. Similarly, exposure of F0 generation pregnant rats to the insecticide DDT (dichlorodiphenyltrichloroethane) induced an epigenetic transgenerational increase in ovarian diseases in the F3 generation, which was comprized of primordial follicle loss and increased rates of ovarian cysts [16]. Ancestral exposure to DDT also induced transgenerational changes in F3 generation sperm DNA methylation patterns [16], but epigenetic changes in ovarian somatic cells such as granulosa cell have not been investigated.

The environmentally induced epigenetic transgenerational inheritance requires the germline transmission of epigenetic alterations (epimutations) by either the sperm or egg. The majority of transgenerational studies have examined sperm transmission of epigenetic changes due to limitations in oocyte numbers for efficient analysis. Although DNA methylation has been investigated more extensively, noncoding RNA (ncRNA) expression has also been shown to be involved in epigenetic transgenerational inheritance [17,18]. Differentially expressed ncRNAs have been shown to correlate with increased disease susceptibility originating from the ancestrally exposed male germline [19]. Both long (> 200 nt) and small (< 200 nt) ncRNAs have been implicated as contributing to epigenetic transgenerational inheritance [19,20]. Long noncoding RNAs are hypothesized to maintain epigenetic memory by posttranscriptional regulation and to assist in regulating DNA methylation, chromatin remodeling, and histone modifications [19]. Small noncoding RNAs are known to regulate gene expression by affecting transcript stability and have been shown to be abundant in sperm with a single spermatozoon containing above 20,000 long and short noncoding RNAs [21,22]. Recently, concurrent alterations of DNA methylation, ncRNA, and histone alterations have been identified in sperm mediating the epigenetic transgenerational inheritance of pathology [23,24]. Therefore, the current study investigates alterations in both DNA methylation, ncRNA expression and gene expression.

This study was designed to investigate transgenerational changes to the epigenome of ovarian granulosa cells isolated from F3 generation rats after ancestral vinclozolin or DDT exposure compared to controls. Elucidation of the epigenetic and gene expression changes that occur in the ovary after ancestral exposure to an environmental toxicant provide novel insights into the molecular etiology of the epigenetic transgenerational inheritance of ovarian disease. In addition, this improves our understanding of the risk factors that must be considered when investigating the underlying etiology of ovarian disease in the human population.

Results

Ovarian pathology analysis

Pregnant F0 generation female rats were transiently exposed to vinclozolin, DDT or control vehicle (dimethyl sulfoxide, DMSO) from days 8–14 of

gestation, as described in Methods [25]. The *in utero* exposed offspring (F1 generation rats) were bred to produce the F2 generation, and similarly the F2 generation animals were bred to produce the transgenerational F3 generation. No sibling or cousin crosses were used to avoid inbreeding artifacts. Only the F0 generation rats received the experimental treatments. Granulosa cells were harvested from the ovaries of super-ovulated F3 generation females at 20–22 d of age. Granulosa cells were isolated and analyzed so as to characterize DNA methylation, mRNA gene expression and ncRNA expression as described in Methods. Additional F3 generation vinclozolin, DDT, and control lineage rats were aged to one year and their ovaries subjected to histopathological evaluation to detect signs of ovarian disease.

Ovaries were defined as diseased if there was a decrease in the number of primordial follicles at two standard deviations below those found in controls, and/or if there was an increase in the number of ovarian cysts at two standard deviations above those found in controls (see Methods), Supplemental Figure S1. There was a significant increase in ovarian disease in transgenerational F3 generation DDT and vinclozolin lineage rats at one year of age compared to F3 generation controls (Figure 1). Previous studies have shown that transgenerational increases in ovarian disease were detected following exposures to plastic derived compounds bisphenol A (BPA) and phthalates (DBT & DEHP) [26], dioxin (TCCD) [25], pesticides permethrin and DEET [27], jet fuel hydrocarbons [28], and methoxychlor [29], with nearly 100% disease frequency. Therefore, the transgenerational inheritance of increased ovarian disease can occur after exposure to a variety of environmental toxicants. There was no increase in ovarian disease in direct fetal exposed F1 or germline exposed F2 generation vinclozolin or DDT lineage rats compared to controls [30,31]. Therefore, as previously observed with most exposures, negligible ovarian disease is present following direct exposure [25,27–29], with the exception of BPA and phthalates [26]. This indicates that there was an epigenetic transgenerational increase in susceptibility to ovarian disease in rats ancestrally exposed to DDT or vinclozolin (Figure 1).

DNA methylation analysis

Differences in sites of DNA methylation (i.e., differential DNA methylation regions, DMRs) between F3 generation control, vinclozolin, and DDT lineage rats were characterized for ovarian granulosa cells using an MeDIP-Seq procedure comprized of methylated DNA immunoprecipitation (MeDIP) followed by next-generation

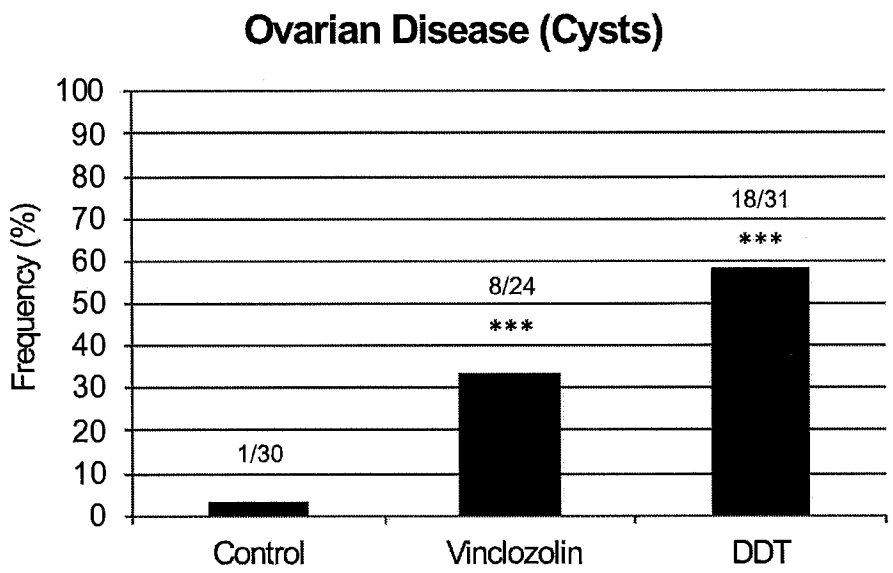

Figure 1. Ovarian pathology frequency. Transgenerational ovarian disease in F3 generation control, vinclozolin and DDT lineage rats at 1 y of age. Numbers for diseased individuals versus the total number of individuals analyzed is shown and (***) indicates statistical significance of $P < 7 \times 10^{-3}$ for vinclozolin and $P < 1 \times 10^{-5}$ for DDT by Fisher's Exact Test. Transgenerational ovarian disease frequency (i.e., presence of ovarian cysts) from control, vinclozolin, and DDT lineage rats at 1 y of age.

sequencing and bioinformatics techniques as described in Methods. A number of *P* value thresholds are assessed. In vinclozolin lineage granulosa cells compared to controls, there are 164 DMRs at a *P* value < 1 x 10^{-6}, of which 33 DMRs are comprized of multiple neighboring genomic windows (Figure 2(A)). A list of these DMRs is presented in Supplemental Table S1. In DDT lineage cells compared to controls there are 293 DMRs at a *P* value < 1 x 10^{-6}, of which 57 DMRs are comprized of multiple genomic windows (Figure 2(B)). A list of these DMRs is presented in Supplemental Table S2. Twenty-one DMRs overlapped between the vinclozolin and DDT lineages (Figure 2(C) and Supplemental Table S3). Chromosomal locations of the DMRs were examined. For vinclozolin lineage cells the DMRs are present on all chromosomes, while for DDT lineage cells the DMRs are present on all chromosomes except the small Y chromosome (Figure 3(A,B)). DMRs are not detected on the mitochondrial genome. The red arrowheads indicate the locations of the DMR and black boxes indicate clusters of DMRs.

Examination of the characteristics of the genomic sites where DMRs reside shows that for F3 generation vinclozolin lineage granulosa most DMRs are present in areas having on average of 1 or 2 CpG sites per 100 base pairs (Figure 4(A)). A CpG is a cytosine adjacent to a guanine on the DNA and it is primarily these cytosine bases that are methylated. For DDT lineage granulosa cells most DMRs are present in areas having on average of 1 to 3 CpG sites per 100 base pairs (Figure 4(C)). This indicates that most of the DMRs identified occur in areas of low CpG density, termed CpG deserts [32]. Most DMRs for both vinclozolin lineage and DDT lineage granulosa cells are shown to be one kilobase (kb) in length (Figure 4(B,D)). Within these 1 kb DMRs small clusters of CpG sites are anticipated to be regulatory as previously described [32].

Granulosa mRNA and noncoding RNA analysis

Differential gene expression and noncoding RNA expression between the granulosa control, DDT, and vinclozolin lineages were determined using RNA-seq as described in the Methods section. Differentially expressed RNAs were reported at a variety of different *P* value thresholds and a $P < 1 \times 10^{-4}$ was selected for subsequent analysis (Figure 5). Both DDT and vinclozolin lineage granulosa cells contained a similar number of differentially expressed mRNAs, while the vinclozolin lineage (Figure 5(A)) had more than twice the number of long noncoding RNAs (lncRNAs) than the DDT lineage (Figure 5(B)) at 123 vs. 51, respectively. In contrast, the DDT lineage contained twice the number of differentially expressed small noncoding RNAs (sncRNAs) at 1,085 compared to the vinclozolin lineage's 492. The classes of differentially expressed RNAs were compared between the two lineages. The 492 sncRNAs from the vinclozolin lineage had a very high degree of overlap with the 483 sncRNAs from the DDT lineage (Figure 5(C)). Eight lncRNAs were similar between the two lineages (Figure 5(D)), while 21 mRNAs were common between DDT and vinclozolin lineage granulosa cells (Figure 5(E)). In addition, the differentially expressed sncRNAs were categorized by class (Figure 6). Notably, piRNAs accounted for nearly all affected sncRNA in both lineages. The high number of common affected sncRNAs between the two lineages (Figure 5(C)) were observed.

The chromosomal locations of the differentially expressed RNAs are presented in Figures 7 and 8 for each RNA type. The vinclozolin lineage's sncRNAs showed a wide chromosomal distribution (Figure 7 (A)). Both the differentially expressed lncRNAs (Figure 7(B)) and the mRNAs (Figure 7(C)) for vinclozolin lineage granulosa are present on all chromosomes except for the Y chromosome and the mitochondrial chromosomes. There was no overlap of vinclozolin lineage DMR with any differentially expressed RNA, and no overlap of the sites of the different classes of differentially expressed RNAs with each other (Figure 7(D)). DDT lineage differentially expressed RNAs of all classes were also widely distributed across chromosomes excepting the Y and the mitochondrial chromosomes (Figure 8(A-C)). In addition, similar to what was seen in the vinclozolin lineage, the DDT lineage DMRs and differentially expressed RNAs had very few overlaps with each other (Figure 8(D)).

Genes and pathway associations

The genes associated with differentially expressed lncRNAs (Supplemental Tables S7 and S8) and

A Vinclozolin Transgenerational Granulosa DMRs

P-value	All Window	Multiple Window
0.001	12109	1587
1e-04	2433	317
1e-05	577	91
1e-06	**164**	**33**
1e-07	68	16

Number of windows	1	2	3	4
Number of DMR	131	24	7	2

B DDT Transgenerational Granulosa DMRs

P-value	All Window	Multiple Window
0.001	17166	3055
1e-04	3855	545
1e-05	1009	155
1e-06	**293**	**57**
1e-07	100	26

Number of windows	1	2	3	4	5	6	9	≥ 10
Number of DMR	236	34	8	6	2	2	1	4

C

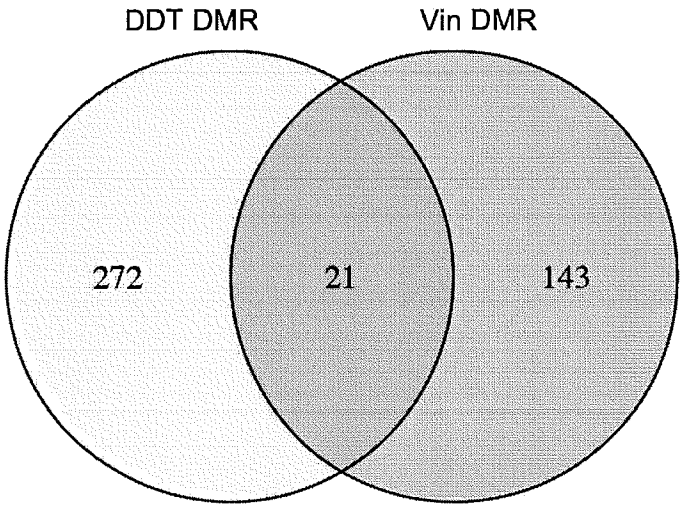

Figure 2. DMR identification. The number of DMRs found using different *P* value cutoff thresholds. The all window column shows all DMRs. The multiple window column shows the number of DMRs containing at least two significant windows. Lower table of each set shows the number of DMR having each specific number of significant windows at a $P < 1x10^{-6}$. (a) Granulosa cell vinclozolin F3 generation DMRs $P < 1x10^{-6}$. (b) Granulosa cell DDT F3 generation DMRs $P < 1x10^{-6}$.

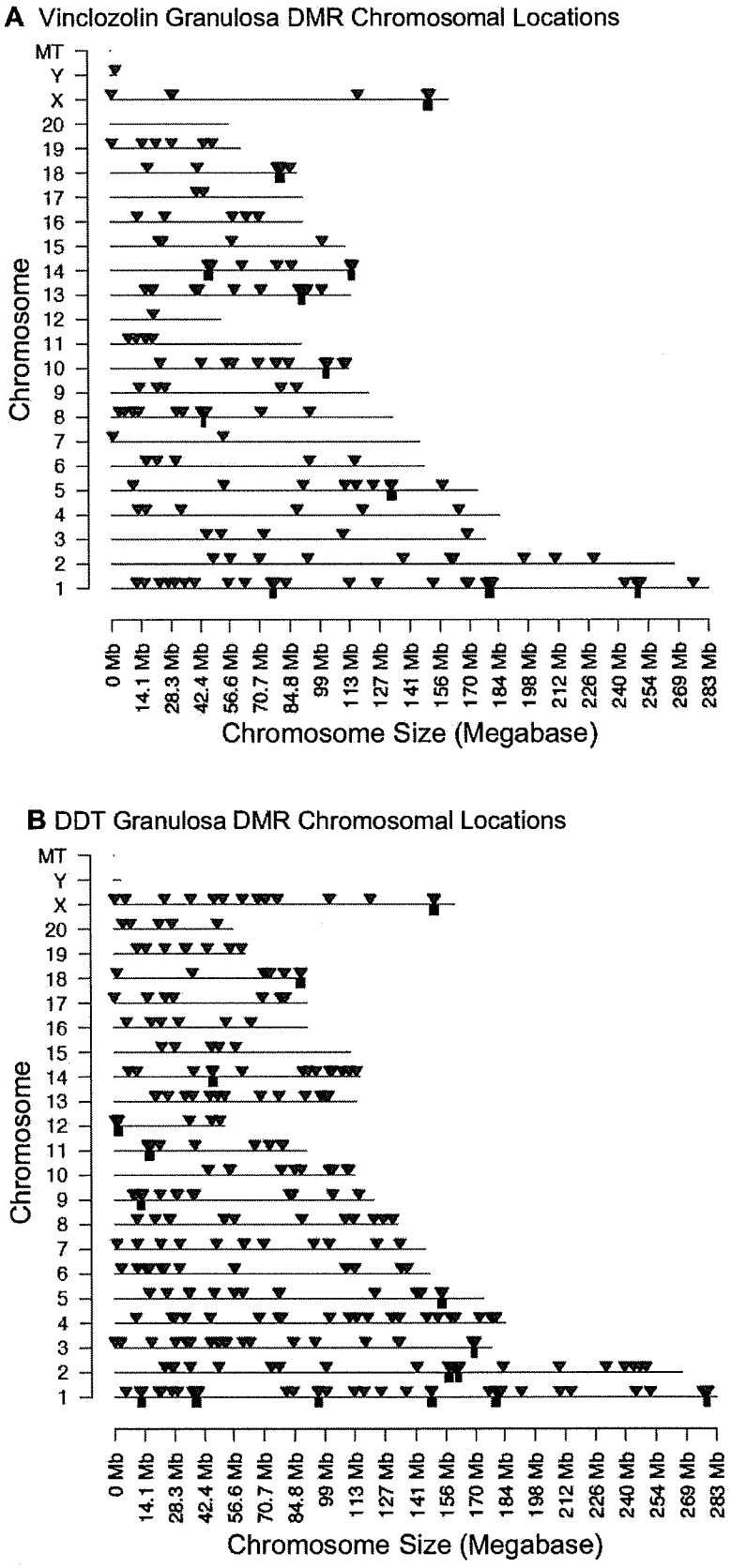

Figure 3. DMR chromosomal locations. The DMR locations on the individual chromosomes for all DMRs at a *P* value threshold of $< 1x10^{-6}$. (a) Vinclozolin F3 generation. (b) DDT F3 generation. Red arrowheads indicate positions of DMRs and black boxes indicate clusters of DMRs.

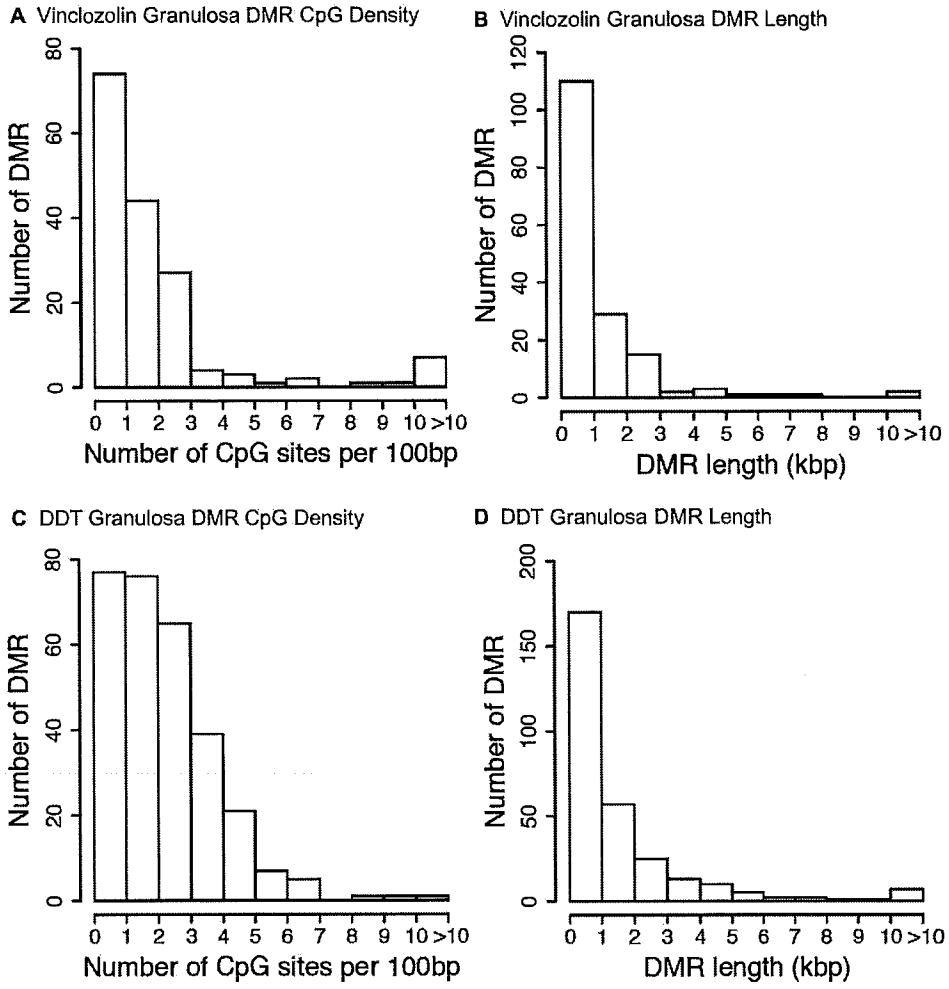

Figure 4. DMR genomic features. (a & c) The number of DMRs at different CpG densities for all DMRs at a p-value threshold of $P < 1x10^{-6}$. (b & d) The DMR lengths for all DMRs are at a P value threshold of $< 1x10^{-6}$. (a & b) Vinclozolin F3 generation. (c & d) DDT F3 generation.

mRNAs (Supplemental Tables S10 and S11) were functionally categorized as described in Methods. The most predominant functional categories for differentially expressed mRNA associated genes are presented in Figure 9(A,B). The top functional category for mRNA associated genes for both the vinclozolin and DDT lineages was transcription (Figure 9(A,B), respectively). Both lineages also had high numbers of differentially expressed genes associated with signaling. Some DMRs occurred in the vicinity (within 10 kb) of known genes, Supplemental Tables S1, S2, S3. These DMR associated genes were categorized and evaluated for potential function. The DDT lineage DMR associated genes were most often involved in signaling and receptor functions (Figure 9(C)) while the vinclozolin lineage DMR associated genes were highest in receptor, metabolism, and transcription functions.

The lists of differentially expressed DMRs and mRNAs are also compared to well-characterized physiological pathways in the KEGG database (http://www.kegg.jp/kegg/kegg2.html). Those pathways having the most DMR associated genes and differentially expressed mRNAs are presented in Figure 10(A,B). Metabolic pathways featured prominently, but since the KEGG metabolic pathway contains hundreds of genes the significance of this is unclear. The DDT lineage DMR associated genes occurred in cell adhesion, axon guidance, focal adhesion, specific signaling pathways and

A Vinclozolin Transgenerational Granulosa Differential Expressed RNA

P-value	Vin mRNA	Vin lncRNA	Vin sncRNA
0.001	439	293	1028
1e-04	174	123	492
1e-05	0	0	252
1e-06	0	0	123
1e-07	0	0	54

B DDT Transgenerational Granulosa Differential Expressed RNA

P-value	DDT mRNA	DDT lncRNA	DDT sncRNA
0.001	467	120	1914
1e-04	212	51	1085
1e-05	0	0	631
1e-06	0	0	370
1e-07	0	0	210

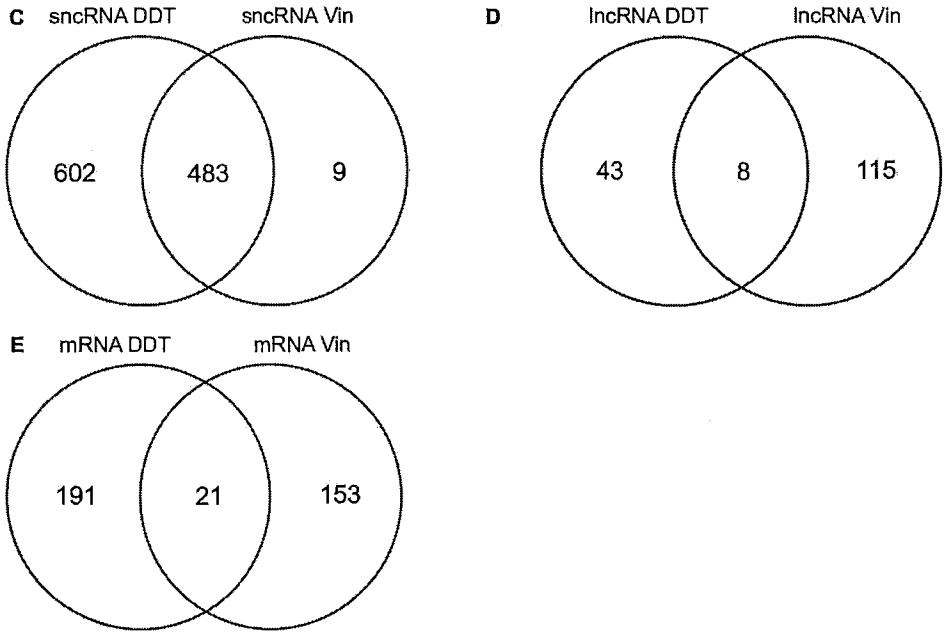

Figure 5. Differential RNA expression at different p-value thresholds for vinclozolin (a) and DDT (b). *P* value < $1x10^{-4}$ was used for subsequent analysis. Venn diagrams show overlap of RNA categories between the two lineages for (c) sncRNA, (d) lncRNA, and (e) mRNA.

several disease-associated pathways. Examination of the genes involved revealed a high proportion of somewhat general-purpose signaling molecules. The vinclozolin lineage DMR associated genes included three olfactory receptors present in the olfactory transduction pathway. The differentially expressed mRNAs were present primarily in specific signaling pathways and disease-associated pathways (Figure 10(C,D)). Differentially expressed vinclozolin lineage mRNAs included the growth factors kit ligand (Kitlg), bone morphogenetic protein 15 (Bmp15), growth differentiation factor 9 (Gdf9), and zona pellucida proteins 1–4 (Zp1, Zp2, Zp3, Zp4). Differentially expressed DDT lineage mRNAs included insulin-like growth factor 1 (Igf1), the receptors platelet derived

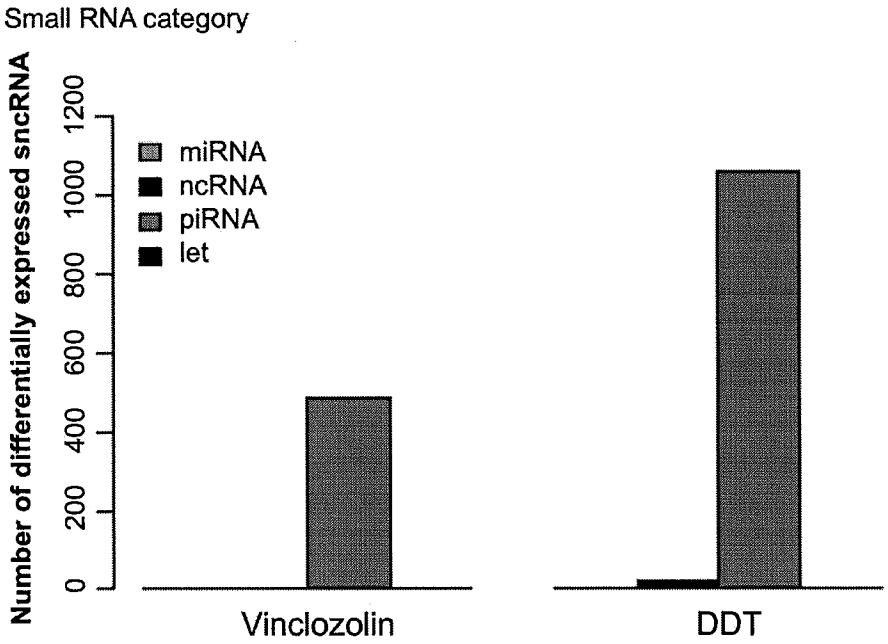

Figure 6. Differentially expressed small, noncoding RNA broken down by class and lineage. *P* value < 1 x 10^{-4} was used for analysis.

growth factor receptor a (Pdgfra), and growth hormone receptor (Ghr), and a selection of collagen and extracellular matrix genes (Col1a1, Col3a1, Col4a1, Col4a5, Col6a1, Col6a2, Col11a1, Itga9, Spp1). The majority of these genes have previously been shown to have functions in the ovary [33].

A final analysis correlated the transgenerational granulosa cell vinclozolin and DDT lineage gene associations with previously identified ovarian disease associated genes. Extensive reviews previously published have summarized the genes that have been associated with ovarian disease [34–41]. These published ovarian disease-associated genes were compiled into a list of 416 genes that are listed in Supplemental Table S13. An overlap of this published ovarian disease gene list with the transgenerational granulosa cell vinclozolin and DDT DMR associated genes (Figure 11(A)) and mRNA (Figure 11(B)) demonstrates several DMR associated genes and 20 mRNA genes overlapping. The specific overlapped genes are presented in Figure 11(C) and Table S14. Therefore, a number of ovarian disease-associated genes previously identified [34–41] were in common with the transgenerational granulosa cell DMRs and mRNA identified.

Discussion

Observations indicate that ancestral exposure to the environmental toxicants vinclozolin or DDT induced an epigenetic transgenerational increase in ovarian disease susceptibility in F3 generation rats. These results are in agreement with previous studies which also found transgenerational increases in susceptibility to ovarian diseases after exposure of F0 generation pregnant rats to vinclozolin [15] or DDT [16]. Experimental exposure of pregnant rats to other environmental toxicants such as jet fuel hydrocarbons, the plastics compounds bisphenol A (BPA) and phthalates, the pesticides permethrin and methoxychlor, and the industrial pollutant dioxin have also been shown to promote a transgenerational increase in ovarian disease [15,42]. This suggests that the ovary may be particularly sensitive to transgenerational epigenetic perturbations that disrupt somatic cell gene expression. Interestingly, these earlier studies demonstrated that the F1 generation direct fetal exposure did not induce ovarian disease later in life (1 y of age), but did promote ovarian disease in the transgenerational F3 generation at 1 y of age [15,42]. The exception was BPA and phthalate exposure that did promote ovarian diseases in both the F1 and F3 generations. In the current study, we also found negligible ovarian disease

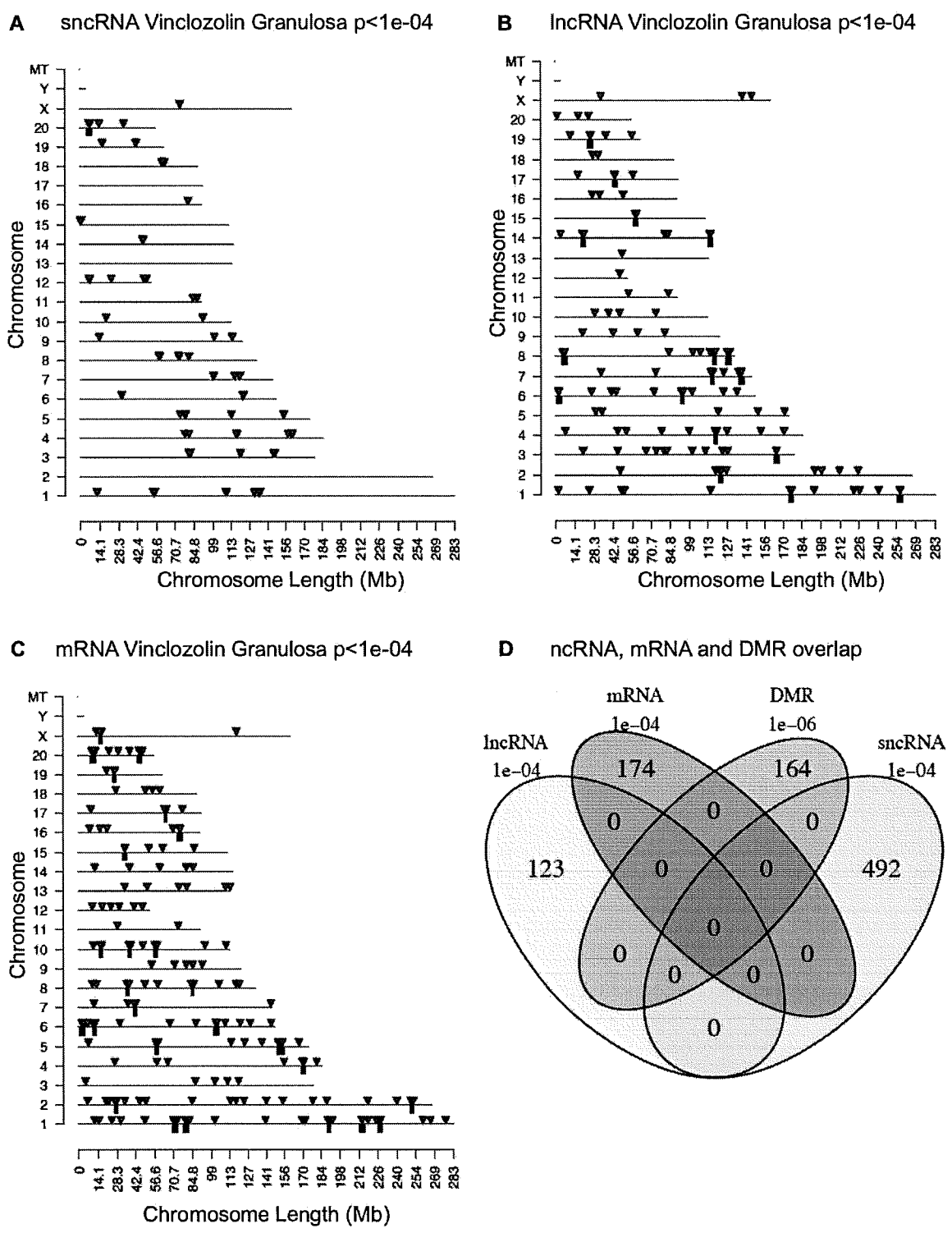

Figure 7. Differentially expressed RNAs from the vinclozolin lineage. Chromosomal locations of differentially expressed sncRNA (a), lncRNA (b), or mRNA (c). Individual RNAs are shown as red arrows and clusters are shown as black boxes. RNAs with unknown locations are not shown. (d) Venn diagram showing overlap of all differentially expressed epigenetic modifications from the vinclozolin lineage.

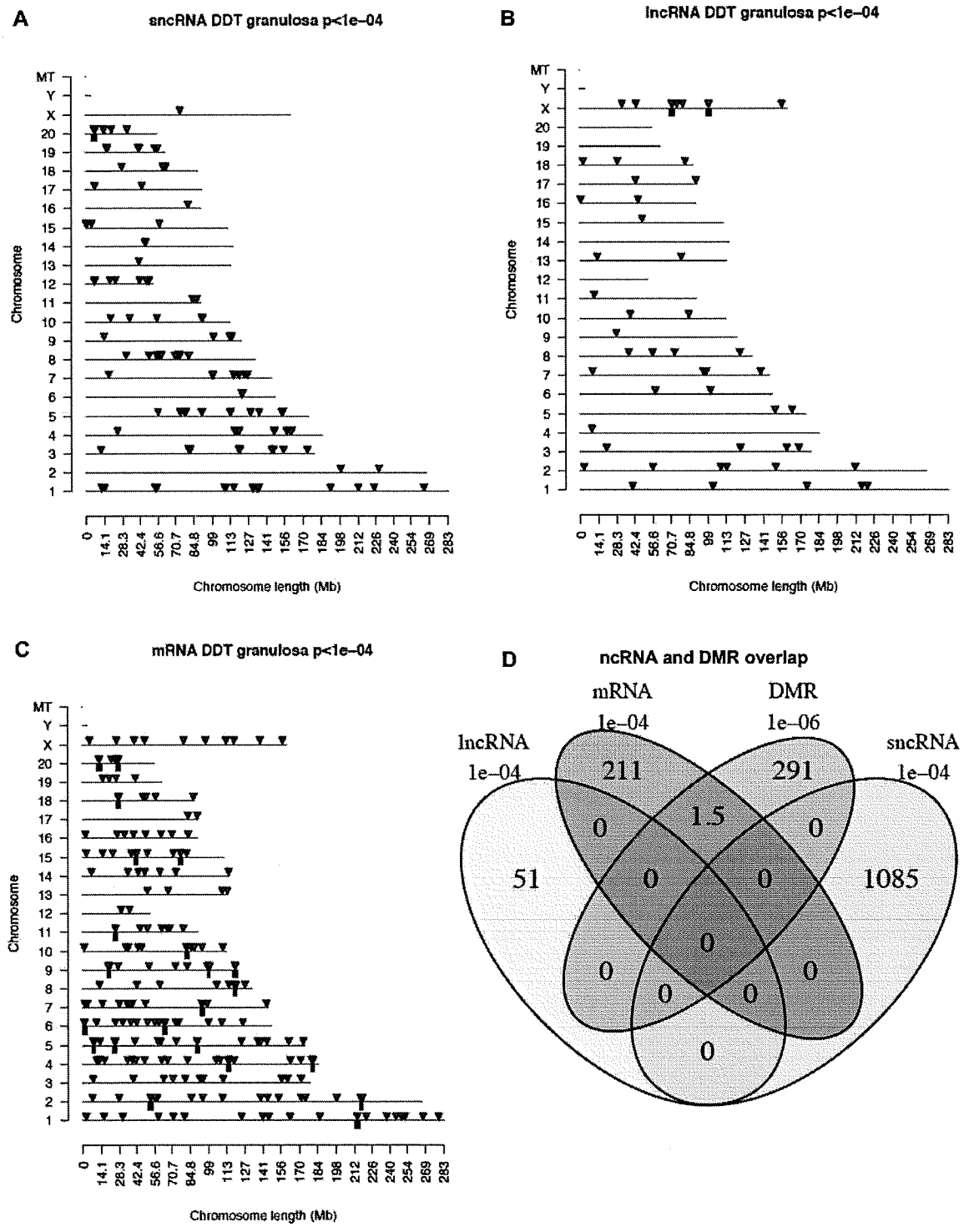

Figure 8. Differentially expressed RNAs from the DDT lineage. Chromosomal locations of differentially expressed sncRNA (a), lncRNA (b), or mRNA (c). Individual RNAs are shown as red arrows and clusters are shown as black boxes. RNAs with unknown locations are not shown. (d) Venn diagram showing overlap of all differentially expressed epigenetic modifications from the DDT lineage.

in the F1 generation, but significant ovarian disease in the F3 generation, Figure 1. When a gestating female is exposed the F0 generation female, the F1 generation fetus, and the germline within the F1 generation fetus

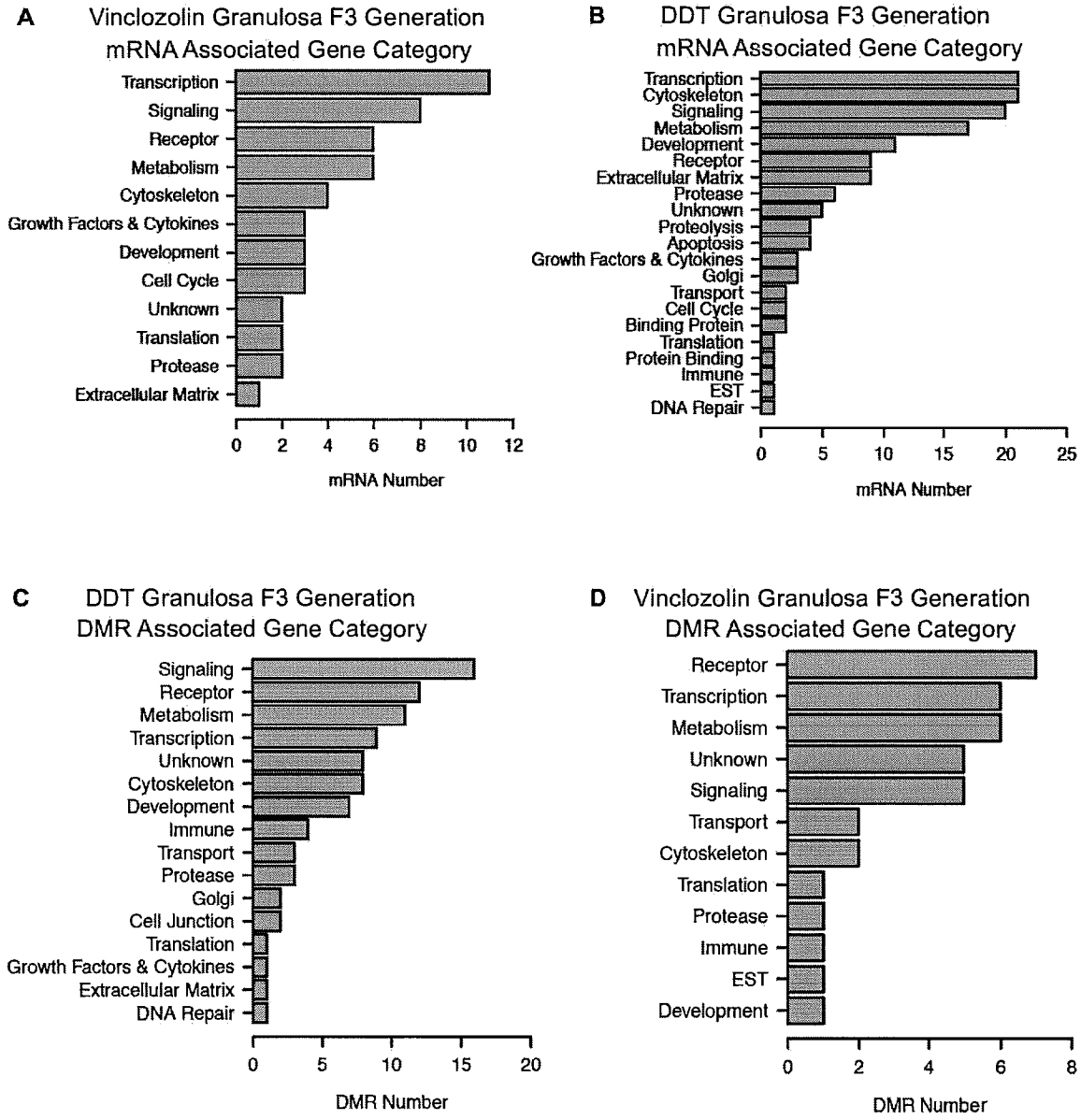

Figure 9. Differentially expressed epigenetic modifications were broken down by predicted associated gene category. Top row: mRNAs from vinclozolin (a) and DDT (b) lineages. Bottom row: DMRs from DDT (c) and vinclozolin (d). Genes or DMRs that could not be assigned a category are not shown.

that will generate the F2 generation are directly exposed to the environmental factor. Therefore, the first transgenerational generation is the F3 generation which has no direct exposure [43]. The direct exposure toxicology involves a signal transduction event and cellular response. The transgenerational molecular mechanism is distinct and involves the germline (sperm or egg) having an altered epigenome that following fertilization may modify the embryonic stem cells epigenome and transcriptome. This subsequently impacts the epigenetics and transcriptome of all somatic cell types derived from these stem cells [43]. Therefore, all somatic cells in the transgenerational animal have altered epigenomes and transcriptomes and those sensitive to this alteration will be susceptible to develop disease. Therefore, the F3

(A) DDT DMR Associated Pathways
1- rno01100 Metabolic pathways (8)
2 - rno04514 Cell adhesion molecules (CAMs) (5)
3 - rno05168 Herpes simplex infection (5)
4 - rno04360 Axon guidance (5)
5 - rno05200 Pathways in cancer (5)
6 - rno04010 MAPK signaling pathway (4)
7 - rno04510 Focal adhesion (4)
8 - rno04151 PI3K-Akt signaling pathway (4)
9 - rno04015 Rap1 signaling pathway (3)
10 - rno05321 Inflammatory bowel disease (IBD) (3)

(B) Vinclozolin DMR Associated Pathways
1 - rno04740 Olfactory transduction (3)
2 - rno01100 Metabolic pathways (2)
3 - rno04714 Thermogenesis (2)
4 - rno04530 Tight junction (2)
5 - rno04380 Osteoclast differentiation (2)

(C) DDT mRNA Associated Pathways
1 - rno04151 PI3K-Akt signaling pathway (12)
2 - rno04510 Focal adhesion (rat) (11)
3 - rno04926 Relaxin signaling pathway (8)
4 - rno05165 Human papillomavirus infection (8)
5 - rno04974 Protein digestion and absorption (8)
6 - rno05166 HTLV-I infection (7)
7 - rno04512 ECM-receptor interaction (7)
8 - rno04933 AGE-RAGE signaling pathway in diabetic complications (6)
9 - rno05410 Hypertrophic cardiomyopathy (HCM) (6)
10 - rno01100 Metabolic pathways (6)

(D) Vinclozolin mRNA Associated Pathways
1 - rno05166 HTLV-I infection (5)
2 - rno04010 MAPK signaling pathway (4)
3 - rno04380 Osteoclast differentiation (4)
4 - rno04151 PI3K-Akt signaling pathway (4)
5 - rno05200 Pathways in cancer (4)
6 - rno05031 Amphetamine addiction (3)
7 - rno04668 TNF signaling pathway (3)
8 - rno05418 Fluid shear stress and atherosclerosis (3)
9 - rno04657 IL-17 signaling pathway (3)
10 - rno05161 Hepatitis B (3)

Figure 10. Associated gene pathways for DMRs (a) DDT and (b) vinclozolin lineages and for mRNA (c) DDT and (d) vinclozolin lineages.

generation can have disease while the F1 and F2 generations do not, due to this difference in the molecular mechanisms involved. When disease is observed in the F1, F2 and F3 generations then the direct exposure

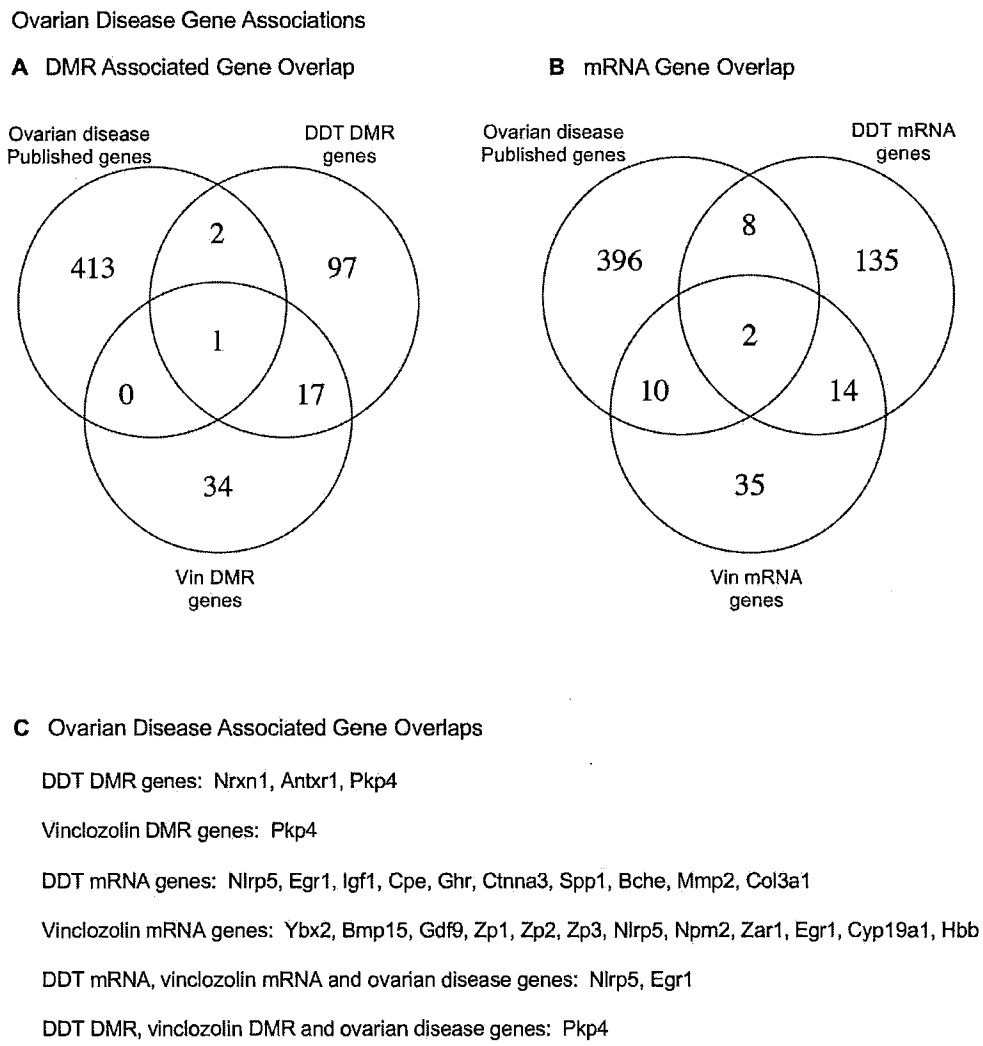

Figure 11. Ovarian disease gene associations. (a) DMR associated gene overlap with published ovarian disease genes. (b) mRNA gene overlaps with published ovarian disease genes. (c) Specific ovarian disease associated gene overlap with DMR associated genes and mRNA genes.

actions and transgenerational actions have similar physiological responses, shown with BPA and phthalate induced ovarian disease [42].

Changes in DNA methylation were observed in F3 generation vinclozolin and DDT lineage granulosa cells compared to the control lineage. The sites of these DMRs were in genomic regions of relatively low CpG density 'CpG deserts' [32]. This finding is consistent with previous work in which transgenerational DMR in sperm were most often found in regions of low CpG density after ancestral toxicant exposure [25–29]. A previous study has also examined changes in DNA methylation in granulosa cells after ancestral exposure to vinclozolin [15]. This investigation used granulosa cells from 5–6-month-old F3 generation rats from vinclozolin and control lineages analyzed using a methylated DNA immunoprecipitation procedure (MeDIP) followed by a gene promoter microarray chip analysis. Similar to the current genome-wide analysis, there were DMRs identified in the vinclozolin lineage granulosa cells compared to controls [15]. In the current investigation the granulosa cells were isolated from 20-day-old rats which is prior to the onset of any clinical signs of ovarian disease. The current study used next-

generation sequencing analysis which allows for a genome-wide investigation of the F3 generation vinclozolin and DDT lineage granulosa cells.

Changes in DNA methylation can affect genome activity and gene expression in concert with other epigenetic factors. DMRs were found in granulosa cells that were associated (within 10 kb) with genes, raising the possibility that these genes might be epigenetically regulated. An investigation of the putative functions of DMR associated genes revealed signaling, transcription, receptor and cytoskeleton genes to be predominant. These classes of genes are important for the interactions between granulosa cells and either oocytes or theca cells that are necessary for normal ovary function. Dysregulation of these functions may promote ovarian disease. In the current study there was limited correspondence between DMR associated genes and differential mRNA expression. However, the differentially expressed mRNAs were evaluated in granulosa cells collected from the healthy ovaries of young animals. The epigenetic changes observed may as the animals age activate associated gene expression to promote the dysregulation and increase disease susceptibility later in life. Alternatively, the DMR epimutations can influence distal gene expression through ncRNA.

Examination of the noncoding RNAs showed that F3 generation vinclozolin and DDT lineage granulosa cells differed in their classes of differentially expressed ncRNAs altered. The vinclozolin lineage had fewer differentially expressed lncRNAs and more sncRNAs when compared to the DDT lineage. Surprisingly, there was a significant overlap between the differentially expressed sncRNAs of each lineage accounting for 98% of the sncRNAs of the vinclozolin lineage and 45% of the DDT lineage. The differentially expressed sncRNAs of the vinclozolin lineage are a subset of those of the DDT lineage. The significance of this is unclear and warrants further investigation. However, looking into the functions of differentially expressed ncRNAs and mRNAs that are in common between the vinclozolin and DDT lineage granulosa cells may shed light on the underlying causes of the disease phenotypes.

Several growth factor and receptor mRNAs that were differentially expressed in F3 generation vinclozolin or DDT lineage granulosa cells have been previously implicated in normal ovarian function (Kitlg, Bmp15, GDF9, Pdgfra) [44–47]. In addition, abnormalities in Gdf9 and BMP15 expression are associated with polycystic ovarian syndrome (PCOS) and primary ovarian insufficiency (POI) in humans [48–50]. The receptor Scarb1 was differentially expressed in DDT lineage granulosa and has been associated with POI [51]. The growth factor IGF1 and the receptors Pdgfra and Ghr were also differentially expressed in the DDT lineage and have been implicated in PCOS [52–54]. Therefore, differentially expressed genes observed in the F3 generation of vinclozolin and DDT lineage granulosa cells have been correlated with PCOS and POI.

Correlations of genes previously identified to be associated with ovarian disease [34–41] (Supplemental Table S13) with the transgenerational granulosa cell genes of this study identified a number of genes associated with ovarian disease, Figure 11 and Supplemental Table S14. A gene that was present in the DDT and vinclozolin DMR associated genes and ovarian disease associated genes was Pkp4, plakophilins 4 [34,55]. The mRNA genes that were present in the DDT and vinclozolin mRNA genes and ovarian disease associated genes were Nlrp5 and Egr1 [34,56,57]. The Nlrp5 is associated with mitochondrial function in oocytes and embryo [56]. The Egr1 is associated with granulosa cell apoptosis during atresia through the NF-KB pathway [57]. The majority of transgenerational granulosa cell DMR associated genes and differentially expressed mRNA were not in common with these previously identified ovarian disease associated genes [34–41]. A more complete list of ovarian disease genes would likely have greater overlap. Observations demonstrate some genes previously shown to be involved in ovary disease are similar to the transgenerational granulosa cell associated genes identified.

The chromosomal locations for differentially expressed RNAs and DMRs for both vinclozolin and DDT lineages are generally genome-wide. There was a marked lack of overlap between the different epimutations in either lineage. It will become important to determine the gene targets of these epimutations to establish the mechanism

behind the granulosa associated transgenerational disease. Interestingly, the epimutations and gene expression differences observed are present in granulosa cells in the late pubertal female rats at 22–24 d of age, which is long before any visible signs of ovarian disease are detectable. This indicates that the underlying factors that can contribute to adult-onset diseases like PCOS and POI appear to be present early in life. This helps explain the molecular mechanisms behind the developmental origins of ovarian disease.

In summary, these studies show that exposure to the environmental toxicants vinclozolin and DDT can promote the epigenetic transgenerational inheritance of ovarian disease susceptibility. Granulosa cells from young F3 generation vinclozolin and DDT lineage animals had epigenetic changes in DNA methylation and ncRNA expression, as well as in mRNA gene expression. These changes appear to contribute to the dysregulation of the ovary that can promote later life disease susceptibility. Future studies will need to translate these observations to investigate similar mechanisms in human females with POI or PCOS. Ancestral exposure to toxicants is now a risk factor that must be considered when investigating the underlying causes of ovarian disease in the human population.

Methods

Animal studies and breeding

Female and male rats of an outbred strain Hsd: Sprague Dawley®™SD®™ (Harlan) at about 70 to 100 d of age were fed ad lib with a standard rat diet and ad lib tap water for drinking. To obtain time-pregnant females, the female rats in proestrus were pair-mated with male rats. The sperm-positive (day 0) rats were monitored for diestrus and changes in body weight. If pregnant, then on days 8 through 14 of gestation [58], the females were administered daily intraperitoneal injections of vinclozolin (100 mg/kg BW/day, Chem Services, Westchester, PA), DDT (dichloro-diphenyl-trichloroethane) (25 mg/kg BW/day, Chem Services), or dimethyl sulfoxide (vehicle) as previously described [42]. Treatment groups were designated 'vinclozolin', 'DDT' and 'control' lineages. The gestating female rats treated were considered to be the F0 generation. The offspring of the F0 generation rats were the F1 generation. Non-littermate females and males aged 70–90 days from F1 generation lineages were bred to obtain F2 generation offspring. The F2 generation rats were bred to obtain F3 generation offspring. Only the pregnant F0 generation rats were treated directly with vinclozolin or DDT. All experimental protocols for the procedures with rats were pre-approved by the Washington State University Animal Care and Use Committee (IACUC approval # 06252).

Histopathology and ovarian disease classification

Rats at 12 months of age were euthanized by CO_2 inhalation and cervical dislocation for tissue harvest. Ovaries were removed and fixed in Bouin's solution (Sigma) followed by 70% ethanol, then processed for paraffin embedding by standard procedures for histopathological examination. Tissue sections (5 μm) were cut and every 30th section was collected and hematoxylin/eosin stained.

The three stained sections (150 μm apart) through the central portion of the ovary with the largest cross-section were evaluated microscopically for number of primordial follicles, small cystic structures and large cysts, as previously described [15]. The mean number of each evaluated structure per section was calculated across the three sections. Follicles had to be non-atretic and have the oocyte nucleus visible in the section in order to be counted. Primordial follicles are in an arrested state and have an oocyte surrounded by a single layer of either squamous or both squamous and cuboidal granulosa cells [59]. Cysts were defined as fluid-filled structures of a specified size that were not filled with red blood cells and which were not follicular antra. A single layer of cells may line cysts. Small cysts were 50–250 μm in diameter measured from the inner cellular boundary across the longest axis. Large cysts were greater than 250 μm in diameter. A cut-off was established to declare a tissue 'diseased' based on the mean number of histopathological abnormalities plus two standard deviations from the mean of control tissues as assessed by each of three individual

observers blinded to the treatment groups. This number was used to classify rats into those with and without ovarian disease in each lineage. A rat tissue section was finally declared 'diseased' only when at least two of three observers marked the same tissue section 'diseased' for the same type of abnormality. Results were expressed as the proportion of affected animals and were analyzed using Fisher's exact test.

Granulosa cell isolation

F3 generation rats from vinclozolin, DDT and control lineages were treated with Pregnant Mare Serum Gonadotropin (Sigma cat, St. Louis, MO) (10 IU PMSG injected IP) at 20–22 d of age. Two days later animals were sacrificed and ovaries removed. The ovarian bursa and its adherent fat were removed from each ovary and the ovaries processed for granulosa cell collection [60]. The ovaries were suspended in Ham's F-12 base medium (Thermo Scientific, Waltham, MA). Following sequential 30-minute incubations at 37 °C in 6 mM EGTA in F-12 (to decrease Ca2+ – mediated cell adhesion) and then 0.5 M sucrose in F-12 (to increase osmotic pressure within follicles), ovaries were returned to F-12. Granulosa cells were released into the medium from antral follicles using 30-gauge needles and gentle pressure. Oocytes were removed by aspiration under a dissecting microscope. Granulosa cells from 4–9 rats from the same treatment group were pooled and collected into 1.5 ml tubes, allowed to settle for 10 minutes and the supernatant discarded. Three pools of granulosa cells were prepared from different animals and ovaries for each treatment group. Samples were stored at −70° until the time of RNA and DNA isolation.

DNA isolation

The granulosa cell pellet was resuspended in 100 µl PBS and then mixed with 820 µl DNA extraction buffer. Then 80 µl proteinase K (20 mg/ml) was added and the sample was incubated at 55°C for 2 hours under constant rotation. Then 300 µl of protein precipitation solution (Promega, Madison, WI, Genomic DNA Purification Kit, A795A) were added, the sample mixed thoroughly and incubated for 15 min on ice. The sample was centrifuged at 17,000xg for 20 minutes at 4°C. One ml of the supernatant was transferred to a 2 ml tube and 2 µl of Glycoblue (Thermo-Fisher AM9515) and 1 ml of cold 100% isopropanol were added. The sample was mixed well by inverting the tube several times then left in −20°C freezer for at least one hour. After precipitation, the sample was centrifuged at 17,000 x g for 20 min at 4°C. The supernatant was taken off and discarded without disturbing the (blue) pellet. The pellet was washed with 70% cold ethanol then centrifuged for 10 min at 4°C at 17,000 x g and the supernatant discarded. The pellet was air-dried at room temperature (about 5 minutes). The pellet was then resuspended in 100 µl of nuclease free water and DNA concentration determined on a NanoDrop.

Methylated DNA Immunoprecipitation (MeDIP)

Methylated DNA Immunoprecipitation (MeDIP) with genomic DNA was performed. The genomic DNA was sonicated to fragment using the Covaris M220. Granulosa cell genomic DNA was diluted to 130 µl with TE buffer (10 mM Tris HCl, pH7.5; 1 mM EDTA) and put into a Covaris tube. The Covaris was set to the 300 bp program and 10 µl of each sonicated DNA was run on 1.5% agarose gel to verify fragment size. The remaining DNA was diluted with TE buffer to 400 µl, heat-denatured for 10min at 95°C, then immediately cooled on ice for 10 min. Then 100 µl of 5X IP buffer and 5µg of antibody (monoclonal mouse anti 5-methyl cytidine; Diagenode #C15200006) were added, and the DNA-antibody mixture was incubated overnight with rotation at 4°C.

The following day 50µl of pre-washed anti-mouse magnetic beads (Dynabeads M-280 Sheep anti-Mouse IgG; Life Technologies 11201D) were added to the DNA-antibody mixture, then incubated for 2 h on a rotator at 4°C. The DNA-antibody-bead mixture was placed into a magnetic rack for 1–2 minutes and the supernatant discarded, then the pellet washed with 1x IP buffer 3 times. The washed bead mixture was then resuspended in 250 µl digestion buffer (5 mM Tris PH8, 10 mM EDT4, 0.5% SDS) with 3.5 µl Proteinase K (20 mg/ml) added. The sample was then incubated for 2–3 hours on a rotator at 55°. Buffered Phenol-

Chloroform-Isoamyl alcohol solution was added (250 µl) to the sample and the tube, vortexed for 30 sec, then centrifuged at 17,000 x g for 5 min at room temperature. The aqueous supernatant was carefully removed and transferred to a fresh microfuge tube. Then, 250 µl chloroform were added to the supernatant from the previous step, vortexed for 30 sec and centrifuged at 17,000 x g for 5 min at room temperature. The aqueous supernatant was removed and transferred to a fresh microfuge tube. To the supernatant 2 µl of Glycoblue (20 mg/ml) (Invitrogen AM9516), 20 µl of 5 M NaCl and 500 µl 100% ethanol were added and mixed well, then precipitated at −20°C for > 1 hour.

The DNA precipitate was centrifuged at 17,000 x g for 20 min at 4°C and the supernatant removed. The pellet was washed with 500 µl cold 70% ethanol and incubated at −20°C for 15 min. then centrifuged again at 17,000 x g for 5 min at 4° C and the supernatant discarded. The pellet was air-dried at room temperature (about 5 min), then resuspended in 20 µl H_2O or TE. DNA concentration was measured using a Qubit (Life Technologies) with ssDNA kit (Molecular Probes Q10212).

MeDIP-seq analysis

The MeDIP DNA was used to create libraries for next generation sequencing (NGS) using the NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB #E7530S) (San Diego, CA) starting at step 1.4 of the manufacturer's protocol to generate double stranded DNA. After this step the manufacturer's protocol was followed. Each pool or individual sample received a separate index primer. NGS was performed at WSU Spokane Genomics Core using the Illumina HiSeq 2500 with a PE50 application, with a read size of approximately 50 bp and approximately 45 million reads per pool. Five to six libraries were run in one lane.

RNA isolation and sequencing

Granulosa cell pellets were stored in 1.2 ml of Trizol reagent (Thermo Fisher) at − 80°C until use. Total RNA was extracted using Trizol reagent following the manufacturer's protocol with one exception: during RNA precipitation, 1 ml of isopropanol was added to recover small RNAs. RNA was eluted in 50 µL of water and 0.5 µl murine RNase inhibitor (NEB) was added. The final RNA concentration was determined using the Qubit RNA High Sensitivity Assay Kit (Thermo Fisher), and quality control analysis was performed using an RNA 6000 Pico chip on the Agilent 2100 Bioanalyzer.

Large RNA libraries (noncoding and messenger RNA) were constructed using the KAPA RNA HyperPrep kit with RiboErase according to the manufacturer's instructions with some modifications. NEBNext Multiplex Oligos for Illumina was used for the adaptor and barcodes. Libraries were incubated at 37°C for 15 minutes with the USER enzyme (NEB) before the final amplification. qPCR was used to determine cycle number with the KAPA RealTime Library Amplification Kit. Size selection (200–700 bp) was done using KAPA Pure beads. Quality control analysis was done with the Agilent DNA High Sensitivity chip and final concentration was determined with the Qubit dsDNA high sensitivity assay. Pooled libraries+ were sequenced with paired-end 100 bp sequencing on the Illumina HiSeq 4000 sequencer.

Small RNA libraries were constructed with the NEBNext Multiplex Small RNA Library Prep Set for Illumina and were barcoded with NEBNext Multiplex Oligos for Illumina. Purification and size selection were done with the KAPA Pure beads following the protocol. An additional size selection (115–160 bp) was performed using the Pippin Prep 3% gel with marker P (Sage Science). Concentration was determined using the Qubit dsDNA high sensitivity assay (Thermo Fisher) and quality control was done with Agilent DNA High Sensitivity Chip. Libraries were pooled and concentrated using KAPA Pure beads (2.2X), and sequenced with a custom sequencing primer: 5-ACA CGT TCA GAG TTC TAC AGT CCG A-3 on the Illumina HiSeq 4000 sequencer (single-end 50 bp).

DMR statistics and bioinformatics

The basic read quality was verified using summaries produced by the FastQC program. The new

data was cleaned and filtered to remove adapters and low-quality bases using Trimmomatic [61]. The reads for each MeDIP sample were mapped to the Rnor 6.0 rat genome using Bowtie2 [62] with default parameter options. The mapped read files were then converted to sorted BAM files using SAMtools [63]. To identify DMRs, the reference genome was broken into 100 bp windows. The MEDIPS R package [64] was used to calculate differential coverage between control and exposure sample groups. The edgeR *P* value [65] 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 DMRs. The DMR edges were extended until no genomic window with an edgeR p-value less than 0.1 remained within 1000 bp of the DMR. CpG density and other information was then calculated for the DMR based on the reference genome. DMR clusters were identified as previously described [66].

DMRs were annotated using the biomaRt R package [67] to access the Ensembl database [68]. The genes that overlapped with DMR were then input into the KEGG pathway search [69,70] to identify associated pathways. The DMR associated genes were then sorted into functional groups by consulting information provided by the DAVID [71], Panther [72], and Uniprot databases incorporated into an internal curated database (www.skinner.wsu.edu under genomic data).

All molecular data has been deposited into the public database at NCBI (GEO # GSE118381 and SRA # PRJNA472849) and R code computational tools available at GitHub (https://github.com/skinnerlab/MeDIP-seq) and www.skinner.wsu.edu.

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Disclosure statement

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Feature Review

Epigenetic Transgenerational Inheritance of Obesity Susceptibility

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The prevalence of obesity and associated diseases has reached pandemic levels. Obesity is often associated with overnutrition and a sedentary lifestyle, but clearly other factors also increase the susceptibility of metabolic disease states. Ancestral and direct exposures to environmental toxicants and altered nutrition have been shown to increase susceptibility for obesity and metabolic dysregulation. Environmental insults can reprogram the epigenome of the germline (sperm and eggs), which transmits the susceptibility for disease to future generations through epigenetic transgenerational inheritance. In this review, we discuss current evidence and molecular mechanisms for epigenetic transgenerational inheritance of obesity susceptibility. Understanding ancestral environmental insults and epigenetic transgenerational impacts on future generations will be critical to fully understand the etiology of obesity and to develop preventative therapy options.

Highlights

The prevalence of obesity has increased dramatically over the past 30 years, and cannot be explained by genetics, diet, and exercise alone.

A variety of early life and *in utero* exposures to environmental insults can change metabolic outcomes through developmental epigenetic reprogramming.

Epigenetic transgenerational inheritance of obesity has been observed following ancestral exposure to a high-fat diet, malnutrition, and several environmental toxicants.

Unique obesity-specific sperm epimutation signatures have been identified in the transgenerational F3 generation of animals ancestrally exposed to environmental toxicants.

Numerous genes modified by DNA methylation in a variety of phenotypes and ancestral exposures have been found to be potential novel modulators of adipocyte (fat cell) metabolism and function.

Origins of the Obesity Epidemic

Obesity is rapidly increasing in prevalence worldwide, and has become a public health crisis of pandemic proportions. In 2016, 650 million adults over the age of 18 were considered to be obese, with an overall prevalence of 13%[i]. Within the USA, almost 40% of adults and 18.5% of children are obese [1]. The worldwide prevalence of obesity in adults tripled between 1975 and 2016, and the next generation of children are strongly affected. Global obesity rates in children increased from under 1% in 1975 to 6% in girls, and 8% in boys for 2016[i]. Obesity is defined as a body mass index (BMI) of ≥30 kg/m^2, and is associated with several pathologies, including type 2 diabetes, cardiovascular disease, osteoarthritis, nonalcoholic fatty liver disease, kidney disease, and certain cancers [2]. Sixty five percent of the world lives in a country where obesity has a higher mortality rate than malnutrition[ii]. Elevated BMI contributed to 4 million deaths in 2015, and has an estimated global annual cost of US$2 trillion[iii] [3]. Each five-unit increase in BMI above 25 kg/m^2 also increases the overall mortality risk by 29% [4]. Given the public health consequences of obesity, it is imperative to investigate the etiology and pathogenesis of the disease.

There have been several proposed etiologies for the obesity pandemic. Traditionally, obesity has been attributed to overnutrition and a sedentary lifestyle; however, over the past decade, it has become clear that additional factors are involved [5]. Between 1998 and 2006, BMI increased by 2.3 kg/m^2 on average in the USA when controlling for dietary intake and exercise levels [6]. In addition, Brown *et al.* [6] found that leisure time spent on exercise in fact increased between 1988 and 2006, indicating that other causes should be investigated [6]. Other potential direct causes of obesity proposed include changes in the gut microbiome [7], effects of air conditioning on thermogenesis [8], chronic sleep deprivation [9], and certain pharmaceutical drugs inducing weight gain [5]. Numerous genome-wide association studies (GWAS) have also been performed to identify a genetically based increased susceptibility to obesity; however, obesity-related genetic variants are limited in predictive power and only account for ~3% of BMI variance [10,11].

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In addition, while BMI is commonly used to assess obesity in population-wide studies, the accuracy of BMI in assessing metabolic and cardiovascular health has come into question [12], as discussed further in Box 1.

Epigenetics and the Developmental Origins of Health and Disease

Several recent studies have implicated that a variety of exposures in early life and *in utero* can change metabolism. Barker and colleagues made observations of this phenomenon with several epidemiological studies showing that infants born small for gestational age have an increased susceptibility to cardiovascular disease and metabolic dysfunction [13,14]. The 'thrifty phenotype hypothesis' was then proposed from observations of increased adiposity and decreased fat mobilization following poor fetal nutrition [15]. Environmental insults during early development can influence cellular plasticity, thereby increasing the risk of chronic diseases later in life,

Box 1. Challenges in the Assessment of Obesity and Disease Risk

According to the WHO[i], obesity is defined as excess fat accumulation that may impact health. Therefore, it is imperative that current methods of determining obesity accurately assess risk factors for cardiovascular and metabolic comorbidities. BMI is calculated by weight in kilograms divided by height in meters squared (Equation I) [2]:

$$BMI = weight/height^2 \quad [I]$$

Although a BMI of ≥30 kg/m^2 is often used as a threshold to define obesity [2], there are potential weaknesses in exclusively utilizing height and weight as anthropometric measurements. For example, BMI does not compensate for body composition differences associated with age, sex, or ethnicity [124–126]. Specifically, the phenotype of individuals with the same BMI is highly heterogeneous, with vast differences in the anatomical distribution of fat as well as cardiometabolic health [127].

Recently, several attempts have been made to formulate novel anthropometric measures that more accurately predict cardiovascular and metabolic risk. The body adiposity index (BAI) attempts to measure the percentage of body fat by using a modified ratio of hip circumference to height (Equation II) [128]:

$$BAI = hip/height^{1.5} \quad [II]$$

The Clínica Universidad de Navarra-Body Adiposity Estimator (CUN-BAE) similarly attempts to estimate total body fat percentage by using a modified BMI equation that accounts for both age and sex as risk factors [129]. Both equations are built on the principle that excess adiposity, as opposed to excess body weight, correlates better to cardiometabolic risk; however, these measurements do not account for fat depot-specific risk. White adipose tissue is categorized as subcutaneous or visceral adipose tissue [130]. Subcutaneous adipose tissue lays directly under the skin, while visceral adipose tissue involves fat pads that lay deep in the abdomen and next to visceral organs [130]. Visceral adiposity has been linked to increased risk of cardiovascular and metabolic diseases compared with subcutaneous adiposity [131]. Therefore, it is important to consider visceral or abdominal adiposity as a measurement of cardiometabolic risk in a clinical setting.

Several cohorts have used waist circumference (WC) or waist-to-height ratio (WHtR) as alternative anthropometric measures for abdominal adiposity because they correlate significantly better with cardiovascular risk factors compared with BMI [12,132]. To further refine these measurements, a body shape index (ABSI) was developed to adjust WC for height and weight (Equation III) [133]:

$$ABSI = WC/BMI^{2/3}\ height^{1/2} \quad [III]$$

ABSI predicted mortality risk in different age, sex, and weight using a population data set from the USA, although the measurement had weaknesses in accuracy depending on ethnic group [133]. A recent review of 66 studies using 25 different novel anthropometric parameters including a variety of ethnic and socioeconomic demographics found that these new measurements were generally positively correlated with risk factors and disease outcomes [134]. The authors also cautioned that specific cut-off values may not be applicable to all populations and further research is needed to determine the accuracy of these measurements [134]. BMI should still be utilized in obesity studies due to ease of measurement and historical use to determine trends in obesity data. However, future cohorts should consider including various measurements being used in these novel anthropometric formulas, such as thigh, waist, and hip circumference, to account for the cardiometabolic risk associated with variance in fat deposition. Perhaps an epigenetic biomarker for obesity susceptibility and/or obesity will be developed in the future.

Glossary

DNA methylation: addition of a methyl group to a cytosine in a 5′-cytosine-phosphate-guanine-3′ (CpG) dinucleotide residue sequence to form 5-methylcytosine.
Epigenetic processes: DNA methylation, histone modification, ncRNAs, RNA methylation, and chromatin structure.
Epigenetic transgenerational inheritance: germline-mediated inheritance of epigenetic information between generations in the absence of continued direct environmental influences that leads to phenotypic variation.
Epigenetics: molecular factors and processes around the DNA that regulate genomic activity independent of DNA sequence, and are mitotically stable.
Epimutations: mitotically stable epigenetic alterations, such as an environmentally induced DNA methylation, at a specific CpG site.

including obesity and its comorbidities [14,16–18]. The epigenetic mechanisms behind this developmental reprogramming are discussed in Box 2.

One of the most relevant examples of this phenomenon comes from a cohort of patients with severe acute malnutrition between 1963 and 1993 at the University of the West Indies in Kingston, Jamaica, a population used to study the potential origins of malnutrition phenotypes. Severe malnutrition in childhood can lead to differing body composition comprising edematous (i.e., swollen with excessive accumulation of fluid) (kwashiorkor or marasmic kwashiorkor phenotype) or nonedematous (marasmus phenotype). In the Jamaican population, children who developed kwashiorkor had increased birthweight compared with children with marasmus, which implies that early developmental factors may contribute to the different phenotypes from the same nutritional stressor [19]. The individuals in the study who experienced either marasmus or kwashiorkor were followed into adulthood to determine any metabolic changes. Adult survivors of marasmus had increased postchallenge glucose levels, reduced glucose sensitivity, and worsened β cell function compared with adult survivors of kwashiorkor [20]. A study of adult survivors of malnutrition identified a reduction in BMI and bone mineral density in marasmus survivors compared with kwashiorkor survivors. When admitted as infants, marasmus survivors were identified as having a reduced gestational age at birth, birth weight, weight and height for age, neutrophil count, and lymphocyte count compared with kwashiorkor survivors. **DNA methylation** (see Glossary) analysis was performed on muscle biopsies from survivors of severe acute malnutrition and identified differential DNA methylation patterns associated with metabolic pathways, which may influence the phenotypic origins of kwashiorkor and marasmus [21]. In summary, when faced with an extreme

Box 2. Epigenetics and the Developmental Origins of Health and Disease

A variety of exposures in early life and *in utero* can change metabolic outcomes in adulthood. In 1934, Kermack *et al.* observed a significant drop in mortality rate within the UK and Sweden between 1751 and 1930, which the authors attributed to an improved early-life environment [135]. In 1960, Widdowson and McCance observed that rats born to small litters (n = 3) with greater maternal nutrition access grew more rapidly, reached sexual maturity earlier, and maintained a larger body size into adulthood compared with rats from larger litters (n = 15–20) [136]. These results provided evidence that there are critical windows of development susceptible to changes in nutrition that have long-lasting effects in adulthood. In 1962, J.V. Neel developed what is now known as the 'thrifty genotype' hypothesis from the observations that babies born from women with diabetes often have macrosomia and develop diabetes in adulthood. Neel postulated that this genotype is more efficient at energy intake and efficiency, evolutionarily gaining an energy reserve advantage during famine conditions, which may be detrimental under nutritional surplus [137].

Later studies concluded that the timing of the critical window (i.e., the specific trimester or early childhood) in addition to changes in environment in later life may change the phenotype. In 1976, Ravelli *et al.* investigated prenatal and postnatal nutrition levels during the 1944–1945 Dutch famine and the potential for increased susceptibility to obesity. If individuals experienced famine during the last trimester of pregnancy or within the first few months of life, the rates of obesity significantly decreased. However, if individuals experienced famine during the first half of pregnancy, they had a significantly increased rate of obesity [138]. In 1977, Forsdahl hypothesized that individuals born in poverty who experienced affluence in later life had a reduced tolerance to a HFD, increasing the susceptibility to arteriosclerosis compared with individuals who never experienced poverty [139]. In 1985, Wadsworth *et al.* identified a similar correlation between cardiovascular disease and socioeconomic status [140]. Both men and women who came from families with the lowest socioeconomic class had significantly higher mean systolic blood pressures compared with those from the highest socioeconomic status [140]. Interestingly, men who grew up in the lowest socioeconomic status but rose in socioeconomic status had even higher mean systolic blood pressure compared with those that stayed within the same socioeconomic class in adulthood [140]. Barker and colleagues made observations related to this phenomenon, with several epidemiological studies showing that infants born small for gestational age had an increased susceptibility to cardiovascular disease and metabolic dysfunction [13,14]. Derived from Neel's 'thrifty gene hypothesis' [137], the 'thrifty phenotype hypothesis' was proposed from observations of increased adiposity and decreased fat mobilization following poor fetal nutrition [15].

Thus, the molecular mechanisms involved in developmental origins of health and disease phenomena will be affected by environmental influences on the epigenome, with early-life developmental origins of epigenetic alterations generating later-life impacts on health and disease.

stressor such as severe acute malnutrition, individuals who had a low birth weight had a more benign phenotype of marasmus compared with individuals with a higher birth weight who developed the edematous kwashiorkor. However, while individuals who had a low birth weight and the marasmus phenotype fared better in famine conditions, they had adverse metabolic outcomes as adults outside of famine conditions compared with kwashiorkor survivors. Therefore, environmental insults during early development can influence the adaptive response to metabolic challenges and increase the risk of metabolic disease in later life.

Epigenetic Developmental Reprogramming

Epigenetic developmental plasticity allows an organism to respond to the surrounding environment during cell differentiation, which changes the phenotype and gene expression without modifying the genetic code [22]. **Epigenetics** involves molecular factors and processes around the DNA that regulate genomic activity independent of the DNA sequence, and are mitotically stable [23,24]. Epigenetic changes involve both DNA and chromatin molecular modifications that change the expression of genes and genome activity [25,26]. Epigenetic modifications include DNA methylation of CpG dinucleotide residues, histone modification, most noncoding (nc)RNAs, RNA methylation, and chromatin structure [23] (Figure 1). DNA methylation of CpG dinucleotides is one of the most well-characterized epigenetic marks, and is generally stable and enduring in somatic cells [24]. However, during critical windows of development, the epigenome goes through cycles of methylation changes to accommodate for specific gene expression patterns needed for embryogenesis and fetal development [27]. For example, reduced methylation state is required to obtain a pluripotent stem cell state during development. Changes in environmental conditions during these critical windows of development, such as nutritional imbalances and environmental toxicants, can disrupt these processes, and permanently alter the DNA methylation patterns of the fetal and subsequent somatic cell epigenomes [27,28]. The history of epigenetics and epigenetic inheritance is presented in Box 3.

Epigenetic Transgenerational Inheritance Mechanisms

Changes in methylation patterns in the germline due to environmental insults can induce a transgenerational phenotype. **Epigenetic transgenerational inheritance** is the germline-mediated inheritance of epigenetic information between generations in the absence of continued direct environmental influences that leads to phenotypic variation [24,29]. There are two main exposure mechanisms behind epigenetic transgenerational inheritance. Environmental exposures can induce an altered cascade of epigenetic change, such as DNA methylation, in the fetus of

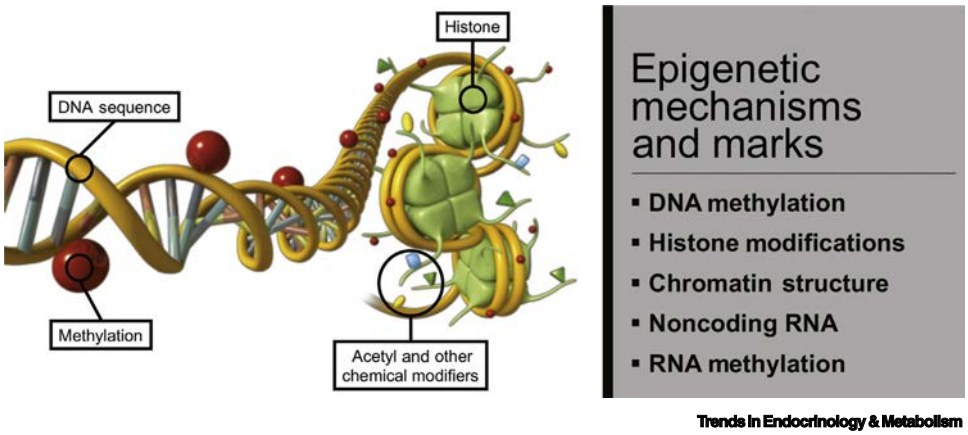

Figure 1. Epigenetic Processes and Marks. Modified from [149].

Box 3. History and Mechanisms of Epigenetics and Epigenetic Inheritance

During the 1940s, Conrad Waddington coined the term 'epigenetics' in reference to environment–gene interactions that could not be explained by classic Mendelian genetics [141]. Waddington found that heat shock could induce a change in *Drosophila* wing structure phenotype that was heritable for 16 generations [40]. Three decades later, one of the first epigenetic marks identified was DNA methylation, the addition of a methyl group to a CpG DNA residue [142–144]. Other epigenetic processes were identified in the following decades. During the 1980s and 1990s, histone modifications were found to be associated with changes in gene expression [145]. Eventually, ncRNA, chromatin structure, and RNA modifications were identified as epigenetic factors [23]. As both the field and technology advances, it is likely that other epigenetic molecular marks and factors will be identified.

Several historic observations have suggested the presence of non-Mendelian inheritance processes, including observations by Mendel with peas and by Krammerer during the early 1900s with the midwife toad [74]. These observations were not generally accepted during the early 1900s due to the rediscovery of genetics. The first person to help establish the field of epigenetics was Waddington [40,141] who coined the term and again observed non-Mendelian inheritance phenomenon with *Drosophila*. Subsequent observations with paramutation in plants supported this process, but was suggested to be a genetic phenomenon [146]. The first indirect observations of the link of epigenetics and inheritance came from imprinted genes. Imprinted genes involve monoallelic gene expression, are transmitted in a parent-of-origin (maternal or paternal) manner in the germline, and involve modifications of DNA methylation, histone modification, and ncRNA expression [76,147,148]. The control and inheritance of genomic activity involves interdependent mechanisms of several epigenetic and genetic processes.

One of the first studies associating changes in epigenetic marks and transgenerational inheritance correlated ancestral exposure to vinclozolin with DNA methylation changes in the germline in 2005 [34]. Epigenetic transgenerational inheritance requires the germline transmission of these imprinted-like epigenetic modifications by altering the epigenome of developing embryonic stem cells in the next generation [76]. Within 15 years, the field expanded rapidly and current research has demonstrated that the phenomenon of epigenetic transgenerational inheritance can occur in a variety of species and ancestral exposures [76].

The term 'epigenetic inheritance' refers to any epigenetic effect on subsequent generations, namely a combination of direct multigenerational exposures and transgenerational exposures [29]. This is distinct from epigenetic transgenerational inheritance, which requires the transmission of germline information between generations in the absence of any continued direct exposure [76]. A distinction between multigenerational exposure or intergenerational epigenetic inheritance and epigenetic transgenerational inheritance is required due to the distinct mechanisms and biological impacts of the two processes. Therefore, to distinguish the two, the term 'epigenetic transgenerational inheritance' is used as originally proposed [34].

gestating females during the developmental period of gonadal development and primordial germ cell migration (Figure 2). Aberrant DNA methylation of the germline can be heritable and is referred to as germline **epimutations** [30]. The exposure of an F0 generation gestating female to an environmental insult also exposes the developing F1 generation embryo (Figure 3). In addition, alterations of the epigenome in the developing germ cells within the F1 generation fetus can influence the F2 generation. If the altered DNA methylation patterns are heritable to the subsequent F3 generation, the transmission of these epimutations is considered epigenetic transgenerational inheritance [29,31]. Preconception exposure-mediated epigenetic transgenerational inheritance can be induced by exposing the F0 generation male or female to an environmental insult that can affect the epigenome of the germline. The germline, which eventually becomes the F1 generation, has been directly exposed to the environmental exposure, and is not considered to be transgenerational. Therefore, the F2 generation is considered to be the first nonexposed transgenerational offspring in this preconception exposure instance [29] (Figure 3).

The altered germline epigenetics has the potential ability to change the transcriptome and epigenetics of the totipotent cells in the early embryo. This can subsequently alter the epigenetics of all somatic cell types derived from these stem cells. During cellular and tissue differentiation, a cascade of gene expression changes occurs simultaneously with a cascade of epigenetic changes (Figure 2). The early stages of development are more susceptible to environmental insults that alter this cascade of epigenetic change. Therefore, the environmentally altered differential

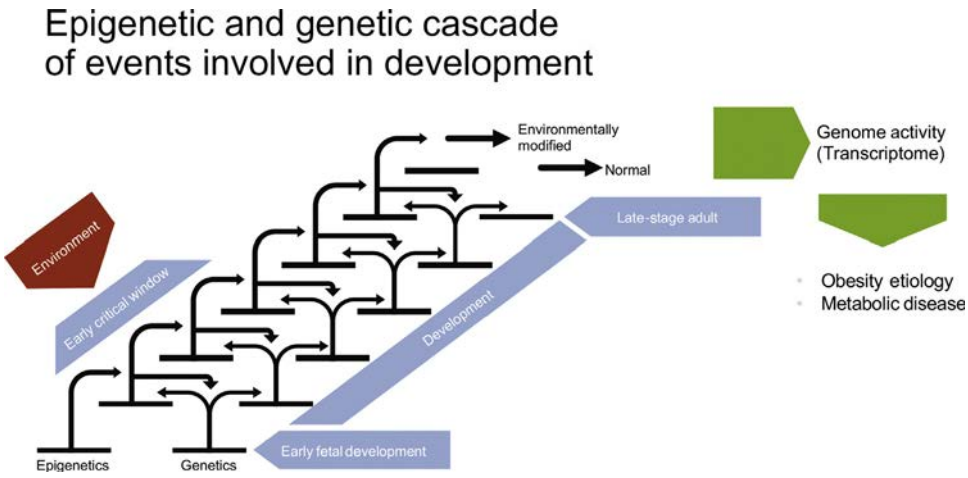

Figure 2. Epigenetic and Genetic Cascade of Events Involved in Development. Development and cellular differentiation are dependent on the impacts of a cascade of genetic and epigenetic changes. Early life environmental exposures have an increased impact on the transcriptome and physiology of an organism compared with exposures later in development. In organisms that have finished development, most cells have already been fully differentiated. However, early developmental exposures can affect stem cells and cell differentiation to increase the susceptibility to altered transcriptomes and impact disease etiology and phenotypic variation. Modified from [24].

epigenetic state can influence genome activity and the cell type-specific differentiated transcriptome to subsequently increase susceptibility for diseases, such as obesity (Figure 2). The integrated genetic and **epigenetic processes** that occur through these developmental periods

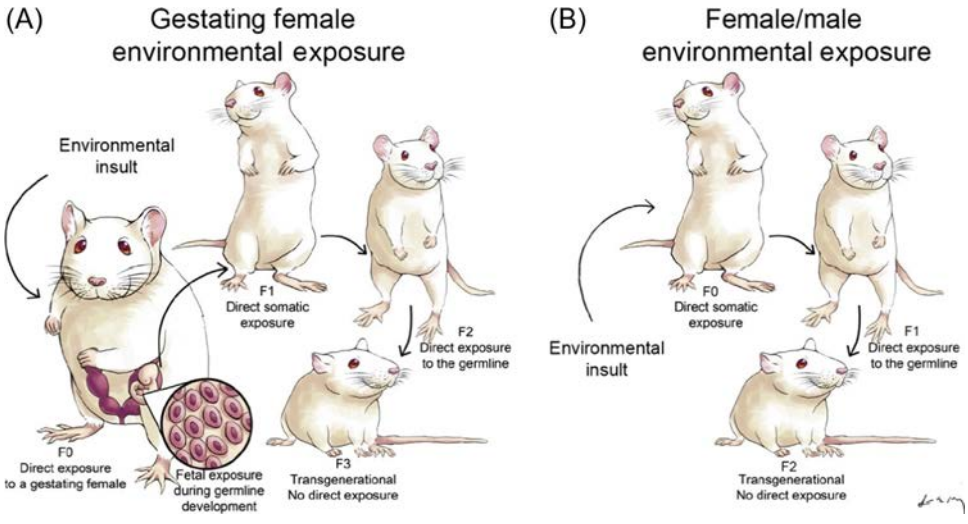

Figure 3. Exposure Mechanisms of Environmentally Induced Epigenetic Transgenerational Inheritance. (A) The exposure of an F0 generation gestating female to an environmental insult also exposes the developing F1 generation embryo. In addition, alterations of the epigenome in the developing germ cells can also influence the F2 generation if the altered methylation patterns are heritable by the subsequent F3 generations. The transmission of these epimutations is considered epigenetic transgenerational inheritance. (B) Preconception exposure-mediated epigenetic transgenerational inheritance can be induced by exposing the F0 generation to an environmental insult that can affect the epigenome of the germline. The germline, which eventually becomes the F1 generation, has been directly exposed to the environmental toxicant and is not considered to be transgenerational. Therefore, the F2 generation is considered to be the first transgenerational offspring.

establish the physiology and susceptibility to disease in later life stages [23]. Therefore, the environmentally induced epigenetic transgenerational inheritance of disease susceptibility, such as lifestyle and diet, then promotes the susceptibility for diseases, such as obesity.

Evidence for Epigenetic Transgenerational Inheritance

Although non-Mendelian forms of inheritance have been observed over the past century, such as Kammerer's midwife toad [32] and Waddington's heat-induced fly wing structure alteration [33], the molecular mechanisms involved were unknown and observations were not considered distinct from genetic inheritance. This was not demonstrated until recently, when more molecular information was available regarding epigenetics (Box 3). One of the first observations of environmentally induced epigenetic transgenerational inheritance was the exposure of gestating rats to the agricultural fungicide vinclozolin, which was found to promote transgenerational male testis disease and germline DNA methylation changes [34]. The number of examples of epigenetic transgenerational inheritance has increased dramatically over the past few decades, and the phenomenon has now been demonstrated in both plants and animals (Figure 4). In plants, partial reprogramming of epigenetic marks in both male and female gametes occurs immediately after fertilization in plants [35]. Both temperature and drought have been shown to promote epigenetic transgenerational phenotypic changes in both flowering and growth characteristics [36,37]. A well-known plant example of epigenetic transgenerational inheritance involves a change in symmetry in *Linaria vulgaris* flowers [38]. Increased DNA methylation in the promoter region of the *Lcyc* locus changed the floral symmetry phenotype from bilateral to radial, and was transgenerationally transmitted for >100 generations [39].

In non-mammalian animals, there are also a variety of species that have demonstrated the capacity for epigenetic inheritance. The model insect *Drosophila melanogaster* has demonstrated epigenetic transgenerational inheritance in many studies [40–42]. Other members of the phylum Arthropoda, such as *Artemia* [43] and *Daphnia magna* [44], have also been shown to exhibit

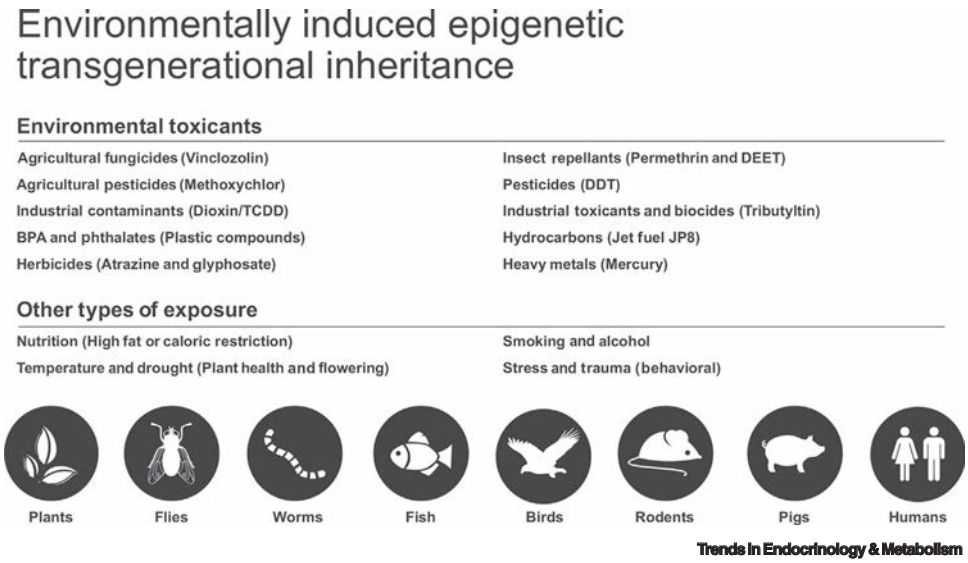

Figure 4. Environmentally Induced Epigenetic Transgenerational Inheritance. The potential for epigenetic transgenerational inheritance is induced by various environmental insults, including environmental toxicants. This phenomenon has been observed in a variety of organisms, including plants and animals. Adapted from [19]. Abbreviations: BPA, bisphenol-A; DEET, diethyltoluamide; DDT, dichlorodiphenyltrichloroethane; TCDD, dioxin.

epigenetic transgenerational inheritance. The nematode, *Caenorhabditis elegans,* has shown transgenerational inheritance of both histone modification and DNA methylation [45,46]. Several species of fish have also demonstrated epigenetic inheritance, such as the zebrafish [47–49] and the pipefish *Syngnathus typhle* [50]. Birds, such as quail [51] and the Muscovy duck [52], have also exhibited environmentally induced heritable changes. While most mammalian research is performed on rodents [24], there have been other examples of epigenetic transgenerational inheritance occurring in domestic pigs [53] and the common marmoset [54]. Several epidemiological studies, such as the Dutch and Swedish Famine Cohorts, have also identified transgenerational inheritance in humans [55,56]. Therefore, epigenetic transgenerational inheritance appears to be a highly conserved adaptive response among various species from plants to mammals (Figure 4).

Numerous environmental insults (Table 1) have been identified to induce epigenetic transgenerational inheritance, including heat exposure [40,43,57,58], salt stress [57], drought [59], stress and trauma [60–63], a high-fat diet (HFD) [64–66], nutritional deprivation [55,56], diabetes and/or prediabetes [67,68], folate [69], smoking [70,71], and alcohol [72,73] (Figure 4).

Table 1. Environmental Insults That Can Induce Epigenetic Transgenerational Inheritance

Exposure	Effects	Refs
Vinclozolin	Testis disease, prostate disease, kidney disease, age of puberty, male infertility, immune system abnormalities, tumor development	[29,34,76,89]
Methoxychlor	Kidney disease, ovary disease, obesity, male infertility	[34,82]
Permethrin/DEET	Pubertal abnormalities, testis disease, ovary disease	[81]
Dioxin	Prostate disease, ovary disease, kidney disease, uterine disease, testis disease, increased risk of preterm birth	[86,87,95]
BPA/phthalates	Pubertal abnormalities, testis disease, obesity, ovarian disease	[80]
BPA	Heart disorders, reduced fertility, changes in social behavior	[79,150,151]
Hydrocarbon mixture (jet fuel)	Ovary disease, obesity	[83]
DDT	Obesity, testis disease, ovary disease, kidney disease	[107]
Benzo[a]pyrene	Behavioral changes, infertility, increased BMI	[48,84]
Tributyltin	Obesity	[85,105,114]
Glyphosate	Obesity, testis, kidney, ovary, and prostate disease	[78]
Mercury	Behavioral changes	[47]
Caloric restriction	Cardiovascular mortality, increased chronic disease, increased BMI	[55,102,103]
High-fat diet	Increased adiposity, mammary cancer, hyperglycemia	[64–66]
Folate		[69]
Stress	Depressive-like behaviors, increased risk taking, and glucose dysregulation; reduced anxiety and serum cortisol; reduced growth and delayed behavioral development	[57,60–63]
Drought	Changes in DNA methylation	[36,59]
Heat/salt stress	Accelerated flowering, increased tolerance	[57]
Prediabetes/diabetes	Impaired insulin sensitivity	[67,68]
Smoking	Abnormal pulmonary function, increased fat mass	[70,71]
Alcohol	Neurological defects	[72,73]
Heat stress	Increased tolerance to heat stress in plants; wing structure change in *Drosophila melanogaster*	[43,58]

Several studies have revealed that environmental toxicants, including the fungicide vinclozolin [34,74–76]; the herbicides atrazine and glyphosate [77,78]; plasticizers, such as bisphenol A [79,80] and phthalates [80]; the pesticides diethyltoluamide (DEET) with permethrin [81] and methoxychlor [82]; the hydrocarbons jet fuel (JP8) [83] and benzo[a]pyrene [84]; the antifouling agent tributyltin [85]; mercury [47]; and dioxins [86–88], promote increased rates of disease and sperm epimutations. Ancestral exposure to environmental insults have been shown to induce a variety of diseases and phenotypic abnormalities. Transgenerationally increased rates of disease include testis abnormalities [80,89–94], prostate disease [86,89], ovarian disease [80–83,86,92], uterine disease [87,95], kidney disease [82,86,89,96,97], immune system abnormalities [89], and tumor development [89]. More details regarding the history of epigenetics and epigenetic transgenerational inheritance are provided in Box 3.

Epigenetic Transgenerational Inheritance of Susceptibility for Obesity and Metabolic Dysfunction

Transgenerationally increased susceptibility to obesity and its comorbidities has been observed following ancestral exposure to several environmental insults (Table 2). Most studies have investigated the potential for transgenerational inheritance of obesity and metabolic dysfunction through exposure to a HFD and caloric restriction. Ancestral exposure to a maternal HFD was shown to result in paternal transmission of increased body size in F3 generation female mice [64]. The F2 generation male offspring of mice, ancestrally exposed to neonatal lactational overnutrition, developed glucose intolerance and fasting hyperglycemia [98]. Paternal HFD in the F0 generation of mice was associated with increased adiposity and with alterations in sperm miRNA, with a reduction in total global germ cell methylation in F2 generation offspring [99]. In another study, F2 generation offspring ancestrally exposed to a paternal HFD had reduced birth weight and resistance to weight gain, with adult females developing glucose intolerance. Additionally, the ancestral paternal HFD was also associated with differential expression of the

Table 2. Environmental Insults That Can Transgenerationally Increase the Susceptibility to Obesity and Its Comorbidities

Exposure	Effects	Refs
Maternal HFD	Increased body size in females	[64]
Paternal HFD	Increased adiposity and sperm miRNA changes; reduced birth weight, resistance to weight gain with glucose intolerance; increased adiposity and serum leptin	[99–101]
Paternal HFD and prediabetes	Impaired insulin sensitivity and glucose intolerance	[68]
Paternal overnutrition	Glucose intolerance and fasting hyperglycemia in males	[98]
Maternal famine	Increased rate of chronic disease	[102]
Paternal famine	Increased BMI; increased incidence of cardiovascular disease and diabetes	[55,103]
BPA/phthalates	Increased adiposity and sperm methylation changes associated with obesity genes	[80]
DDT	Increased adiposity and sperm methylation changes associated with obesity genes	[107]
Methoxychlor	Increased adiposity	[82]
Tributyltin	Increased fat:lean tissue ratio, increased weight gain on a HFD, decreased weight loss when fasting, changes in chromatin structure and DNA methylation, increased expression of leptin	[85,114]
Glyphosate	Increased adiposity and adipose size, obesity and sperm DNA methylation	[78]

let-7c miRNA in sperm, and subsequent expression in the adipose tissue of the offspring [100]. Paternal HFD also demonstrated an increase in adiposity and serum leptin in F2 generation males [101]. Ancestral exposure to paternal HFD and streptozotocin induced prediabetes, and also predisposed the F2 generation to impaired insulin sensitivity and glucose intolerance through germline-mediated epigenetic transgenerational inheritance [68]. The daughters of women who experienced the Dutch Hunger Winter of World War II *in utero* had 1.8 times more chronic diseases as adults compared with nonexposed women [102]. The F2 generation grand-offspring of fathers who experienced the Dutch Hunger Winter of World War II had significantly increased BMI compared with exposed mothers or the unexposed population [55]. A preconception paternal exposure transgenerational impact appears in the F2 generation (Figure 3). In Överkalix, an isolated town in Northern Sweden, paternal ancestor nutrition appeared to significantly influence the incidence of cardiovascular disease and diabetes in their grand-offspring [103]. These results indicate that ancestral exposure to HFD and malnutrition (caloric restriction) can promote germline epimutations that can induce a transgenerational obesity and metabolic dysregulation phenotype in future generations.

A recent study by Risal *et al.* [104] observed a fivefold increase in polycystic ovary syndrome (PCOS) in daughters whose mothers also had PCOS. Daughters of women with PCOS had increased free androgen levels as well as metabolic dysfunction-related symptoms, such as increased BMI, waist circumference, and diastolic blood pressure. To determine whether prenatal exposure to androgens can promote PCOS-like symptoms in the transgenerational F3 generation [104], F0 generation mice were exposed to either dihydrotestosterone, a high fat and high sucrose diet, dihydrotestosterone plus a high fat and high sugar diet, or vehicle control. F3 generation androgenized lineage females had increased weight and fat mass, and reduced energy expenditure. PCOS-like reproductive and metabolic dysfunctions were observed in the F3 generation of the androgen lineage, including increased weight, fat mass, adipocyte size, liver triglyceride concentrations, and altered adipogenesis [104]. In the high fat, high sugar diet lineage, metabolic dysfunction was observed in the F1 generation, but was less substantial in the F2 and F3 generations. In the androgen plus high fat and high sugar diet lineage, the embryonic development of the F2 generation was detrimentally affected to the extent that only one female survived and no F3 offspring were obtained [104]. These results indicate that androgen exposure, as well as a high fat, high sugar diet, can promote metabolic dysfunction in the F3 generation. A combination of both exposures appears to be mostly fatal by the F2 generation [104]. Interestingly, single cell RNA-seq identified transcriptomic alterations in the oocytes of F1 through F3 generations of both the obesity and androgen lineages, which may be a potential mechanism for the inheritance of these phenotypes [104]. Further investigation is needed to determine whether the altered transcriptome is associated with epigenetic alterations in the germline required for epigenetic inheritance [23].

Environmental toxicants have also been indicated in the epigenetic transgenerational inheritance of obesity susceptibility. In 2006, Grün and Blumberg developed the term 'obesogen' to refer to environmental toxicants that can induce obesity [105]. Several environmental toxicants have since been shown to induce a transgenerational obese phenotype in the F3 generation of rats following ancestral prenatal exposure. A recent study showed that rats ancestrally exposed to a cadmium and mercury mixture demonstrated increased abdominal adiposity and impaired glucose tolerance through the F4 generation [106]. Ancestral exposure to a mixture of plastics derived endocrine disruptors [bisphenol-A (BPA), bis(2-ethylhexyl)phthalate (DEHP), and dibutyl phthalate (DBP)] increased the susceptibility to obesity in the F3 generation of male and female rats [80]. Additionally, several differential DNA methylated regions (DMR) in the sperm of the F3 generation plastics lineage males were associated with genes previously shown to be relevant

to obesity [80]. A transgenerational obese phenotype was observed in the F3 generation of males and females ancestrally exposed to jet fuel hydrocarbons as well as to the pesticide methoxychlor [82,83]. These studies identified germline epimutation signatures that contribute to the obesity phenotype. However, further investigations into the molecular mechanisms behind the transgenerational inheritance of obesity susceptibility are needed.

Ancestral exposure to the pesticide dichlorodiphenyltrichloroethane (DDT) was shown to dramatically increase the susceptibility to obesity in F3 generation male and female rats [97,107]. DDT was historically one of the most commonly used pesticides against insect vectors of disease, with widespread use starting during the 1940s and 1950s [108]. In 1973, the compound was banned in the USA following health and environmental concerns; however, the World Health Organization (WHO) has continued to recommend indoor use to combat malaria[iv]. In 2013, Skinner *et al.* identified a 50% incidence of obesity in rats ancestrally exposed to DDT, and several F3 generation DDT sperm DMR were associated with known obesity genes [107]. A follow-up study utilizing an expanded assessment of obesity identified similar patterns, as well as a unique obesity-specific sperm epimutation signature [97]. F3 generation DDT lineage rats were bred to the F4 generation with wild-type rats in a maternal outcross (MOC) and paternal outcross (POC). The obesity phenotype present in the F3 DDT lineage generation females did not appear in either F4 generation POC or MOC outcrossed females however, the F4 generation males had significantly increased obesity in both outcross lineages [109]. The results indicate that the male obesity phenotype may be inherited through either the male or female germline, whereas the female obesity phenotype may require inheritance of both paternal and maternal alleles. Interestingly, the great-grandchildren (F3 generation) of many of the F0 generation human females exposed to DDT during pregnancy are adults today. Therefore, ancestral exposures to environmental toxicants such as DDT should be considered a potential component of the current obesity epidemic (Figure 5).

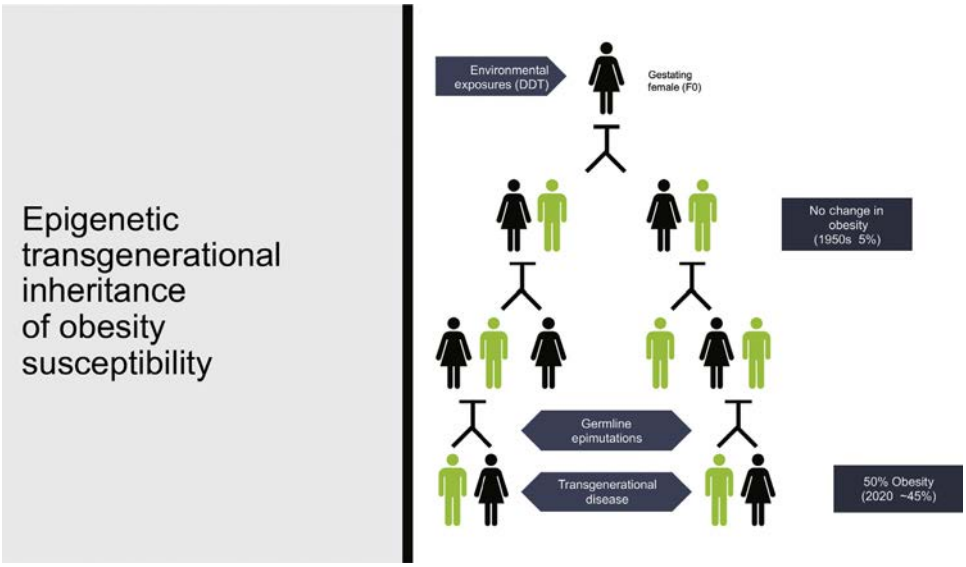

Figure 5. Environmentally [e.g., Dichlorodiphenyltrichloroethane (DDT)]-Induced Epigenetic Transgenerational Inheritance of Obesity. Obesity susceptibility epigenetically inherited from ancestral exposures. During the 1950s, the entire North American population was exposed to high levels of the pesticide DDT, when the obesity rate was <5% of the population. Three generations later, the obesity frequency in North America is now ~45% of the population.

Another obesogen capable of inducing transgenerational obesity is tributyltin, an organotin antifouling agent. Tributyltin acts as an agonist of peroxisome proliferator-activated receptor γ (PPARγ) and retinoid X receptor (RXR), which are important activators of adipocyte differentiation from mesenchymal stem cells [110–112]. Interestingly, *in utero* exposure of tributyltin increased gonadal fat deposition, but did not increase overall body weight in the F1 generation, because there was a change in the lean: fat mass ratio [113]. This effect was also transmitted to the F3 generation of mice ancestrally exposed to tributyltin, as evidenced by increased adipose tissue deposition, adipocyte size, and adipocyte number without a change in body weight. Ancestral tributyltin exposure transgenerationally reprogrammed F3 generation mesenchymal stem cells to increase adipogenesis and attenuate the osteogenesis pathway [85]. A 'thrifty phenotype' appeared in F4 generation males ancestrally exposed to tributyltin, characterized by increased potential for weight gain when fed a HFD, and decreased capacity to lose weight during fasting compared with controls [114]. These metabolic alterations were coupled with changes in chromatin structure and DNA methylation associated with increased expression of the leptin gene in gonadal white adipose tissue [115]. These observations provide insights into the molecular etiology of epigenetic transgenerational inheritance of obesity and, thus, further studies into the molecular mechanisms of other obesogens are necessary.

To build on previous transgenerational obesity research, King *et al.* used a purified cell population of mature white adipocytes for epigenetic analysis rather than adipose tissue [116]. Epigenetic modifications regulate gene expression in a cell-specific manner, and individual cell types have their own differential epigenetic pattern [117–119]. Adipose tissue has many varying cell types, and the cell composition is highly plastic [120]. Mature adipocytes comprise one-third of the cell population, while other cells include preadipocytes, endothelial precursor cells, mesenchymal stem cells, and immune cells at varying population levels [121]. Therefore, investigating an individual cell type, such as mature white adipocytes or preadipocytes, may provide a more metabolically relevant epigenome. Optimally, analysis of epimutations in a purified cell population such as white adipocytes, brown adipocytes, preadipocytes, and/or mesenchymal stem cells, as well as the germline, are needed to provide insight into potential epigenetic changes associated with obesity etiology. Elucidation of the impacts of ancestral exposures on metabolically relevant cells, such as adipocytes, will help elucidate the molecular etiology of obesity.

In the 2019 study by King *et al.*, adipocytes were isolated from the gonadal fat pad of lean, normal, and obese rats ancestrally exposed to the herbicide atrazine (lean phenotype) [122], the pesticide DDT (obese phenotype) [123], or vehicle control [116]. DNA methylation analysis identified unique adipocyte DMR patterns specific to control lineage obesity and DDT lineage obesity, indicating the potential for a unique molecular etiology to environmental toxicant-mediated obesity. Additionally, there were unique adipocyte DMR patterns identified between the F3 generation DDT lineage males and females, which suggests a potential sex-specific effect. Interestingly, a comparison of epigenetic alterations at a reduced stringency identified an extensive set of common potential genes and pathways affected by changes in DNA methylation between the lean and obese phenotypes. The overlapping DMR identified were associated with genes previously correlated with obesity, type 2 diabetes, and metabolic syndrome. The most commonly identified genes included *Caln1*, *Ikzf1*, *Iqsec3*, *Kcnma1*, *Ksr2*, *Mycbp2*, *Myo16*, *Negr1*, *Nr1h5*, *Rbms3*, and *Tmem236*. These genes, commonly modulated by DNA methylation in adipocytes, should be further investigated with transcriptomic analysis to determine whether there are gene expression changes associated with their methylation. This study also only examined DNA methylation, and subsequent analysis of other epigenetic marks, such as ncRNA and histone modifications, would be valuable. Additionally, further studies are needed to determine whether similar epigenetic mechanisms are present in the visceral adipocytes of lean

and obese humans, which may identify potential therapeutic targets for metabolic pathologies [116]. The regulatory impacts of these genes should be further investigated as novel modulators of adipocyte metabolism and function. Epigenetic transgenerational inheritance is a novel mechanism to consider in the etiology of obesity, and further research and eventual human studies may provide insight into potential therapeutics for metabolic diseases.

Concluding Remarks and Future Directions

Obesity has become a worldwide public health crisis, with a rapid increase in prevalence over the past 30 years. Although poor diet and an increasingly sedentary lifestyle have been traditionally indicated as the cause for this increase in obesity, it is clear that other factors also increase susceptibility to obesity. Various environmental insults have been shown to modify the germline epigenome and induce a transgenerational phenotype in future generations in the absence of continued exposure. Epigenetic transgenerational inheritance is a highly conserved mechanism for adaptive response to the environment, and has been identified in a variety of both plant and animal species. Several ancestral environmental exposures can transgenerationally increase the susceptibility to obesity and adult onset diseases. A number of different studies have investigated heritable epigenomic changes in the male germline [80,82,97,99,100,107] and, recently, a study attempted to identify a potential obesity-specific epigenetic signature in sperm [97]. This research should be developed further to determine the plausibility of a preconception biomarker of increased susceptibility to obesity in offspring. Identification of biomarkers in the sperm may aid in early development interventions to prevent adverse metabolic outcomes.

Unfortunately, little is known about the transgenerational effects in the female germline. Although molecular analysis of oocytes has proven challenging due to the inability to collect adequate numbers of cells [123], there have been attempts to identify female-germline specific disease phenotype inheritance through parent-of-origin allelic transmission [82,107,109]. Maternal and paternal outcrosses of DDT and vinclozolin lineage rats identified transgenerational disease phenotypes that may require both male and female germline-mediated changes [109]. Recent advancements in single cell or low cell count sequencing technologies are under development and have started to be applied to recent transgenerational studies [104,123]. Applying single cell sequencing technology to transgenerational studies will not only give a more accurate understanding of the molecular etiology of the inheritance of obesity, but also has the potential to identify novel mechanisms of epigenetic inheritance.

Many studies are attempting to identify the germline-mediated mechanisms behind the transgenerational inheritance of obesity; however, few focus on identifying the molecular changes at the tissue or somatic cell level. Previous studies have shown that epigenetic transgenerational inheritance of obesity following ancestral exposure can modify adipocyte differentiation and the epigenome of adipose tissue and adipocytes [85,114,116]. These studies have identified epigenetic modifications of certain genes and pathways that have the potential to be used as pharmaceutical targets. Further studies are needed to investigate whether similar epigenetic changes are present in humans (see Outstanding Questions).

Outstanding Questions

Few environmental insults have been assessed for the effects of ancestral exposure. What other environmental insults have the capacity to induce the transgenerational inheritance of obesity?

How does the epigenetic profile related to obesity in sperm influence the physiology of adipose tissue? How do other epigenetic marks, such as histone retention and ncRNA, in the sperm come into play in obesity phenotypes?

How is the development of adipose tissue affected in the epigenetic transgenerational inheritance of obesity? Are there effects on brown (thermogenic) adipose tissue?

Are there epigenetic changes in adipocyte precursor cell types, such as preadipocytes and mesenchymal stem cells?

Given that obesity and metabolic disorders are multifaceted and likely have multiple causes, are other somatic tissues involved in these transgenerational obesity phenotypes? Many of the animals in these studies are fed *ad lib*. Could there be transgenerational neurological changes, such as hyperphasia and changes in satiation, related to these phenotypes? Could transgenerational inheritance of endocrine changes, such as hypothyroidism, be involved?

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Resources

[i]www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight

[ii]http://apps.who.int/iris/bitstream/10665/148114/1/9789241564854_eng.pdf

[iii]www.mckinsey.com/industries/healthcaresystems-and-services/our-insights/how-the-world-could-better-fightobesity

[iv]http://apps.who.int/iris/bitstream/10665/69945/1/WHO_HTM_GMP_2011_eng.pdf

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Environmental induced transgenerational inheritance impacts systems epigenetics in disease etiology

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Environmental toxicants have been shown to promote the epigenetic transgenerational inheritance of disease through exposure specific epigenetic alterations in the germline. The current study examines the actions of hydrocarbon jet fuel, dioxin, pesticides (permethrin and methoxychlor), plastics, and herbicides (glyphosate and atrazine) in the promotion of transgenerational disease in the great grand-offspring rats that correlates with specific disease associated differential DNA methylation regions (DMRs). The transgenerational disease observed was similar for all exposures and includes pathologies of the kidney, prostate, and testis, pubertal abnormalities, and obesity. The disease specific DMRs in sperm were exposure specific for each pathology with negligible overlap. Therefore, for each disease the DMRs and associated genes were distinct for each exposure generational lineage. Observations suggest a large number of DMRs and associated genes are involved in a specific pathology, and various environmental exposures influence unique subsets of DMRs and genes to promote the transgenerational developmental origins of disease susceptibility later in life. A novel multiscale systems biology basis of disease etiology is proposed involving an integration of environmental epigenetics, genetics and generational toxicology.

Abbreviations

DMRs	DNA methylation regions
WGCNA	Whole genome coexpression network analysis
GWAS	Genome-wide association studies
DOHAD	Developmental origins of health and disease
ncRNA	Non-coding RNA
EWAS	Epigenome-wide association studies
MeDIP	Methylated DNA immunoprecipitation
MeDIP-Seq	Methylated DNA immunoprecipitation followed by next generation sequencing
JP8	Jet fuel
DEET	Diethyltoluamide
BPA	Bisphenol A
PCA	Principal component analysis
AHR	Aryl hydrocarbon receptor

Chronic disease has been shown to impact over 75% of the world-wide human population[1]. The frequency of disease has increased dramatically in the past decades at all ages, suggesting a major environmental impact on disease etiology[1,2]. Each generation continues to have an extended life span due to our more effici t medical capacity, but the percentage of disease continues to increase at all ages within the population[1,2]. Although currently we have a high incidence of infectious disease[3], this is far less than the level of chronic disease in the human population[1,2]. Environmental factors such as diet, lifestyle and pollutants are considered to be the risk factors for this increased generational occurrence of disease within the population. Since the vast majority of environmental factors cannot act as mutagens and directly change DNA sequence[4], our understanding of disease etiology needs to be expanded.

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The classic current paradigm for the molecular basis of disease etiology involves genetics[5]. The development of genetic mutations has been, and is still, thought to be the causal factor for phenotypic variation and disease development. Genome-wide association studies (GWAS) have demonstrated in large population-based studies that most diseases have specific associated gene mutations. The issue is that generally less than 1% of the specific diseased population have the associated genetic mutations[5], so these specific mutations have negligible impact on the specific disease population. Environmental factors such as toxicant exposures have been shown to promote disease development, but these exposures generally do not have the capacity to directly induce genetic mutations[6,7]. There is a growing appreciation that a combination of environmental factors and epigenetics are integrated in disease etiology[8,9]. Advancing age has been shown to correlate in the etiology of nearly all pathologies. The concept that early life exposures promote molecular alterations to induce the developmental origins of health and disease (DOHAD) has been established[10]. Studies to examine the impacts of race, ethnic background and regional alterations of disease etiology have suggested that environmental factors such as diet, exercise, toxicants, and lifestyle are the primary elements inducing the disease frequency differences, rather than genetics[6,11].

Epigenetics is defined as "molecular factors and processes around DNA that regulate genome activity independent of DNA sequence, and are mitotically stable"[12]. The epigenetic factors such as DNA methylation, non-coding RNA (ncRNA), histone modifi ations, chromatin structure alterations, and RNA methylation have the ability to integrate with genetics to impact all areas of biology[6,13]. When environmental factors influence the epigenetics of the germ cells (sperm and egg), the environmental factors have the potential to promote the epigenetic transgenerational inheritance of phenotypic variation and disease[6,14]. Environmental epigenetics provides a molecular mechanism to explain the developmental origins of health and disease (DOHAD) theory, phenotypic variation and adaptation associated with evolutionary biology, and the etiology of disease[6,13]. The integration of epigenetic and genetic molecular mechanisms is required for most biological processes and associated disease. However, the current paradigms in science generally focus only on genetics.

A large number of environmental factors have been shown to induce the epigenetic transgenerational inheritance of pathologies and disease[6,15]. The fi st observation involved the use of the agricultural fungicide vinclozolin[14], followed by a stress-induced behavior alteration transgenerationally[16]. Since then, a large variety of environmental toxicants from dioxin[17] to glyphosate[18], or nutritional abnormalities[19], or more recently infectious disease[20] have been shown to promote the epigenetic transgenerational inheritance of disease[6]. Th s non-genetic form of environmentally induced disease in subsequent generations needs to be considered as a component in disease etiology. The rapid increase in specific disease frequency within the population[1,2] will likely involve this environmentally induced epigenetic transgenerational inheritance of pathology phenomenon.

Recently, a number of epigenome-wide association studies (EWAS) have been shown to identify epigenetic alterations (i.e., epimutations) associated with diseases. Sperm epimutations involved in the transgenerational inheritance of specific pathologies have been identified[21–27]. Transgenerational sperm epimutations associated with kidney, prostate, puberty, testis, obesity, and multiple pathologies have been identifi d for a variety of environmental toxicants including dioxin[21], plastics[22], pesticides[23], glyphosate[24], methoxychlor[25], atrazine[26], and jet fuel[27] in animal studies. The transgenerational sperm epimutations for exposure and disease-specific epimutations have been identified in these EWAS studies[21–27] and in EWAS human studies[28,29].

The current study used the sperm samples and histological sections from these previous toxicant-induced epigenetic transgenerational inheritance EWAS rat studies to identify with more advanced protocols epigenetic alterations for specific diseases and associate with the genetics of the specific diseases. The common and distinct differential DNA methylation regions (DMRs) for the different exposure lineage diseases were correlated with known disease associated genes. Observations provide new insights into the integration of epigenetics and genetics in disease etiology. A novel systems epigenetics and multiscale framework is suggested to explain the apparent stochastic genetic events and variation within a disease population.

Results

Previously, a variety of distinct environmental toxicant exposures have been used to promote the epigenetic transgenerational inheritance of a number of pathologies and phenotypic variation. Th s initially involved an outbred rat model, but has now been found in all organisms examined from plants to humans[6]. Gestating female F0 generation rats were exposed transiently to a specific toxicant during the period of fetal gonadal sex determination (i.e., embryonic days 8–14 in the rat). The F1 generation off pring were obtained and aged to 3 months to be bred within the exposure lineage and avoid any inbreeding to obtain the F2 generation grand offspring. The F2 generation was then bred at 3 months of age to generate the F3 generation great-grand off pring. Interbreeding unrelated males and females within the exposure lineages was used to avoid any inbreeding and optimize the pathology observed by obtaining both maternal and paternal lineage contributions to the F3 generation, as previously described[30]. All animals were aged to 1 year in order to assess pathology and disease phenotypes, and the sperm collected to assess epigenetic (DNA methylation) alterations. This was previously accomplished with jet fuel (JP8)[31], pesticides (permethrin and diethyltoluamide (DEET))[32], plastics (bisphenol A (BPA) and phthalates)[33], dioxin[17], methoxychlor[34], glyphosate[18], and atrazine[26]. Control populations of animals were also prepared for comparison to identify the exposure-specific disease and epigenetic alterations of differential DNA methylation regions (DMRs) in the sperm, Supplemental Fig. S1. For the current study, the previous studies archived histology tissue slides and archived frozen (− 80 °C) sperm were reanalyzed to identify exposure specific pathologies (Supplemental Tables S1–S8) exposure specific DMRs (Supplemental Tables S9–S15) and control population disease specific DMR sets in the sperm for each of the F3 generation males with a single specifi disease or multiple disease (Supplemental Tables S16–S20). The reanalysis was performed with more advanced histology and pathology analysis of the slides and with more advanced technology for the sperm DMR analysis, as described in the Methods. Th s includes the use of updated methylated DNA immunoprecipitation (MeDIP)

procedure and bioinformatics[35], in comparison to tiling arrays and the earlier MeDIP procedures used in the past. More advanced reagents were used to improve reproducibility and accuracy of the MeDIP and generation of sequencing libraries, as described in the Methods (i.e., MeDIP-Seq Analysis). A 1 kb DMR size was used instead of 100 bp to improve the bioinformatics, as described in the Methods. The updated histopathology procedure used three histologists blinded to the slide identity and the counting of larger tissue section regions than used previously[17,18,26,27,31–33]. The analysis of larger regions allowed more effici t detection of various abnormal histology and pathology. Only animals with a specific disease were utilized for that specific disease. The disease specifi DMRs of transgenerational animals for jet fuel[27], pesticides[23], plastics[22], dioxin[21], methoxychlor[25], glyphosate[24], and atrazine[36] were previously reported, so are not included in the Supplemental Tables. Those males that only had an individual specific disease (i.e., no other disease present) were used to identify the pathology specifi sperm epigenetic biomarkers for that disease. Animals with multiple diseases were used and referred to as "multiple disease" groups. The current study was designed to compare the different toxicant exposures and assess the transgenerational disease specific DMRs to provide insights into the role of epigenetics in disease etiology and generational toxicology.

As previously observed[17,18,31–34,36], the control lineage F3 generation generally does not have appreciable pathology. The pathologies investigated were testis disease, prostate disease, kidney disease, obesity, pubertal onset abnormalities, and tumors. The specific histology analysis and tissue-specific pathology analysis are described in the Methods section. Th ee different individuals blinded to the slide identity separately assessed the pathology as described[21–27] in the Methods. Due to the reduced number of pathologies in the control lineages, all the different previous study controls were combined for the current study to identify potential control lineage epigenetic biomarkers for disease between individuals without and with specifi pathologies, Supplemental Table S1. The pathologies observed were testis disease, prostate disease, kidney disease, obesity and multiple disease, with no tumors observed and negligible pubertal abnormalities, Supplemental Table S1. Th s allowed for sufficient numbers of individuals and the ability to identify a control lineage epigenetic biomarker for each of the pathologies, Supplemental Fig. S1. The reanalysis of the F3 generation exposure specific pathologies is presented in Supplemental Tables S2–S8. As can be seen in Supplemental Tables S1–S8, here are generally sufficient numbers of animals with a single disease, such that individual disease epigenetic biomarkers can be identifi d. Only animals with a specifi disease were used to identify DMR for the specifi disease. Those animals with multiple disease were grouped and designated "multiple disease" and the associated epigenetic biomarkers identifi d. The DMRs in sperm were assessed for each of the individual pathologies at $p < 1e{-}04$ (Supplemental Fig. S1A), and the negligible overlap was observed for the various disease specific DMRs (Supplemental Fig. S1B). The disease control lineage DMR lists, and gene associations are presented in Supplemental Tables S16–S20. The principal component analysis (PCA) of the different control disease specific and non-disease specific and non-disease DMR comparison are presented in Supplemental Fig. S1, showing generally good separation of the DMRs. This combined F3 generation control disease specifi information is used to compare with the various toxicant exposure disease DMR biomarkers previously identified[21–27].

The reanalysis of the archived sperm from the F3 generation males of all the different exposures used a combined set of control samples, except for glyphosate and atrazine. Due to the lower number of transgenerational DMRs with glyphosate and atrazine, only the original control sets were used. A more advanced technology of methylated DNA immunoprecipitation (MeDIP) followed by DNA sequencing for an MeDIP-Seq procedure was used, which examines over 95% of the genome DNA methylation sites[35]. Th s is in contrast to the tiling array technology used previously for several of the exposures. The specific exposure DMRs at an edgeR p value of $p < 1e{-}06$, in all but glyphosate, is shown in Fig. 1A. Due to the low DMR numbers with glyphosate exposure, the imbalance was addressed by using $p < 1e{-}04$ for glyphosate. The exposure-specific DMR lists that provide chromosomal position, CpG density, log-fold change for the increase or decrease in DNA methylation, statistical p value and gene associations for only the DMR with associated genes are presented (Supplemental Tables S9–S20). An overlap of the different exposure DMRs at $p < 1e{-}06$, except for glyphosate at $p < 1e{-}04$, demonstrated minimal overlap among all exposure lineages, Fig. 1B, but some overlap was observed between specific exposures. An extended overlap with a comparison of the $p < 1e{-}06$, or $p < 1e{-}04$ for glyphosate, with others at $p < 0.05$ demonstrated higher (17–85%) overlaps, such as plastics and pesticides and lower overlaps with the dioxin and glyphosate, Fig. 1C. This is likely in part due to common or distinct signal transduction of the various exposures. For example, dioxin and jet fuel at 85% overlap both use the aryl hydrocarbon receptor (AHR) system, or methoxychlor and plastics at 82% overlap both use the estrogen receptor system. Therefore, the different environmental toxicants promoted the epigenetic transgenerational inheritance of common and exposure-specific DMRs in sperm from the F3 generation males.

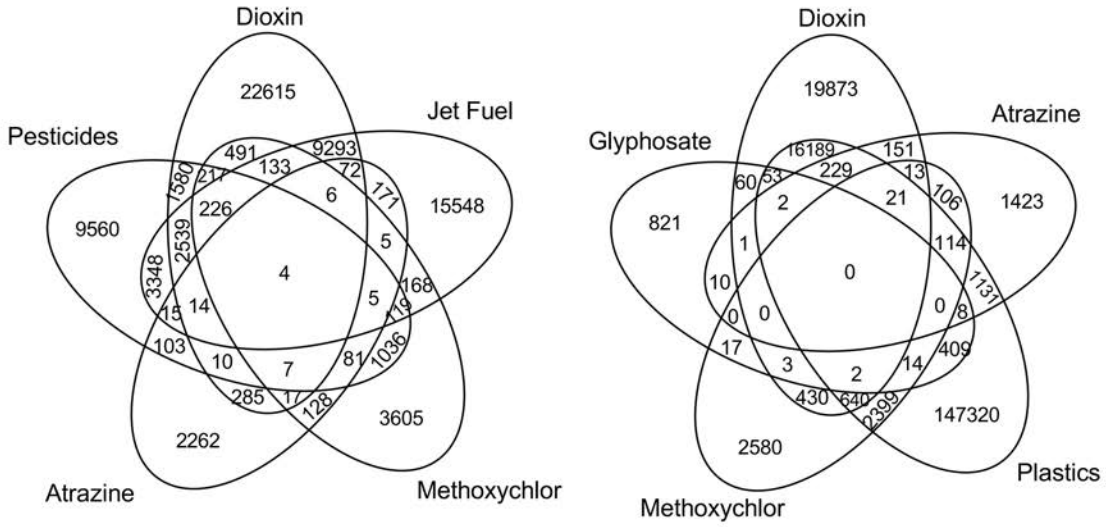
Each of the environmental toxicant exposures promoted transgenerational disease and pathology, Supplemental Tables S2–S8, that was common between the different exposures, Fig. 2. As previously described, the individuals with only a single specific disease were used to identify potential sperm epigenetic biomarkers for disease[21–27], Supplemental Tables S2–S8. A summary of the different exposures for each disease is presented in Fig. 2. The sperm DMRs at $p < 1e{-}04$ are presented for all 1 kb windows throughout the genome for each kidney disease (Fig. 2A), prostate disease (Fig. 2B), puberty abnormalities (Fig. 2C), testis disease (Fig. 2D), obesity (Fig. 2E), and the presence of multiple pathology (Fig. 2F). The overlap of the various disease-specific DMRs for each exposure were found to be distinct with negligible overlap (Fig. 2G–L), for all the different pathologies. An expanded overlap of the exposure-specific DMRs at $p < 1e{-}04$ with a less stringent threshold of $p < 0.05$ also demonstrated negligible overlap with less than 10% overlap observed, Fig. 3. Th s was similar for all the different pathologies and diseases. Therefore, the different toxicant exposures promoted similar pathology, but the disease-specific epigenetic biomarkers were distinct. A further analysis examined the chromosomal locations of the sperm DMRs in the rat genome for each of the different exposures for each of the different pathologies, Supplemental Fig. S2. A genome-wide distribution of the exposure's disease-specific DMRs were observed for

Exposure Specific DMRs

A Exposure Induced F3 Generation Sperm DMRs at edgeR p-value

Jet fuel	Pesticides	Plastics	Dioxin	Methoxy	Glyphosate	Atrazine
p<1e-06	p<1e-06	p<1e-06	p<1e-06	p<1e-06	p<1e-04	p<1e-06
31,517	18,791	168,543	37,475	6188	1395	3166

B Overlap of Exposure DMRs



C Extended Overlap of Exposure DMRs

p<0.05 / p<1e-06*	Dioxin	Glyphosate	Pesticides	Plastics	Methoxychlor	Jet Fuel	Atrazine
Dioxin	37475 (100%)	6538 (17%)	21567 (58%)	28064 (75%)	19035 (51%)	31972 (85%)	16641 (44%)
Glyphosate (p<1e-04)*	623 (45%)	1395 (100%)	541 (39%)	903 (65%)	554 (40%)	620 (44%)	525 (38%)
Pesticides	15096 (80%)	3992 (21%)	18791 (100%)	17250 (92%)	15397 (82%)	15928 (85%)	9217 (49%)
Plastics	106771 (63%)	31553 (19%)	92450 (55%)	168543 (100%)	78569 (47%)	103990 (62%)	73896 (44%)
Methoxychlor	4158 (67%)	1702 (28%)	5299 (86%)	5086 (82%)	6188 (100%)	3804 (61%)	4324 (70%)
Jet Fuel	29173 (93%)	5823 (18%)	22837 (72%)	25314 (80%)	16773 (53%)	31517 (100%)	13990 (44%)
Atrazine	2082 (66%)	649 (20%)	1519 (48%)	2502 (79%)	2163 (68%)	1681 (53%)	3166 (100%)

Figure 1. Exposure specific MRs. (**A**) Exposure DMRs at using a $p < 1e{-}6$ edgeR p value threshold for everything except glyphosate at $p < 1e{-}4$. (**B**) Overlap of Exposure DMRs ($p < 1e{-}06$), *except glyphosate at $p < 1e{-}04$. (**C**) Extended Overlap of Exposure DMRs. The overlapping DMRs and percent overlap are indicated.

the kidney disease (Supplemental Fig. S2A), prostate disease (Supplemental Fig. S2B), puberty pathology (Supplemental Fig. S2C), testis disease (Supplemental Fig. S2D), obesity (Supplemental Fig. S2E), and multiple pathologies (Supplemental Fig. S2F). Clusters of DMRs on the chromosomes were also observed for each, but were distinct between the exposures and disease.

Observations with differential DNA methylated regions (DMRs) identifi d exposure-specific DMR sets that correlated with similar transgenerational disease, but each disease had unique DMR biomarkers and associated

Disease Specific DMRs

A Kidney Disease

Exposure	p-value	All DMRs
Control	p<1e-04	84
Dioxin	p<1e-04	130
Pesticide	p<1e-04	463
Plastic	p<1e-04	276
Glyphosate	p<1e-04	180
Methoxychlor	p<1e-04	216
Atrazine	p<1e-04	261
Jet Fuel	p<1e-04	174

B Prostate Disease

Exposure	p-value	All DMRs
Control	p<1e-04	377
Dioxin	p<1e-04	177
Glyphosate	p<1e-04	242
Methoxychlor	p<1e-04	190
Pesticide	p<1e-04	218

C Puberty Pathology

Exposure	p-value	All DMRs
Atrazine	p<1e-04	322
Jet Fuel	p<1e-04	280

D Testis Disease

Exposure	p-value	All DMRs
Control	p<1e-04	283
Dioxin	p<1e-04	123
Pesticide	p<1e-04	163
Plastic	p<1e-04	49
Atrazine	p<1e-04	693

E Obesity

Exposure	p-value	All DMRs
Control	p<1e-04	89
Jet Fuel	p<1e-04	174
Dioxin	p<1e-04	165
Glyphosate	p<1e-04	250
Methoxychlor	p<1e-04	180

F Multiple Pathology

Exposure	p-value	All DMRs
Control	p<1e-04	629
Pesticide	p<1e-04	2100
Plastic	p<1e-04	131
Glyphosate	p<1e-04	345
Methoxychlor	p<1e-04	404
Atrazine	p<1e-04	336
Jet Fuel	p<1e-04	463

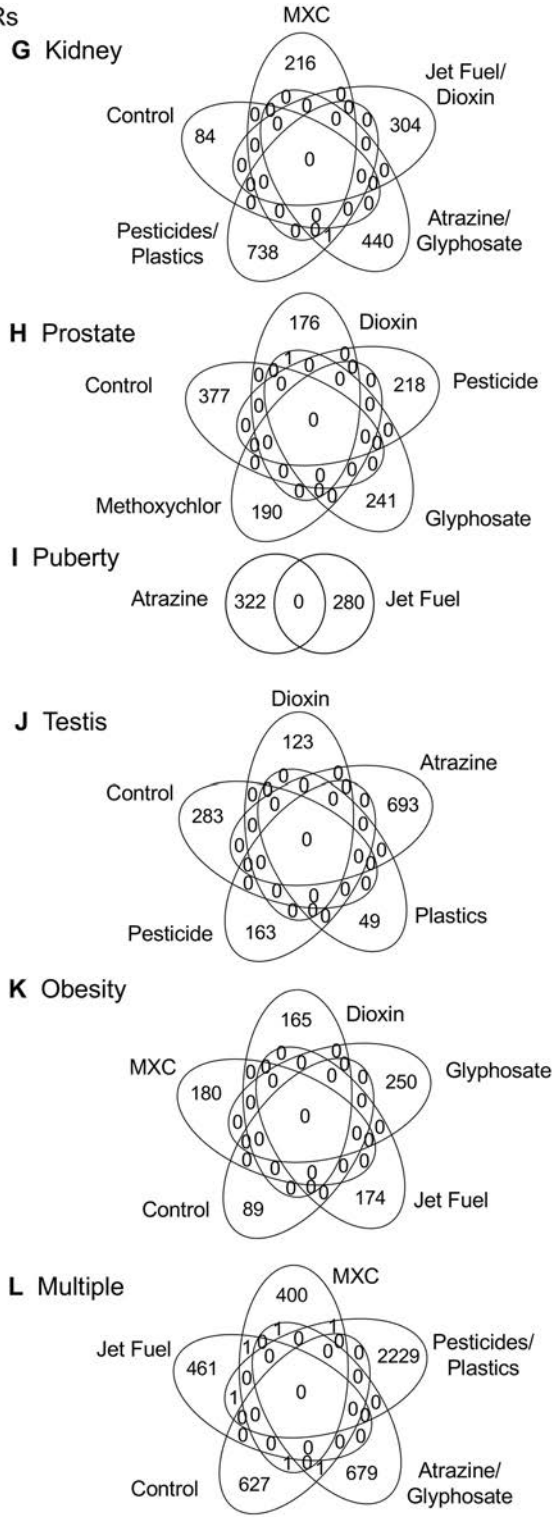


Figure 2. Specific d sease DMRs. (**A**) Kidney disease; (**B**) Prostate disease; (**C**) Puberty pathology; (**D**) Testis disease; (**E**) Obesity; (**F**) Multiple pathology. The exposure, *p* value and number of DMR are presented. Venn diagram overlaps for each exposure DMR set are shown for (**G**) Kidney; (**H**) Prostate; (**I**) Puberty; (**J**) Testis; (**K**) Obesity; (**L**) Multiple.

genes for the specific pathology, Supplemental Tables S21–S26. Therefore, similar transgenerational pathologies and diseases were induced by the various toxicant exposures with exposure and pathology distinct DNA methylation alterations. An additional analysis that was used to help elucidate this phenomenon was weighted

Extended Overlap

A Kidney

p<0.05 / p<1e-04	Dioxin Kidney	Control Kidney	MXC Kidney	Jet Fuel Kidney	Atrazine Kidney	Pesticides Kidney	Glyphosate Kidney	Plastics Kidney
Dioxin Kidney	130 (100.00%)	6 (4.62%)	4 (3.08%)	9 (6.92%)	7 (5.38%)	4 (3.08%)	7 (5.38%)	7 (5.38%)
Control Kidney	3 (3.57%)	84 (100.00%)	3 (3.57%)	2 (2.38%)	4 (4.76%)	0 (0.00%)	2 (2.38%)	2 (2.38%)
MXC Kidney	9 (4.17%)	4 (1.85%)	216 (100.00%)	6 (2.78%)	6 (2.78%)	21 (9.72%)	9 (4.17%)	30 (13.89%)
Jet Fuel Kidney	1 (0.57%)	5 (2.87%)	4 (2.30%)	174 (100.00%)	4 (2.30%)	7 (4.02%)	2 (1.15%)	15 (8.62%)
Atrazine Kidney	10 (3.83%)	6 (2.30%)	11 (4.21%)	10 (3.83%)	261 (100.00%)	6 (2.30%)	11 (4.21%)	13 (4.98%)
Pesticides Kidney	24 (5.18%)	18 (3.89%)	25 (5.40%)	26 (5.62%)	12 (2.59%)	463 (100.00%)	18 (3.89%)	40 (8.64%)
Glyphosate Kidney	6 (3.33%)	4 (2.22%)	12 (6.67%)	6 (3.33%)	6 (3.33%)	6 (3.33%)	180 (100.00%)	15 (8.33%)
Plastics Kidney	12 (4.35%)	6 (2.17%)	9 (3.26%)	5 (1.81%)	15 (5.43%)	12 (4.35%)	14 (5.07%)	276 (100.00%)

B Prostate

p<0.05 / p<1e-04	Dioxin Prostate	Control Prostate	Methoxychlor Prostate	Glyphosate Prostate	Pesticides Prostate
Dioxin Prostate	177 (100.0%)	11 (6.2%)	6 (3.4%)	9 (5.1%)	12 (6.8%)
Control Prostate	19 (5.0%)	377 (100.0%)	9 (2.4%)	19 (5.0%)	13 (3.4%)
Methoxychlor Prostate	18 (9.5%)	10 (5.3%)	190 (100%)	14 (7.4%)	8 (4.2%)
Glyphosate Prostate	15 (6.2%)	11 (4.5%)	14 (5.8%)	242 (100.0%)	11 (4.5%)
Pesticides Prostate	8 (3.7%)	17 (7.8%)	5 (2.3%)	14 (6.4%)	218 (100.0%)

C Puberty

p<0.05 / p<1e-04	Jet Fuel Late Puberty	Atrazine Late Puberty
Jet Fuel Late Puberty	280 (100.0%)	14 (5.0%)
Atrazine Late Puberty	15 (4.7%)	322 (100.0%)

D Testis

p<0.05 / p<1e-04	Dioxin Testis	DOD Control Testis	Pesticides Testis	Plastics Testis	Atrazine Testis
Dioxin Testis	123 (100.00%)	2 (1.63%)	2 (1.63%)	1 (0.81%)	9 (7.32%)
Control Testis	13 (4.59%)	283 (100.00%)	8 (2.83%)	7 (2.47%)	24 (8.48%)
Pesticides Testis	6 (3.68%)	12 (7.36%)	163 (100.00%)	7 (4.29%)	17 (10.43%)
Plastics Testis	1 (2.04%)	3 (6.12%)	0 (0.00%)	49 (100.00%)	3 (6.12%)
Atrazine Testis	35 (5.05%)	55 (7.94%)	26 (3.75%)	16 (2.31%)	693 (100.00%)

E Obesity

p<0.05 / p<1e-04	Dioxin Obesity	MXC Obesity	MXC Control Obesity	Jet Fuel Obesity	Glyphosate Obesity
Dioxin Obesity	165 (100.0%)	10 (6.1%)	9 (5.5%)	4 (2.4%)	13 (7.9%)
MXC Obesity	10 (5.6%)	180 (100.0%)	12 (6.7%)	10 (5.6%)	4 (2.2%)
MXC Control Obesity	2 (2.2%)	1 (1.1%)	89 (100.0%)	1 (1.1%)	3 (3.4%)
Jet Fuel Obesity	5 (2.9%)	2 (1.1%)	10 (5.7%)	174 (100.0%)	3 (1.7%)
Glyphosate Obesity	10 (4.0%)	4 (1.6%)	12 (4.8%)	16 (6.4%)	250 (100.0%)

F Multiple

p<0.05 / p<1e-04	Control Multiple	MXC Multiple	Jet Fuel Multiple	Atrazine Multiple	Pesticides Multiple	Glyphosate Multiple	Plastics Multiple
Control Multiple	629 (100.0%)	23 (3.7%)	34 (5.4%)	68 (10.8%)	43 (6.8%)	35 (5.6%)	11 (1.7%)
MXC Multiple	28 (6.9%)	404 (100.0%)	21 (5.2%)	20 (5.0%)	35 (8.7%)	19 (4.7%)	12 (3.0%)
Jet Fuel Multiple	48 (10.4%)	38 (8.2%)	463 (100.0%)	24 (5.2%)	48 (10.4%)	22 (4.8%)	17 (3.7%)
Atrazine Multiple	34 (10.1%)	9 (2.7%)	24 (7.1%)	336 (100.0%)	7 (2.1%)	11 (3.3%)	10 (3.0%)
Pesticides Multiple	172 (8.2%)	183 (8.7%)	234 (11.1%)	84 (4.0%)	2100 (100.0%)	109 (5.2%)	152 (7.2%)
Glyphosate Multiple	82 (23.8%)	19 (5.5%)	66 (19.1%)	15 (4.3%)	37 (10.7%)	345 (100.0%)	9 (2.6%)
Plastics Multiple	16 (12.2%)	7 (5.3%)	7 (5.3%)	6 (4.6%)	10 (7.6%)	6 (4.6%)	131 (100.0%)

Figure 3. Extended disease specific MR overlap at $p < 1e{-}04$ versus $p < 0.05$. (**A**) Kidney; (**B**) Prostate; (**C**) Puberty; (**D**) Testis; (**E**) Obesity; (**F**) Multiple. The DMR number and percentage overlap presented. The horizontal row overlap identifies MR number and percentage for each exposure.

genome coexpression network analysis (WGCNA)[37,38]. Th s bioinformatics procedure takes all the genome-wide sequencing data from the MeDIP-Seq analysis for each transgenerational exposure and pathology to assess DNA methylation patterns in the genome that correlate with the exposures and disease. Although this WGCNA has not been used extensively with DNA methylation, it has been extensively used to assess transcriptomes for gene correlations[39]. Previously, we have used this approach for gene predictions for developmental systems[40], so the current study extends this to epigenetic alterations correlated with associated genes. In the current study, all

MeDIP-Seq data was used in the WGCNA (https://doi.org/10.1186/1471-2105-9-559) to initially establish dendrograms of correlated DNA methylation site information to identify coexpression clusters, Supplemental Fig. S3. The exposure and control data generated large clusters of correlation specific DNA methylation data, but the pathology specific DNA methylation data generated smaller clusters throughout the genome, which often associated with the exposure clusters, Supplemental Fig. S3. Due to computational limitations (i.e., extended > 7 day periods), the 100,000 1 kb genomic windows with the highest total read depth were selected for inclusion in the WGCNA analysis. Genomic windows were clustered into modules based on methylation levels in all samples. These modules were then correlated with the disease and exposure characteristics of the samples. A summary with correlation coefficient and *p* value statistics is presented in Fig. 4. The various pathologies and exposures are correlated to a number of modules of DNA methylation site data. The modules are identified with different colors listed and the number of DNA methylation sites for each listed next to the color module, Fig. 4. Black outlined boxes identify significant correlations for the pathologies and exposures that were selected for further analysis. For the exposures, two of the top statistically significant modules were identified. Generally, the exposures had much higher levels of correlations, and in general each exposure had correlations with different modules. Some overlap is observed, such as between the control and glyphosate modules, Fig. 4. The pathology correlations had lower statistical significance, but correlations with one or two modules were observed, with multiple pathology having three correlated modules, Fig. 4. The DNA methylation sites within each of the WGCNA modules were associated with genes within 10 kb of the site(s) to incorporate distal and proximal promoter regions, Supplemental Tables S27–S32, similar to exposure specific DMR associated genes within 10 kb, Supplemental Tables S21–S26. Only the DMR sites or DNA methylation sites with associated genes are presented in Supplemental Tables S9–S20. This is an underestimate of potential regulatory sites due to not considering distal ncRNA regulation. The WGCNA provided DNA methylation correlations with exposure and pathology that had gene associations for further analysis, Supplemental Tables S27–S32.

A summary of the exposure DMR associated pathology genes is presented in Fig. 5A–F. The number of genes associated with the exposure DMRs that have been shown to correlate to specific disease are provided. A list of the pathology associated genes in the various exposure DMRs is presented in Supplemental Table S21–S26. Figure 5 presents the gene numbers for kidney disease (A), prostate disease (B), puberty abnormalities (C), testis disease (D), obesity (E), and multiple pathologies (F). Each of the exposure toxicants that promote the pathology has associated pathology genes, as indicated. Although the exposure DMRs are distinct, all the exposures have known pathology associated genes. A summary of the WGCNA module DNA methylation associated genes is also presented in Fig. 5. The DNA methylation site modules that correlate with specific pathologies are presented and the number of associated genes within 10 kb of the DMR are listed for each, Fig. 5G–L. The WGCNA module DNA methylation site associated genes for each pathology are listed in Supplemental Table S27–S32. Although different exposures were found to have different correlated modules, all had specific disease or pathology associated genes. Further network analysis of the DMR associated disease specific processes are presented for kidney disease in Fig. 6. Each of the different exposure kidney disease associated DMR associated genes are identified with a different color, Fig. 6. Unique subsets of genes specific for the different exposures are identified. Similar analyses of the other disease network analyses are presented in Supplemental Fig. S4 for prostate disease (Fig. S4A), puberty abnormality (Fig. S4B), testis disease (Fig. S4C), and obesity (Fig. S4D). The multiple disease associated genes were not analyzed for a network due to presence of multiple pathologies. In each of the disease DMR associated gene networks, there were exposure specific genes identified that were distinct. Therefore, the exposure specific epigenetic alterations were associated with unique subsets of disease specific genes.

Discussion

Over the past fifty years, there have been many observations that suggest the environment has significant impacts on disease etiology, but the vast majority of the environmental impacts cannot directly alter DNA sequence or promote genetic mutations. This includes regional impacts on disease frequencies[1,41], the low frequency of associated genetic mutations within a disease population[5], the dramatic increase in disease frequency over the last several decades[1,2], discordant monozygotic twin disease as the twins age[42], and direct impacts of environmental factors such as diet and toxicants on disease etiology[9,43]. The current paradigm primarily considered in disease etiology involves genetic determinism, where familial inheritance or random genetic mutations promote altered gene expression to promote abnormal cell or tissue biology to induce disease, Fig. 7A. Although this has developed over the past century, the sequencing of the human genome in the early 2000s significantly expanded support for this paradigm. The inability of this genetic determinism paradigm to incorporate the growing literature observations on environmental impacts on disease etiology suggests an additional molecular component needs to be incorporated into a new scientific paradigm for disease etiology.

Epigenetics provides an additional molecular mechanism that is now known to be essential for gene expression and is environmentally responsive. The incorporation of environmental epigenetics into a new paradigm for disease etiology will address the limitations of the more classic genetic determinism disease etiology paradigm. The observations that environmentally induced epigenetic alterations in the germline can promote the epigenetic transgenerational inheritance of disease allows a more generational component of disease etiology to be considered. This non-genetic form of inheritance is known to be influenced by a variety of environmental factors, from nutrition to toxicants, in all species investigated from plants to humans[6]. The current study used a number of previously published toxicant induced epigenetic transgenerational inheritance studies[17,18,31–34,36] to compare and develop greater insights into the molecular mechanisms involved in disease etiology.

The various environmental toxicant exposures of a gestating female rat around the time of gonadal sex determination during fetal development all promoted the epigenetic transgenerational inheritance of disease to the F3 generation great-grand offspring. The male sperm transmitting these transgenerational disease phenotypes

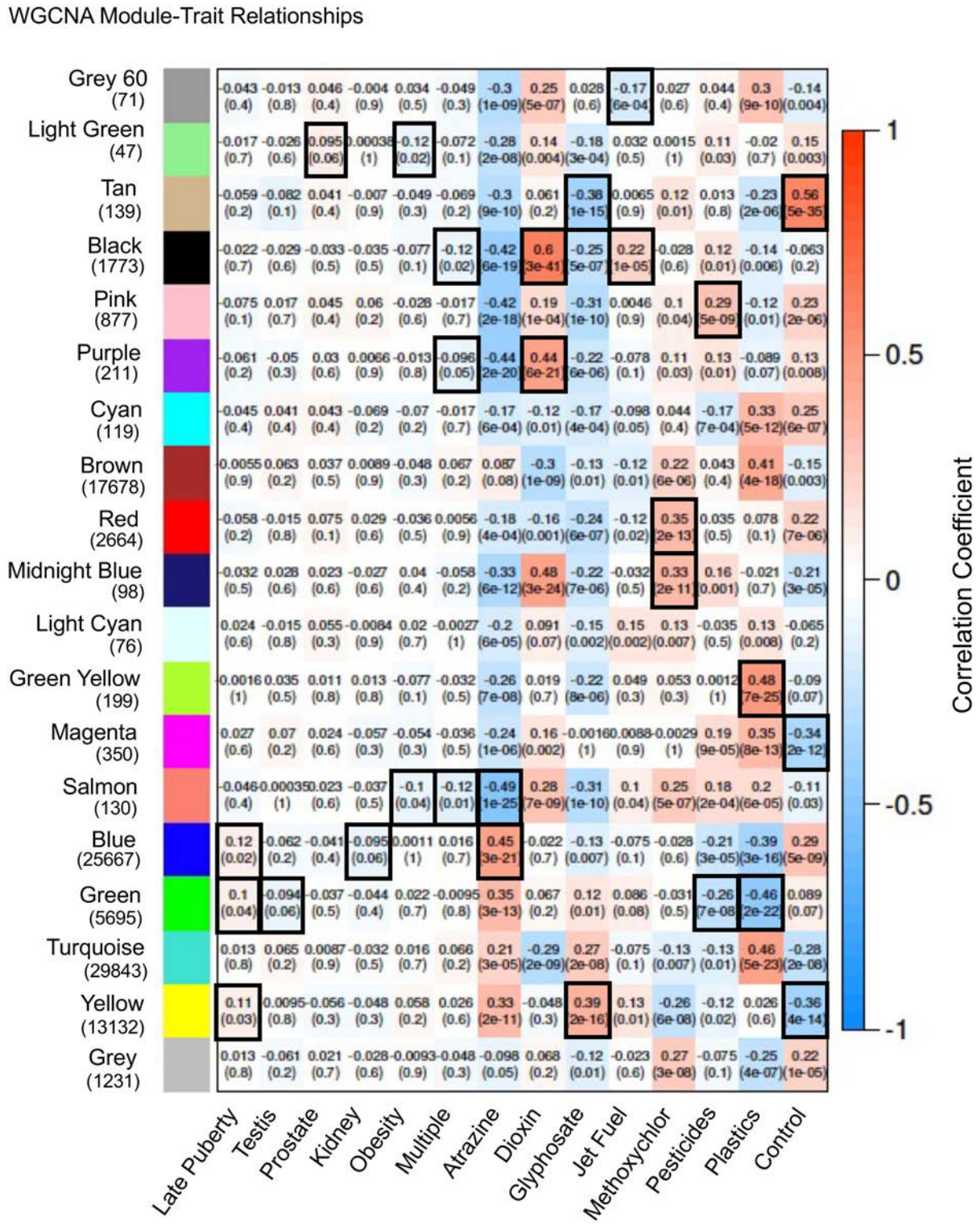


Figure 4. Weighted co-expression network analysis WGCNA module-trait relationships. Module colors and genomic window numbers listed correlate to specific d seases and exposures. The Correlation Coeffici t and *p* value (brackets) for each presented. Black outline correlations used for subsequent analysis.

all had exposure specific epigenetic DNA methylation alterations called epimutations (Fig. 1). Although the different toxicants promoted distinct subsets of epigenetic alterations, the disease and pathology phenotypes observed were similar for the different exposures, Fig. 2. Therefore, the alterations of various exposure subsets of unique differential DNA methylation regions (DMRs) were observed and associated with unique subsets of genes to promote altered transcriptomes associated with similar disease phenotypes, Fig. 7B.

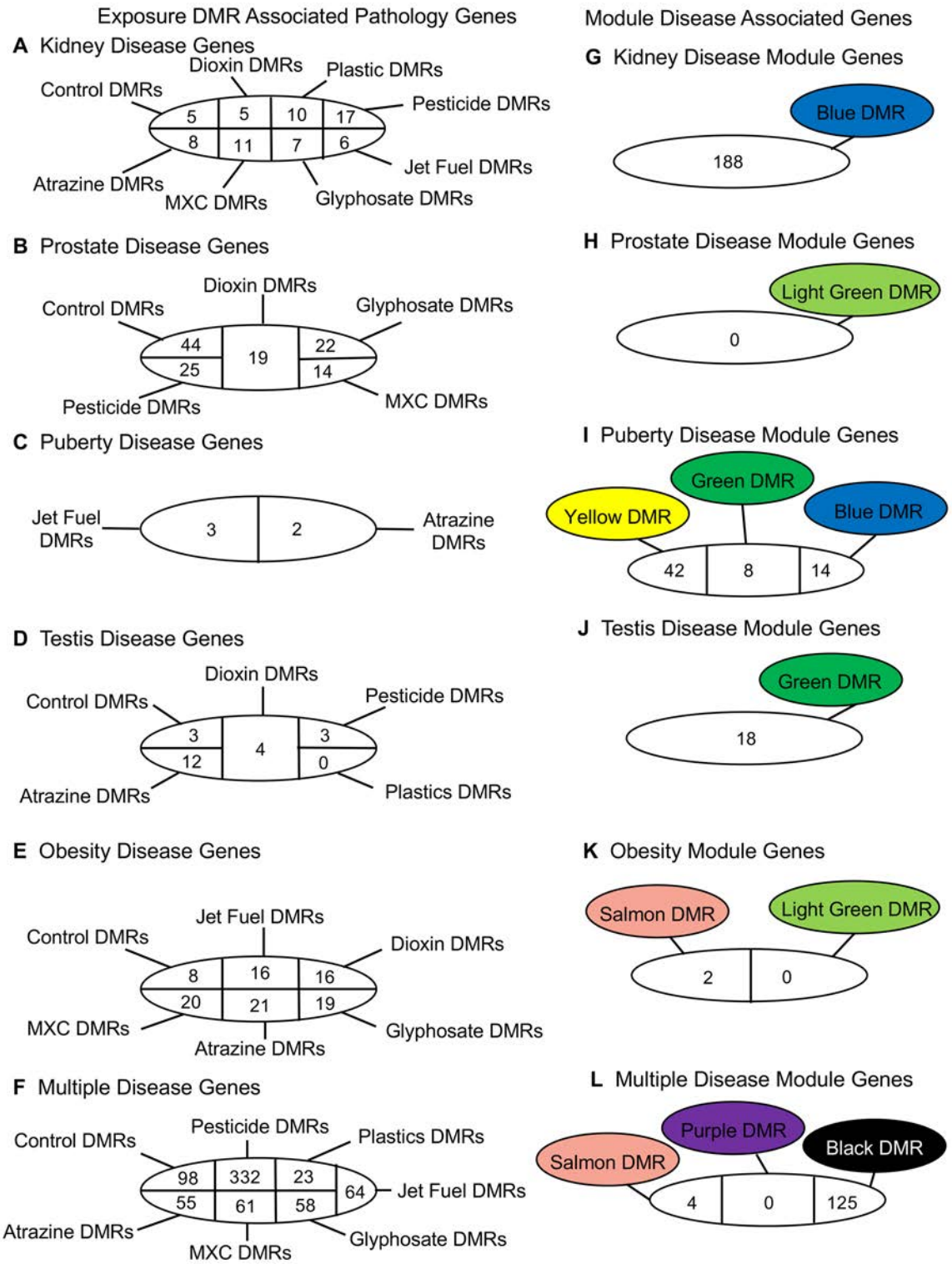


Figure 5. Exposure DMR associated pathology genes. Pathologies and specific number of DMR per exposure: (**A**) Kidney; (**B**) Prostate; (**C**) Puberty; (**D**) Testis; (**E**) Obesity; (**F**) Multiple. Module disease associated genes; (**G**) Kidney disease module associated genes; (**H**) Prostate disease module associated genes; (**I**) Puberty disease module associated genes; (**J**) Testis disease module associated genes; (**K**) Obesity disease module associated genes. (**L**) Multiple disease module associated genes.

The exposure-induced epigenetic transgenerational inheritance of disease etiology was further investigated with an examination of pathology specific epigenetic biomarkers for disease, Fig. 2. The various toxicants were

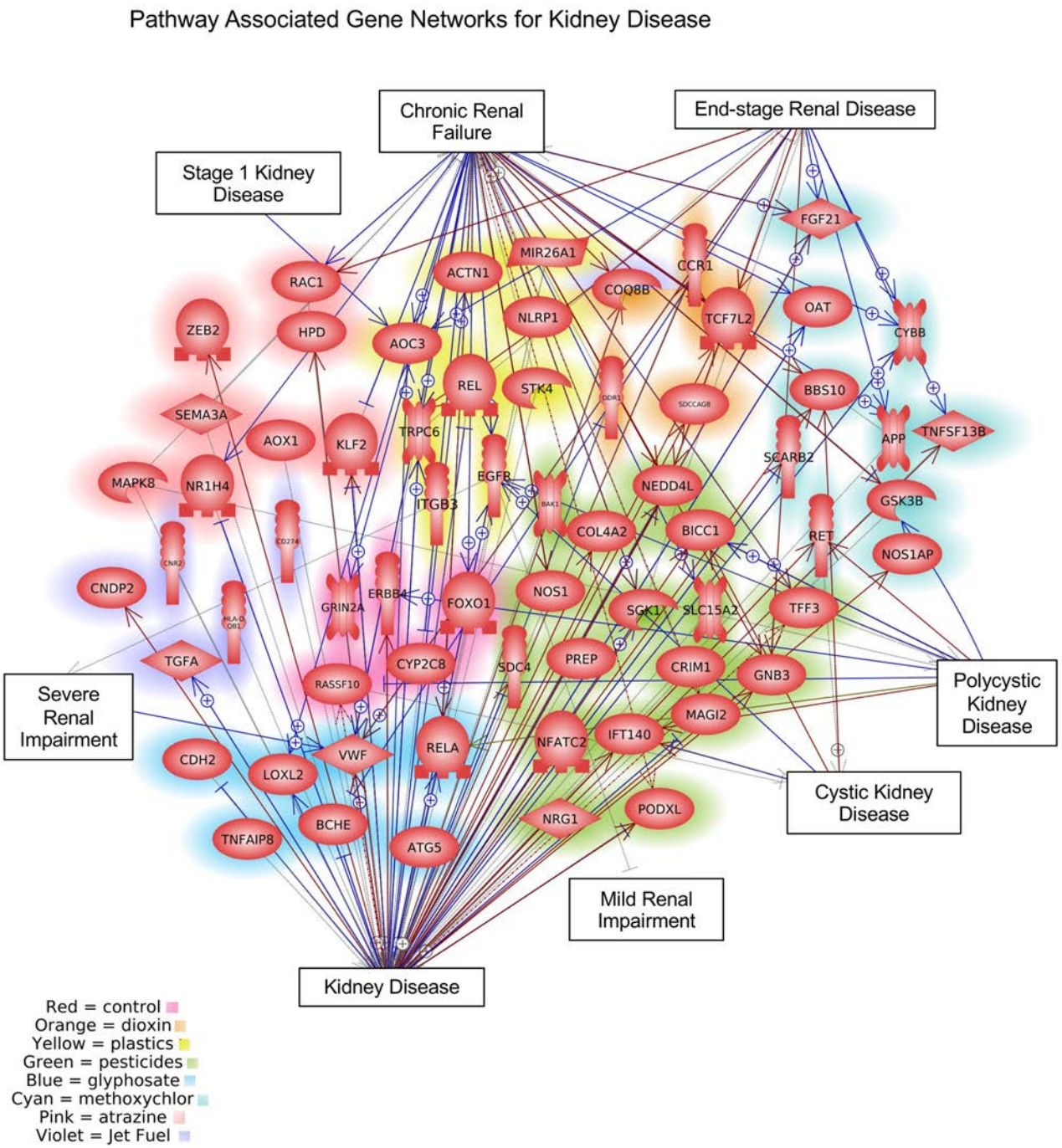


Figure 6. Kidney disease DMR associated genes and network. The index presents the color and exposure group for DMR associated genes. The disease pathways and processes with gene links identifi d.

found to promote similar transgenerational disease and pathologies[21–27]. Comparison of the exposure-specific disease DMR biomarkers demonstrated negligible overlap, Fig. 2. Therefore, the different exposure disease versus non-disease DMR sets were found to be primarily distinct and associated with unique genes, but promote similar disease phenotypes. When the associated genes were identifi d for specific diseases and pathologies, unique subsets of genes previously associated with the specific pathologies were identifi d, Fig. 6, Supplemental Fig. S4, and Fig. 7B. Observations suggest large numbers of genes are associated with specific pathologies and small subsets induced through environmental epigenetics can promote disease susceptibility and etiology. These observations are in contrast to the classic genetic determinism concept where a limited number of specific genetic mutations and associated genes are the primary regulators of disease etiology.

Analysis of control animals in the absence of any environmental toxicant exposures also had similar pathologies when sufficient numbers of animals were combined, due to the low frequency of pathology in the control populations, Supplemental Table S1 and Fig. S1. The epigenetic DMR biomarkers for the specific pathologies

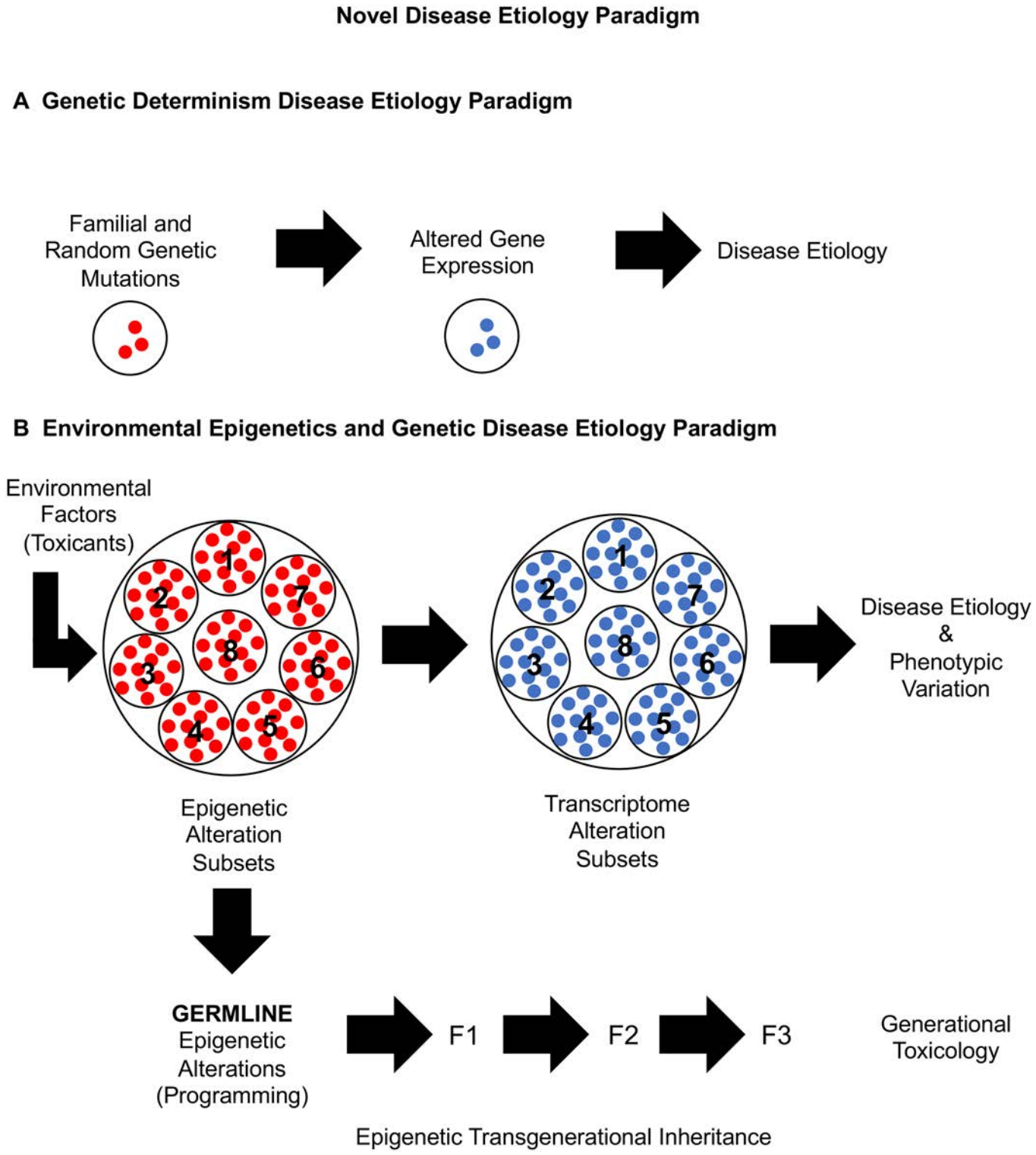


Figure 7. Novel disease etiology paradigm. (**A**) Genetic determination disease etiology paradigm. (**B**) Environmental epigenetics and genetic disease etiology paradigm. Environmental exposures promote subsets of distinct subsets of DMR associated sites that are associated with subsets of DMR linked genes that when altered promote disease etiology and phenotypic variation. The subsets of altered epimutations are transmitted to subsequent generations through the germline to promote generational toxicology.

were compared with the toxicant exposure diseased vs non-diseased transgenerational DMR sets. The control populations also had a unique subset of epimutations that were associated with genes previously shown to be

involved in those pathologies, Supplemental Table S1. This observation suggests any exposure or natural environment impacts that alters specific subsets of epimutations will then alter small subsets of genes associated with specific pathologies to increase disease susceptibility and etiology, Fig. 7B.

The alternate approach used for the analysis examined the correlations of DNA methylation sites throughout the genome, in contrast to DMR analysis. The weighted genome coexpression network analysis (WGCNA) is useful to assess genome-wide trends and patterns in the genome to correlate with genomic characteristics[37,38,44]. Th current study extends this to the epigenome (i.e., DNA methylation) and compared this with the gene associations. The WGCNA identifi d strong correlations with the exposures and different modules of DNA methylation site information, Fig. 4. Although the pathology data from all the exposures was also found to correlate, this was at a reduced significance compared to the exposures. The modules with significant correlations were identified, and the associated DNA methylation site genes identifi d. As was observed with DMRs, the WGCNA module data had exposure specific patterns, as well as pathology specific module patterns. When the module associated genes for specific pathologies were examined, subsets of DNA methylation sites were identifi d. Therefore, as was observed with the DMR analysis data in Figs. 1 and 3, the WGCNA data also support the disease etiology paradigm of environmental epigenetics and genetics, Fig. 7B. Environmentally induced epigenetic alterations promote subsets of epimutations that impact expression of subsets of genes that promotes disease susceptibility and pathologies. Examples are provided for specific disease in Fig. 6 and Supplemental Fig. S4.

The current study focused on transgenerational disease observed in the F3 generation following the exposure of gestating females in the F0 generation[17,18,31–34,36]. The epigenetic transgenerational inheritance of disease supports this novel disease etiology paradigm of the inclusion of epigenetics and genetics, Fig. 7. The direct exposure of toxicants to promote epigenetic alterations and later life disease etiology involves the same disease etiology paradigm as the transgenerational model. The inclusion of environmental epigenetics in disease etiology is required to integrate with the classic genetic determinism paradigm. Observations demonstrate that the toxicants not only affect the first generation exposed, but also transmit this through the germline to subsequent generations. Th s is referred to as "generational toxicology"[18]. Previously, this has not been considered in disease etiology nor in toxicology. The ability of an environmental toxicant to promote pathologies in subsequent generations dramatically impacts the hazards of toxicants[6]. Exposures such as glyphosate, atrazine, or pesticides had negligible effects on the F1 generation, so negligible direct exposure toxicity, but had dramatic effects at later generations, such as the great grand-offspring F3 generation, that does not have any direct exposure[18,32,36]. The proposed environmental epigenetics disease etiology paradigm, Fig. 7B, helps explain this generational toxicology phenomenon, and further supports the integration of environmental epigenetics in disease etiology.

The current study used seven different environmental toxicants to induce the epigenetic transgenerational inheritance of various diseases and pathologies. Comparison of the DMRs demonstrated subsets of distinct epimutations in sperm that had subsets of distal gene associations that promoted similar pathologies. Th s was also assessed in a WGCNA and demonstrated DNA methylation site modules that supported the same inclusion of environmental epigenetics and genetics in disease etiology, Fig. 7B. Therefore, environmental epigenetics impacts subsets of epimutations, that impact subsets of gene expression alterations, that promote disease susceptibility and etiology. This novel paradigm for disease etiology incorporates epigenetics and genetics to address the limitations of the classic disease etiology paradigm of genetic determinism. In addition, this helps clarify the molecular mechanisms of generational toxicology.

Methods

Animal studies and breeding. As previously described[17,18,31–34,36], 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. All animal cages were housed in the same room and environment with gestating females and females with litters being housed individually within cages. Conditions were designed to minimize differences that would cause maternal effects. The breeding of unrelated males and females within specific exposure lineages (interbreeding) was used to optimize the maternal and paternal lineage contributions to the phenotypes observed[30]. No inbreeding within the colonies was performed. Generally, six unrelated breeding pairs at the F0 generation were used to generate the subsequent generations. Timed-pregnant females were mated and on embryonic days 8 through 14 (E8–E14) of gestation were administered daily intraperitoneal injections of the treatment compounds (Dioxin TCDD 100 ng/kg BW/day; glyphosate (25 mg/kg BW/day; JP-8 hydrocarbon jet fuel 500 mg/kg BW/day; permethrin 150 mg/kg BW/day and insect repellent DEET 40 mg/kg/BW/day; BPA 50 mg/kg BW/day, phthalate DEHP 750 mg/kg BW/day and phthalate DBP 66 mg/kg/BW/day; methoxychlor 200 mg/kg BW/day; atrazine 25 mg/kg BW/day) or vehicle control dimethyl sulfoxide (DMSO) or in the case of glyphosate, phosphate buffered saline (PBS), as previously described[17,18,31–34,36].

The gestating female rats exposed were designated as the F0 generation. F1–F3 generation control and exposure 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 exposure or control lineages were bred within their treatment group to obtain F2 generation off pring. 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 exposure 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 & 6931). All methods were performed in accordance with the relevant guidelines and regulations. The excess sperm samples and paraffi tissue sections stored (i.e., archived) from the previous studies[17,18,31–34,36] were used and reanalyzed for the current study.

Tissue harvest and histology processing. As previously described[17,18,31–34,36], at 12 months of age, rats were euthanized by CO_2 inhalation and cervical dislocation for tissue harvest. Testis, prostate, and kidney were fi ed in Bouin's solution (Sigma) followed by 70% ethanol, then processed for paraffi embedding, and hematoxylin and eosin (H & E) staining by standard procedures for histopathological examination. Paraffi five micron sections were processed and stained by Nationwide Histology, Spokane WA, USA. Archived glass slides with hematoxylin and eosin stained tissue sections were stored at room temperature for use and reanalysis for the current study.

Histopathology examination and disease classification. Archived histology slides from previous studies[17,18,31–34,36], were reanalyzed and 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 certifi d 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[17,31–34]. 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. The more advanced pathology analysis used involved larger areas of the tissue sections to be analyzed to optimize pathology detection and more consistent multiple readers blinded to the section identity used for assessment of pathology.

Specific descriptions of histopathological analysis and example images were previously reported[17,18,31–34,36]. 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, Supplemental Fig. S5. Prostate histopathology criteria included the presence of vacuoles in the glandular epithelium, atrophic glandular epithelium and hyperplasia of prostatic gland epithelium, Supplemental Fig. S6. Kidney histopathology criteria included reduced size of glomerulus, thickened Bowman's capsule, and the presence of proteinaceous fluid-filled cysts > 50 µm in diameter, Supplemental Fig. S7. 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. Th s 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 fi ally 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, obesity frequency). Groups were analyzed using Fisher's exact test.

Epididymal sperm collection and DNA isolation. The protocol used is as previously described[45]. 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 250 µL NIM buffer and stored at − 80 °C for further processing. An appropriate amount of rat sperm suspension (approximately 50 µl) was used for DNA extraction. Previous studies have shown mammalian sperm heads are resistant to sonication unlike somatic cells[46,47]. Somatic cell contamination and debris were removed by brief sonication (Fisher Sonic Dismembrator, model 300, power level 25), which destroys the somatic cells, then centrifugation and washing 1–2 times in 1× PBS. The resulting purifi d sperm 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×*g* 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 (MeDIP). The archived sperm samples were prepared as previously described[45]. Genomic DNA was sonicated and run on 1.5% agarose gel for fragment size verifi ation. The sonicated DNA was then diluted with 1× 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 one to several hours. The DNA precipitate was pelleted, washed with 70% ethanol, then dried and resuspended in 20 µl H_2O or 1× TE. DNA concentration was measured in a Qubit apparatus (Life Technologies) with the ssDNA analysis kit (Molecular Probes Q10212). The more advanced protocol used involved the new reagent kits and optimal procedures for the MeDIP[30].

MeDIP-Seq analysis. 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[30]. After this step, the manufacturer's protocol was followed indexing each sample individually with NEBNext Multiplex Oligos for Illumina[30]. The WSU Spokane Genomics Core sequenced the samples on the Illumina HiSeq 2500 at PE50, with a read size of approximately 50 bp and approximately 10–20 million reads per pool. Twelve libraries were run in one lane.

Statistics and bioinformatics. The DMR identifi ation and annotation methods follow those presented in previous published papers[36,45]. Data quality was assessed using the FastQC program (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The data was cleaned and filtered to remove adapters and low-quality bases using Trimmomatic[48]. The reads for each MeDIP sample were mapped to the Rnor 6.0 rat genome using Bowtie2[49] with default parameter options. The mapped read files were then converted to sorted BAM files using SAMtools[50]. The MEDIPS R package[51] was used to calculate differential coverage between disease and non-disease sample groups. The edgeR p value[52] was used to determine the relative difference between the two groups for each genomic window. Windows with an edgeR p value less than the selected $p < 1e{-}06$ threshold for all exposures, except glyphosate at $p < 1e{-}04$, 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 signifi ance of the DMR identifi d. A false discovery rate (FDR) analysis for each comparison was performed and provided a $p < 0.1$ for all the exposure comparisons, Supplemental Tables S9–S15. Due to the relatively low number of individuals with one specific disease type, the previous published FDR analysis of the specific disease DMR biomarkers[53–58] were generally between 0.1 and 0.3, depending on the specific exposure diseases[53–58], (Supplemental Tables S16–S20). The toxicant exposure associated disease DMRs were annotated using the biomaRt R package[59] to access the Ensembl database[60], as described in the original publications. The DMR associated genes were then automatically sorted into functional groups using Panther[61] (www.skinner.wsu.edu under genomic data). The exposure DMRs, WGCNA methylation sites, and the control disease DMRs were annotated using a modifi d method. These sites were annotated using NCBI provided gene information. Genes were sorted into categories by converting Panther (25) protein classifi ations into more general groups. 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 # GSE98683 (atrazine), GSE155922 (jet fuel), GSE157539 (dioxin), GSE158254 (pesticides), GSE158086 (methoxychlor), GSE163412 (plastics), GSE152678 (glyphosate), and GSE198452 (control)] and R code computational tools available at GitHub (https://github.com/skinnerlab/MeDIP-seq) and www.skinner.wsu.edu.

Weighted gene coexpression network analysis (WGCNA). The weighted correlation network analysis (WGCNA)[62] was performed using the WGCNA R package[63]. All MeDIP-Seq genomic windows were ranked by the mean RPKM read depth across all samples. The top 100,000 sites were chosen for inclusion in the analysis. The size of this subset was chosen to allow for a reasonable read depth to be considered and to limit computational time (< 1 week) requirements. WGCNA is a parameter rich analysis and only limited exploration of parameter variations was performed. Modules were calculated using the *blockwiseModules* function with the following parameters: maxBlockSize = 15,000, power = 4, TOMType = "unsigned", minModuleSize = 30, reassignTh eshold = 0, and mergeCutHeight = 0.25. The Pearson correlation was calculated for each development stage and module. The p value for each correlation was calculated using the *corPvalueStudent* function. Sites within each module were annotated using the same methods as the DMRs.

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 & 6931). All methods were performed in accordance with the relevant guidelines and regulations. Th s study was carried out in compliance with the ARRIVE guidelines.

Data availability

All molecular data has been deposited into the public database at NCBI [GEO # GSE98683 (atrazine), GSE155922 (jet fuel), GSE157539 (dioxin), GSE158254 (pesticides), GSE158086 (methoxychlor), GSE163412 (plastics), GSE152678 (glyphosate), and GSE198452 (control)] and R code computational tools available at GitHub (https://github.com/skinnerlab/MeDIP-seq) and www.skinner.wsu.edu.

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Author contributions

D.B.: Bioinformatic analysis, data analysis, wrote and edited manuscript. E.N.: Animal studies, cell isolations, data analysis, edited manuscript. M.B.M.: Molecular analysis, edited manuscript. M.K.S.: Conceived, oversight, obtained funding, data analysis, wrote and edited manuscript.

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Competing interests
The authors declare no competing interests.

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