

RESEARCH ARTICLE

Epigenetic transgenerational inheritance of testis pathology and Sertoli cell epimutations: generational origins of male infertility

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Abstract

Male reproductive health has been in decline for decades with dropping sperm counts and increasing infertility, which has created a significant societal and economic burden. Between the 1970s and now, a general decline of over 50% in sperm concentration has been observed in the population. Environmental toxicant-induced epigenetic transgenerational inheritance has been shown to affect testis pathology and sperm count. Sertoli cells have an essential role in spermatogenesis by providing physical and nutritional support for developing germ cells. The current study was designed to further investigate the transgenerational epigenetic changes in the rat Sertoli cell epigenome and transcriptome that are associated with the onset of testis disease. Gestating female F0 generation rats were transiently exposed during the period of fetal gonadal sex determination to the environmental toxicants, such as dichlorodiphenyltrichloroethane (DDT) or vinclozolin. The F1 generation offspring were bred (i.e. intercross within the lineage) to produce the F2 generation grand-offspring that were then bred to produce the transgenerational F3 generation (i.e. great-grand-offspring) with no sibling or cousin breeding used. The focus of the current study was to investigate the transgenerational testis disease etiology, so F3 generation rats were utilized. The DNA and RNA were obtained from purified Sertoli cells isolated from postnatal 20-day-old male testis of F3 generation rats. Transgenerational alterations in DNA methylation, noncoding RNA, and gene expression were observed in the Sertoli cells from vinclozolin and DDT lineages when compared to the control (vehicle exposed) lineage. Genes associated with abnormal Sertoli cell function and testis pathology were identified, and the transgenerational impacts of vinclozolin and DDT were determined. Alterations in critical gene pathways, such as the pyruvate metabolism pathway, were identified. Observations suggest that ancestral exposures to environmental toxicants promote the epigenetic transgenerational inheritance of Sertoli cell epigenetic and transcriptome alterations that associate with testis abnormalities. These epigenetic

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alterations appear to be critical factors in the developmental and generational origins of testis pathologies and male infertility.

Key words: Sertoli cell; male infertility; testis pathology; transgenerational; vinclozolin; DDT

Introduction

Epimutations were originally defined as ‘heritable epigenetic changes in chromosomes which do not involve changes in the DNA sequence itself’ [1, 2]. A more updated definition of epigenetics is ‘molecular factors and processes that regulate genome activity independent of DNA sequence and are mitotically stable’ and for epimutations is ‘environmentally induced differential presence of epigenetic alterations that can influence genome activity compared to organisms not having the exposure’ [3]. A number of environmental factors can alter epigenetic processes such as DNA methylation, histone modifications (e.g. methylation and acetylation), chromosome structure, noncoding RNA (ncRNA), and RNA methylation to influence gene expression and genome activity. Epigenetic transgenerational inheritance describes a germline transmission of epimutations through generations without continued exposure to or presence of the original exposure beyond the F0 generation [3]. In cases where the germline is exposed during fetal gonadal sex determination, the epigenome in the germline can be modified. These epimutations appear to become ‘imprinted-like’, and potentially escape post-fertilization methylation erasure to allow transmission to the subsequent generations [4, 5]. The hypothesis is that this promotes stem cells in the embryo to develop an altered epigenome and transcriptome. Somatic cells and tissues derived from these epigenetically altered germline and stem cells will potentially develop alterations in cell type-specific epigenomes and transcriptomes [6]. These cell specific altered epigenomes will impact the fate of organs and tissues, and may be early indicators and initiators of the development of disease susceptibility later in life [7–9]. Previous examples of this phenomenon have been observed in prostate epithelial cells for prostate disease [10, 11], and granulosa cells for ovarian disease [12].

Numerous studies have shown epigenetic transgenerational inheritance to occur in different species involving a wide variety of different environmental exposures. Epigenetic transgenerational inheritance has been shown in plants [13], flies [14], worms [15], fish [16], birds [17, 18], rodents [5, 19], pigs [20], and humans [21]. Environmentally induced transgenerational diseases and abnormalities in mammals have been observed such as testis disease [8, 22, 23], prostate disease [9, 24], kidney disease [9, 24, 25], obesity [26], ovarian and uterine disease [22, 25, 27–30], tumor development [9], and behavioral changes [31–36]. A large number of environmental toxicants have been shown to be involved in triggering these transgenerational diseases and abnormalities that include the agricultural fungicide vinclozolin [5, 37–39], the herbicide atrazine [31, 40], plasticizers such as bisphenol A [22, 41] and phthalates [22], insect repellent diethyltoluamide (DEET) with the insecticide permethrin [27], pesticide methoxychlor [25], hydrocarbons (jet fuel) [28], and industrial compounds such as benzo[a]pyrene [42], the biocide tributyltin [43], mercury [16], dioxins [24, 29, 44], and the herbicide glyphosate [45].

The current study was designed to examine the ability of vinclozolin and dichlorodiphenyltrichloroethane (DDT) to induce transgenerationally affected Sertoli cell epigenome and

transcriptome alterations that associate with testis pathology. Sertoli cells play a critical role in spermatogenesis providing structural and nutritional support for the developing germ cells and are involved in the formation of the blood–testis barrier which creates a serum and pathogen free environment for the spermatogenic cells within the seminiferous tubules [46]. Therefore, Sertoli cells synthesize a number of transport binding proteins [47, 48] and provide the primary energy metabolites (i.e. pyruvate and lactate) for the developing germ cells, which are sequestered within the blood–testis barrier and unable to acquire glucose themselves [49]. A disruption in normal Sertoli cell development and function can affect spermatogenesis and promote testis pathology. Abnormal spermatogenesis and low sperm count are often linked to infertility, which can involve testis diseases like cryptorchidism, hypospadias, and testicular cancer [50–52]. Sperm counts have been declining significantly between 1973 and 2011, as shown in a 2017 meta-regression analysis [53]. Environmental factors such as endocrine disrupting chemicals, pesticides, heat, diet, stress, and smoking have been shown to be associated with this sperm count drop, and general testis health problems [53]. The molecular basis for these testis pathologies and generational impacts are investigated in the current study.

Exposure of gestating rats to the environmental toxicants such as DDT and vinclozolin leads to the epigenetic transgenerational inheritance of adult onset diseases, including testis disease and a decrease in sperm count and/or motility [26, 54]. Originally, vinclozolin’s involvement in epigenetic transgenerational inheritance of disease was observed in 2005 by Anway *et al.* [5] who found that vinclozolin exposure of gestating rats leads to epigenetic transgenerational inheritance of unique DNA methylation changes (epimutations) in sperm. Subsequent studies involving vinclozolin and DDT among other environmental toxicants confirmed these findings [26, 39, 55, 56]. Vinclozolin is an agricultural fungicide used in fruit and vegetable production and is an anti-androgenic compound that acts as a competitive antagonist of the androgen receptor [57]. DDT is a pesticide, which was widely used through the 1950s and 1960s in the USA until banned in 1972. It continues to be used in many parts of the world for insect and malaria control. DDT accumulates in the environment and in fatty tissue, and is an estrogen receptor agonist that has estrogenic effects in animals [58].

The epigenetic transgenerational inheritance phenomenon requires the germline transmission of altered epigenetic information between generations [59]. A variety of different environmental factors promoting epigenetic transgenerational inheritance were found to induce exposure specific alterations in sperm DNA methylation [5, 59]. Subsequently, vinclozolin was found to promote alterations in sperm ncRNA transgenerationally [60]. This supported previous studies indicating ncRNA germline alterations are important factors in epigenetic transgenerational inheritance [61, 62]. Recently, we observed that both vinclozolin and DDT cause concurrent alterations in cauda epididymal rat sperm DNA methylation, ncRNA, and histone retention [55, 56]. Therefore, several different epigenetic processes are likely integrated in epigenetic transgenerational inheritance. A 2013 study using vinclozolin determined that

vinclozolin impacts the epigenetic transgenerational inheritance of Sertoli cell DNA methylation and gene expression alterations [8]. This current study extends these findings with genome-wide analyses of DNA methylation and ncRNA alterations, and associated gene expression changes. These transgenerational alterations in Sertoli cell epigenetics correlate to corresponding alterations in testis pathology.

Results

Experimental Design and Testis Pathology

The F0 generation gestating female rats were exposed at approximately 90 days of age to DDT or vinclozolin during gestational days E8–E14, which corresponds to fetal gonadal sex determination and the germline differentiation period of development. The toxicants were dissolved in dimethylsulfoxide (DMSO) and administered by daily intraperitoneal injection during the transient exposure time frame. A separate group of control females was injected on the same schedule with only DMSO as a vehicle control. Each exposure lineage involved six different F0 generation females and was referred to as DDT, vinclozolin, and control lineages, respectively. This study and experimental approach were not designed for risk assessment, but to investigate the transgenerational phenomenon. The F1 generation animals were raised to 90 days of age and then bred within each lineage to obtain the F2 generation animals. The F2 generation animals were bred in the same manner to obtain the F3 generation animals. The only animals directly exposed were the F0 generation females. No sibling or cousin breeding was performed to avoid inbreeding artifacts. The male F3 generation pups were raised to 1 year of age for testis pathology analysis or to 18–22 days of age for isolation of Sertoli cells. The 20-day-old male pups were randomly divided into 3 different groups from different litters for each lineage with each group comprising 6–11 animals depending on litter sizes obtained. Within each group, the testis tissues were combined into one pool for isolation of Sertoli cells. The total Sertoli cell pools from each group were divided into two aliquots and stored as cell pellets at -80°C for DNA and RNA preparations.

The F3 generation Sertoli cells were isolated at 18–22 days of age to obtain optimally purified populations of cells. The purity of the cell preparations is routinely monitored, and has previously been shown to be $>98\%$ Sertoli cells by histology and subsequent culture purity determined with immunocytochemistry for fibronectin containing cells such as peritubular cells, as previously described [8, 63–65]. A cell fraction at this stage of 18–22 days of development allows for efficient analysis, and reduces the impact of testis pathology on the analysis since the majority of pathologies develop between 6 and 12 months of age. Therefore, at this early stage of development, the study is not confounded by the presence of disease. Another group of F3 generation animals was aged to 1 year and used to assess testis pathology. Testes were evaluated by microscopic examination of testis sections. Testis pathology was determined by the presence of atrophy of seminiferous tubules, vacuoles in the seminiferous epithelium, and sloughed spermatogenic cells in the tubule lumens, as described in Methods. Representative histopathologies are presented in [Supplementary Fig. S1](#). In the current study, DDT was found to promote testis disease in 46% of the males compared to 8% in the control lineage animals ([Fig. 1a](#)). Vinclozolin promoted a 44% frequency of testis pathology in the males ([Fig. 1a](#)). Therefore, both vinclozolin and DDT promoted the epigenetic transgenerational inheritance of testis

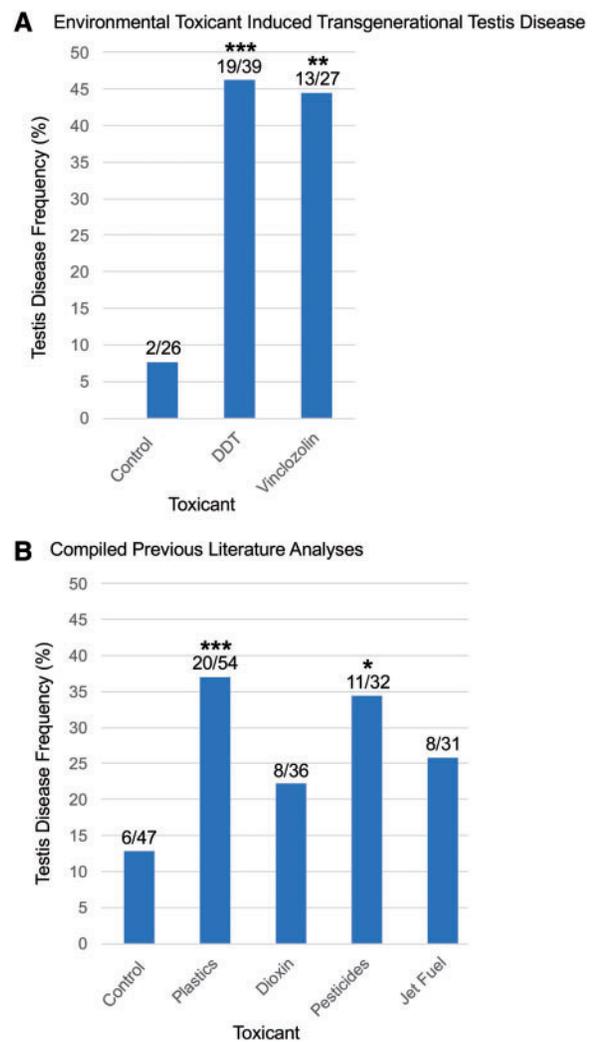


Figure 1: transgenerational induced testis disease frequency. Environmentally induced transgenerational testis disease occurrence in several separate experiments. (a) Current study vinclozolin and DDT-induced transgenerational testis disease with associated control [66, 67]. (b) Previous reports of transgenerational pathology for plastics [22, 41], dioxin [24], pesticides [27], and jet fuel hydrocarbons [28] with associated control frequency. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

pathology, as previously described [66, 67]. The group of animals sacrificed at 18–22 days of age for Sertoli cell preparations were used to investigate the molecular mechanisms in part involved in this transgenerational testis pathology.

Genomic DNA and RNA were isolated separately from the Sertoli cell pool aliquots from each group, and then used for methylated DNA immunoprecipitation (MeDIP) sequencing or RNA sequencing, as described in the Methods. Each of the pools contained 6–11 different animals per pool from different litters. The 9 MeDIP samples (3 control lineage pools, 3 DDT lineage pools, and 3 vinclozolin lineage pools) were enriched for methylated DNA using a MeDIP procedure employing a 5-methylcytosine specific antibody and antibody specific magnetic beads, or were processed to isolate the different RNA categories (total RNA for mRNA and long ncRNA or small ncRNA). Sequencing libraries were created for each MeDIP or RNA pool for next-generation sequencing (NGS) and run on the Illumina 2500 sequencer for MeDIP-Seq or RNA-Seq by the WSU Genomics Core

Laboratory. The procedures and bioinformatics details are described in the Methods section.

Sertoli Cell DNA Methylation Analysis

The MeDIP-Seq information was used to identify differential DNA methylated regions (DMRs) based on comparisons between control and DDT or vinclozolin lineage sequencing data. Read depth comparisons of the NGS reads identified DMRs for each of the analyses with a range of threshold P-values (Fig. 2). The DDT lineage Sertoli cell DMRs were abundant, and a $P < 1e-06$ was selected for further analysis. At $P < 1e-06$ 5359 DMRs were found with the majority of them having a single 100bp statistically significant window, however, 533 DMRs had ≥ 2 significant windows (Fig. 2a). The corresponding false discovery rate (FDR) analysis of 0.001 correlated to the edgeR value of $P < 1e-06$ identified. The vinclozolin lineage Sertoli cell DMRs were less abundant and a $P < 1e-06$ was selected for further analysis. At this P-value 122 DMRs were found with the majority of 108 having a single 100bp significant window DMR (Fig. 2b). All the DMR had an FDR adjusted P-value of less than 0.05. The DDT and vinclozolin lineage Sertoli cell DMRs were primarily distinct from each other with only 29 DMRs in common (Fig. 2c). This is notable, since one-fourth of the small number of DMRs in the vinclozolin lineage overlapped with the DDT lineage DMRs. The lists of DDT Sertoli cell DMRs are presented in Supplementary Table S1 and the vinclozolin Sertoli cell DMRs in Supplementary Table S2. The 29 overlapping DMRs are presented in Supplementary Table S3. The increase or decrease in DNA methylation is presented for each DMR in Supplementary Tables S1 and S2 with a log fold change indicated (exposure/control). A positive fold change is an increase in DNA methylation and negative value a decrease in DNA methylation. For the DDT lineage Sertoli cell DMRs, 70% had an increased log fold change in methylation, while 30% had a decrease in methylation. For the vinclozolin lineage Sertoli cell DMRs, 41% increased and 59% decreased in methylation. The MeDIP procedure does not identify individual CpG site DNA methylation changes, but the mean change for the DMR regions. The majority of the DMRs had 1–3 CpGs per 100 bp and were 1 kb in length so the analysis provides a mean change across the CpGs within the DMR.

The chromosomal locations of the DDT and vinclozolin transgenerational affected Sertoli cell DMRs are presented in Fig. 3. The DDT Sertoli cell DMRs are presented with the ≥ 2 windows number in Fig. 3a due to the inability to present the very large all window number of DMRs. The vinclozolin Sertoli cell DMRs for all windows are presented in Fig. 3b. All chromosomes contain DMRs, except the Y chromosome in the vinclozolin Sertoli cell lineage. Further analysis of the DMRs identified the percentage of intergenic, gene-associated, and repeat element-associated DMRs in regard to chromosomal locations. For DDT DMRs, 49% were apparently intergenic, 51% were associated with genes, and 84% were associated with a short <100bp repeat element within the full DMR length. For vinclozolin DMRs 50% were intergenic, 50% associated with genes, and 80% associated with a short <100bp repeat elements within the DMR region.

The numbers of DMRs at different CpG densities (CpG per 100bp) covering all DMRs at a P-value of $P < 1e-06$ for DDT (Supplementary Fig. S2A) and vinclozolin (Supplementary Fig. S2C) demonstrate a density of 1–4 CpG per 100bp over the entire DMR length (e.g. 1–2 kb). The predominant density is 1 CpG per 100bp, which is a low density CpG region across the DMR total length, and has previously been termed a CpG desert [68].

A DDT Induced F3 Generation Sertoli Cell DMRs

P-value	All Window	Multiple Window
0.001	80128	16038
1e-04	31313	4456
1e-05	12798	1425
1e-06	5359	533
1e-07	2250	247

Number of windows	1	2	3	4	5	6	>7
Number of DMR	4826	454	47	12	4	3	13

B Vinclozolin Induced F3 Generation Sertoli Cell DMRs

P-value	All Window	Multiple Window
0.001	13863	1390
1e-04	2817	211
1e-05	602	45
1e-06	122	14
1e-07	36	9

Number of windows	1	2	3	4	7
Number of DMR	108	10	2	1	1

C Overlap DDT and Vinclozolin DMRs

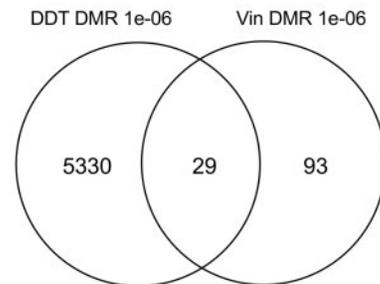


Figure 2: transgenerationally affected Sertoli cell DMRs. The number of DMRs found using different P-value cutoff thresholds. The All Window column shows all DMRs. The Multiple Window column shows the number of DMRs containing at least two significant windows. The number of DMRs with different P-value thresholds presented and the P-value threshold of $1e-06$ highlighted. (a) DDT lineage F3 generation Sertoli cells. (b) Vinclozolin lineage F3 generation Sertoli cells. (c) Venn diagram of the DDT and vinclozolin lineage DMRs at P-value $1e-06$

The length of the DMRs are presented for DDT lineage DMRs in Supplementary Fig. S2B and vinclozolin lineage DMRs in Supplementary Fig. S2D. The DMR length is assessed by extending the 100bp window at $P < 1e-06$ out at 100bp intervals until the $P < 0.05$ stringency is lost, as described in the Methods. The predominant DMR length is 1kb, and all of them cover a range of 1–5 kb. The numbers of DMRs beyond 5kb DMR length are few, but some DDT lineage DMRs appear at 10kb DMR length. Similar observations have been made in previous studies using DDT and vinclozolin lineage sperm DMRs [55, 56].

Sertoli Cell Coding and Noncoding RNA Analysis

Expression profiles of Sertoli cell noncoding RNAs, both long and small, were determined using RNA-Seq and the differential expression was analysed between the control and vinclozolin or DDT exposure lineages. A variety of P-values were used to determine a significance threshold, and $P < 1e-04$ was used for

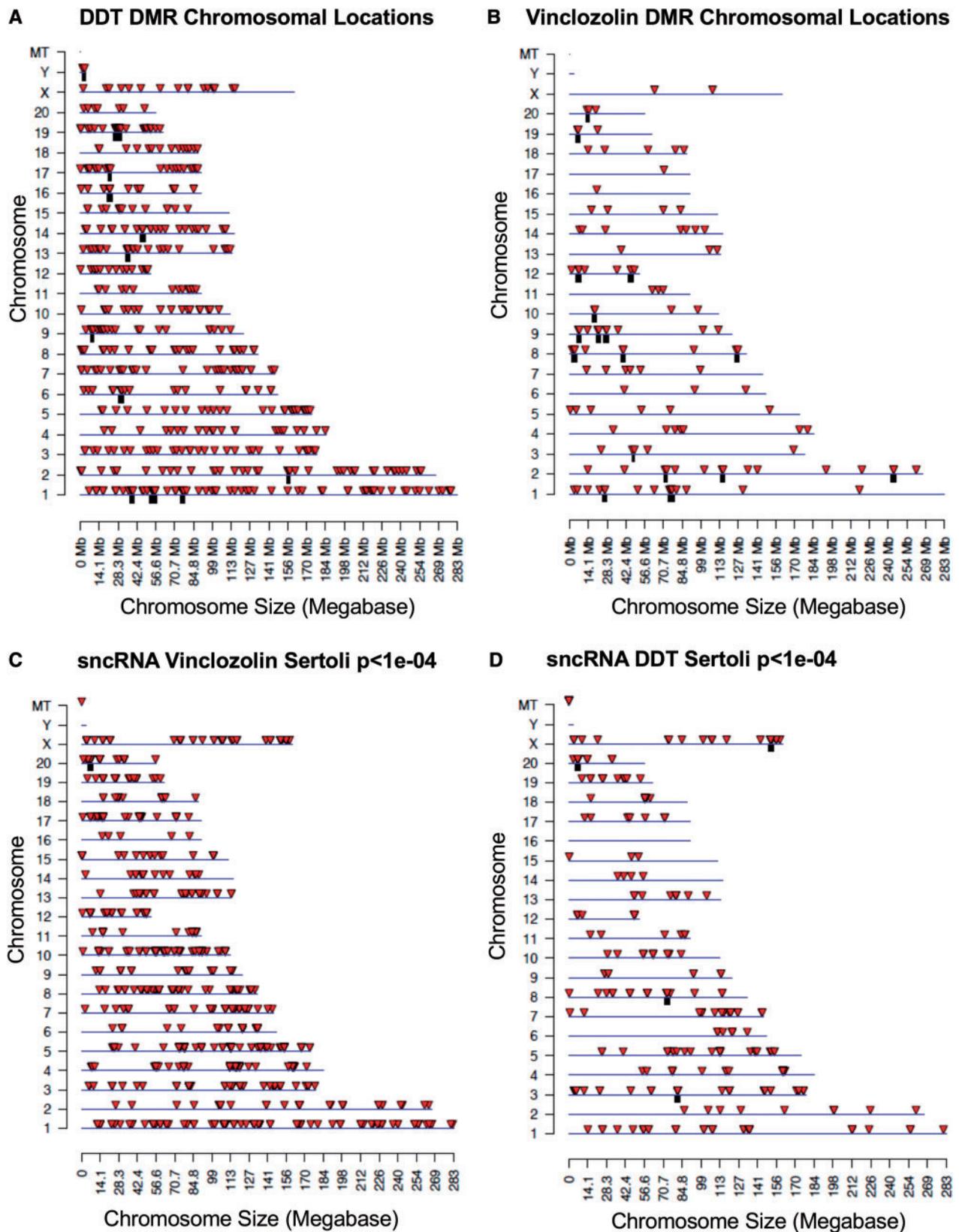


Figure 3: Sertoli cell DMR and snRNA chromosomal locations. (a) The DMR locations on the individual chromosomes. Multiple window DDT lineage DMRs at a P-value threshold of $1e-06$ are shown here. (b) The DMR locations on the individual chromosomes. All vinclozolin lineage DMRs at a P-value threshold of $1e-06$ are shown here. The red arrowheads identify the DMR sites and the black boxes the clusters of DMRs. Chromosomal locations of differentially expressed small noncoding RNAs (snRNAs). (c) Vinclozolin lineage differentially expressed snRNAs, while 220 snRNAs have an unknown location and are not shown. (d) DDT lineage differentially expressed snRNAs, while 31 snRNAs have an unknown location and are not shown. Black boxes represent clusters, while red arrowheads represent individual snRNAs, $P < 1 \times 10^{-4}$

A Differential RNA Expression

Raw.p.value	Vin.mRNA	Vin.lncRNA	Vin.sncRNA
0.001	3091	1578	4771
1e-04	1705	959	2920
1e-05	0	0	1926
1e-06	0	0	1272
1e-07	0	0	871

Raw.p.value	DDT.mRNA	DDT.lncRNA	DDT.sncRNA
0.001	1887	994	1286
1e-04	1050	578	489
1e-05	0	0	241
1e-06	0	0	149
1e-07	0	0	90

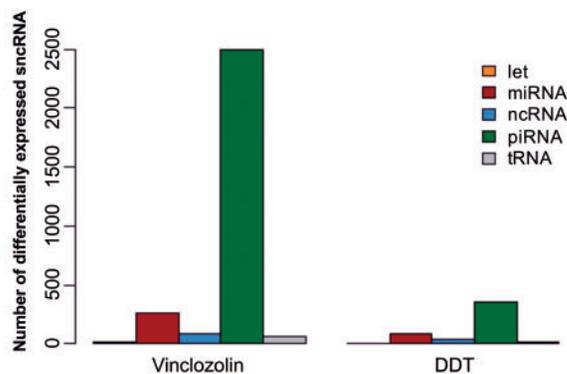
B Small RNA Category

Figure 4: differentially expressed RNAs. (a) The number of differentially expressed mRNAs, long noncoding RNAs (lncRNAs) and small noncoding RNAs (sncRNAs) at different P-value thresholds for vinclozolin (Vin) and DDT lineage F3 generation Sertoli cells. (b) Differentially expressed sncRNA gene categories at $P < 1 \times 10^{-4}$

subsequent analyses (Fig. 4). The vinclozolin lineage contained the highest number of differentially expressed mRNAs compared to the DDT lineage at 1705 and 1050 mRNAs, respectively (Fig. 4a). The differentially expressed long noncoding RNAs (lncRNAs) were almost half that number, while the sncRNAs displayed extensive differential expression. The vinclozolin lineage contained 2920 differentially expressed sncRNAs, while the DDT lineage had 489. These sncRNAs were separated into sncRNA categories by type and both lineages had piRNAs as the most affected small noncoding RNA class (Fig. 4b). The increase or decrease in RNA expression is presented in Supplementary Tables S4–S12 with a log fold change indicated for each differentially expressed RNA. The DDT differential expressed RNA had for mRNA a 49% increase and 51% decrease, for lncRNA a 16% increase and 84% decrease, and for sncRNA 62% increase and 38% decrease expression. The vinclozolin differential expressed RNA had for mRNA a 57% increase and 43% decrease, for lncRNA a 13% increase and 87% decrease, and for sncRNA a 69% increase and 31% decrease in expression.

The differentially expressed RNAs were mapped to their chromosomal locations, and the differentially expressed lncRNAs and mRNAs were found on all chromosomes except for the mitochondrial genome (Fig. 5a–d). In addition, only the DDT lineage showed a differentially expressed mRNA on the Y chromosome (Fig. 5d). The sncRNAs were found on the majority of chromosomes in both lineages. The vinclozolin lineage had differential expression on all chromosomes except for the Y

chromosome, while the DDT lineage had differential expression on all chromosomes except for the Y chromosome and chromosome 16 (Fig. 3c and d). The widespread chromosomal locations of these altered RNAs indicate that ancestral exposure to an environmental toxicant can have genome-wide effects. Finally, an overlap between DMRs and differentially expressed RNAs is shown as a Venn diagram (Fig. 6). In the vinclozolin lineage, only three mRNAs are linked to the DMRs, while the differentially expressed mRNAs only overlapped with two differentially expressed lncRNAs. These weak links suggest a lack of associations among DMRs and altered RNA expression profiles (Fig. 6a). The sncRNAs displayed a sizeable overlap with both the mRNAs and lncRNAs, with two sncRNAs being common to both mRNA and lncRNA, suggesting a potential causative relationship among lncRNAs, sncRNAs, and mRNAs. This is in contrast to the DDT lineage, which had the most overlap between the DMRs and mRNAs, as well as lncRNAs. In addition, there was also some overlap between the sncRNAs and the mRNAs or lncRNAs (Fig. 6b). These observations support the proposal that the exposure-induced epigenetic transgenerational inheritance involves both DMRs and ncRNAs.

Sertoli Cell Gene Association Analysis

DMRs for both DDT and vinclozolin F3 generation lineage Sertoli cells were associated with genes that were within 10 kb distance (i.e. include promoter regions). These DMR-associated genes are presented in Supplementary Tables S1 and S2 and sorted into gene categories in Fig. 7a and b. The overlapping DMR-associated genes between the DDT and vinclozolin F3 generation lineage Sertoli cells are presented in Supplementary Table S3. The main categories for the DDT Sertoli DMR-associated genes are signaling, transcription, metabolism, and receptor, while for the vinclozolin lineage they are signaling, metabolism, and transcription. The differential expressed RNAs for both DDT and vinclozolin F3 generation lineage Sertoli cells are presented in Supplementary Tables S4–S11, and are sorted by gene categories in Fig. 7c and d. The main gene categories for both DDT and vinclozolin altered mRNAs are metabolism, signaling, transcription, cytoskeleton, and receptor. The DMR- and mRNA-associated genes (Supplementary Tables S1, S2, S10, S11) were analysed with a KEGG pathway analysis, and the top gene pathways are presented in Supplementary Fig. S3. Six gene pathways were in common between the vinclozolin DMR-associated gene pathways (Supplementary Fig. S3A) and DDT DMR-associated gene pathways (Supplementary Fig. S3B). They are the cAMP signaling pathway, endocytosis, viral carcinogenesis, pathways in cancer, MAPK signaling pathway, and Ras signaling pathway. Fourteen pathways were in common between the vinclozolin and DDT mRNA-associated gene pathways (Supplementary Fig. S3C and D).

Sertoli Cell, Testis, and Infertility Pathology Associations

Pyruvate and lactate, produced by Sertoli cells, are essential energy metabolites for spermatogenic cells sequestered within the blood–testis barrier. A previous study identified the pyruvate pathway [69, 70] to be associated with vinclozolin-induced Sertoli cell transgenerational DMRs [8]. In the current study, the pyruvate pathway was also found to be affected by both the DDT and vinclozolin Sertoli cell DMR-associated genes and mRNA, as shown in Fig. 8. As shown, the potentially altered DMR-associated genes and RNA may influence pyruvate

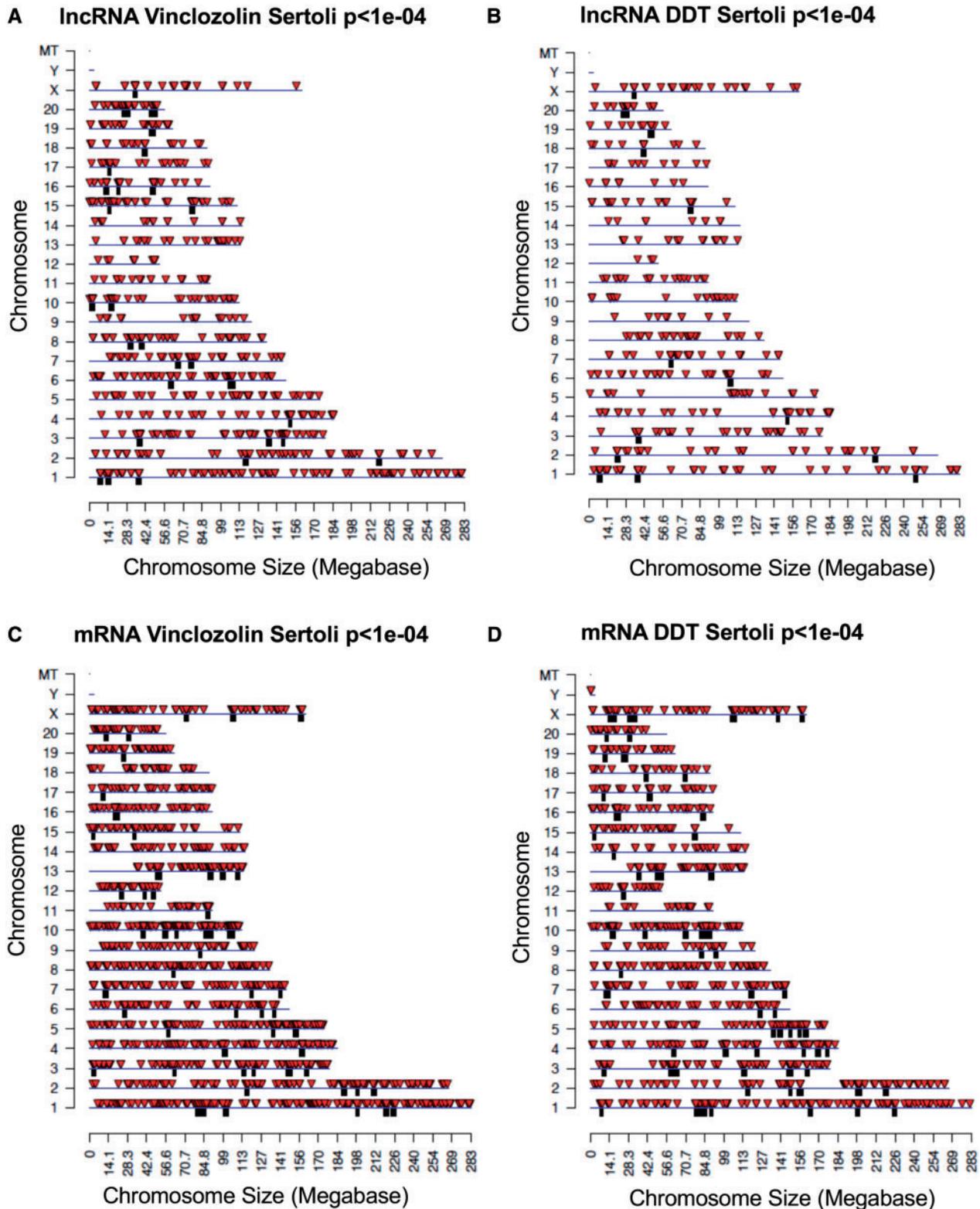


Figure 5: chromosomal locations of differentially expressed lncRNAs and mRNAs. (a) Vinclozolin lncRNA locations and (c) mRNA locations. (b) DDT lncRNAs and (d) mRNA locations. Black boxes represent clusters, while red arrowheads represent individual RNAs, $P < 1 \times 10^{-4}$

production, which would directly impact spermatogenesis in the testis.

The Sertoli cell DMRs and altered mRNAs were examined for potential overlap with previously identified genes shown to be

involved with Sertoli cell, testis, and infertility pathology. The genes previously identified to be associated with abnormal testis pathology were obtained from several exhaustive reviews [71–74]. A list of all 362 genes is presented in [Supplementary](#)

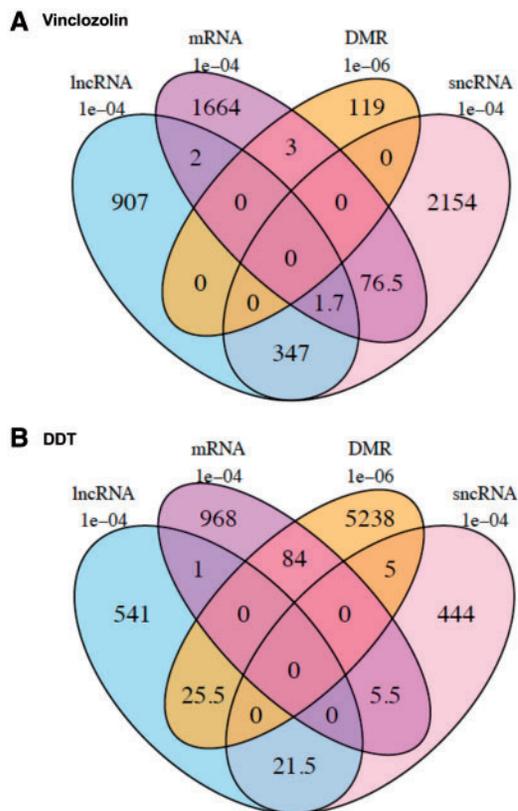


Figure 6: Venn diagram of differentially expressed epigenetic modifications. (a) Vinclozolin lineage. (b) DDT lineage

Table S13. The transgenerationally altered ncRNA had limited gene associations and no overlap observed. The Sertoli cell DMR-associated genes and mRNAs were compared, and the lists of overlapping pathology associated genes presented in [Supplementary Fig. S4](#). These associated genes were categorized as Sertoli cell, testis or male infertility linked pathology genes in [Fig. 9c](#). The vinclozolin DMR-associated genes were not found to overlap with the known pathology genes, but the altered mRNA did contain overlap with the pathology genes ([Fig. 9a and b](#)). In contrast, the DDT DMR-associated genes had 40 overlaps ([Fig. 9a and Supplementary Fig. S4C](#)) and mRNAs had 28 overlaps ([Supplementary Fig. S4B](#)). Interestingly, a large percentage of the DDT and vinclozolin pathology genes were in common, ([Supplementary Fig. S4D](#)). The DDT mRNA overlaps were distinct from the DMR overlaps. The DDT and vinclozolin mRNAs had Sertoli cell genes such as *Daz1*, *Stra8*, *Crem*, *Sox8*, and *DMRT* genes involved. The DDT DMR overlaps had *Adam2*, *Tex14*, *Acur1*, and *Csf1* genes involved. Therefore, the DDT lineage Sertoli cell DMR-associated genes and altered mRNA involved a number of genes previously shown to be involved in Sertoli cell, testis and infertility pathology ([Fig. 9c](#)).

Discussion

The experimental design used the IP injection of a gestating female rat to high environmentally relevant dose exposures for DDT and vinclozolin [25, 26]. Generally, an oral exposure to a lower dose would be used for risk assessment analysis. The current study was not designed for risk assessment, but to further investigate the transgenerational phenomenon. The observations demonstrate an environmental exposure can promote the

epigenetic transgenerational inheritance of disease susceptibility in the testis through molecular alterations in the Sertoli cells. This should not be considered a direct exposure risk assessment study, but further elucidates the epigenetic transgenerational impacts of ancestral environmental exposures.

Environmental toxicants such as DDT and vinclozolin have been shown to promote the epigenetic transgenerational inheritance of adult onset disease [59]. Epigenetic transgenerational inheritance starts with epigenetic modifications in the germline through exposure to environmental factors, which can be transmitted through the sperm and egg to subsequent generations. This germline transmission potentially leads to an altered epigenome in the stem cell of the embryo and subsequently all somatic cells and tissues [6]. The altered epigenomes appear to influence the susceptibility to disease later in life [12]. This study investigates the transgenerational effects of DDT and vinclozolin on the Sertoli cell epigenome and transcriptome. These and a number of other environmental toxicants have been shown previously to affect testis health and spermatogenesis [5, 59]. In the current study, DDT and vinclozolin were both found to promote a transgenerational testis pathology frequency of approximately 45% of the male populations examined compared to 8% in the control population ([Fig. 1a](#)). This compares with the 25–35% testis pathology frequency induced by several other toxicants as observed in previous studies ([Fig. 1b](#)). These studies showed the impacts of plastics (bisphenol A and phthalates) [22, 41], dioxin [24], pesticides (permethrin and DEET) [27], and hydrocarbons (jet fuel) [28] on the induction of transgenerational testis pathology ([Fig. 1b](#)). The testis disease frequency in these previous studies ranged between 20 and 35% compared to 13% in the independent control. The dramatic decline in human sperm concentrations of 50% over the past 50 years and corresponding increase in male infertility [53] suggest an environmental and generational aspect to male testis pathology should be considered. The current study was designed to examine a molecular mechanism for this potential environmental and generational origin of male infertility.

Sertoli cells are an essential somatic cell population in the testis, supporting spermatogenesis nutritionally and structurally. Although other testis somatic cell types such as Leydig cells and peritubular cells have critical functions, the Sertoli cell is the most integrated with spermatogenesis. Abnormal Sertoli cell function can affect testis health and fertility, as has been shown in previous studies [75]. Sertoli cells from prepubertal rats (18–22 days old) can be isolated as a highly purified population of cells. In addition, at this age, there is in general negligible disease present in the animals which would be a confounder in the study. Since the altered Sertoli cell epigenome has already been programmed earlier in development, analysis of the 18–22 days old Sertoli cell will provide insights into the molecular aspects of testis disease susceptibility later in life. Therefore, the late pubertal Sertoli cell is a good model to study the effects of environmental toxicant exposure on epigenetics and transgenerational inheritance of testis pathology. The F0 generation gestating female rats were exposed to DDT or vinclozolin during fetal days E8–14 of development which is the time of gonadal sex determination. The offspring from each generation are bred through the third generation without any further exposure to the toxicants. The F3 generation is the first true transgenerational group since during toxicant exposure of the F0 pregnant female the fetus (F1 generation), as well as the fetus's germline (F2 generation) are exposed [76]. Since the F1 and F2 generation phenotypes and molecular actions can be due to direct exposure, the current study focused only on transgenerational

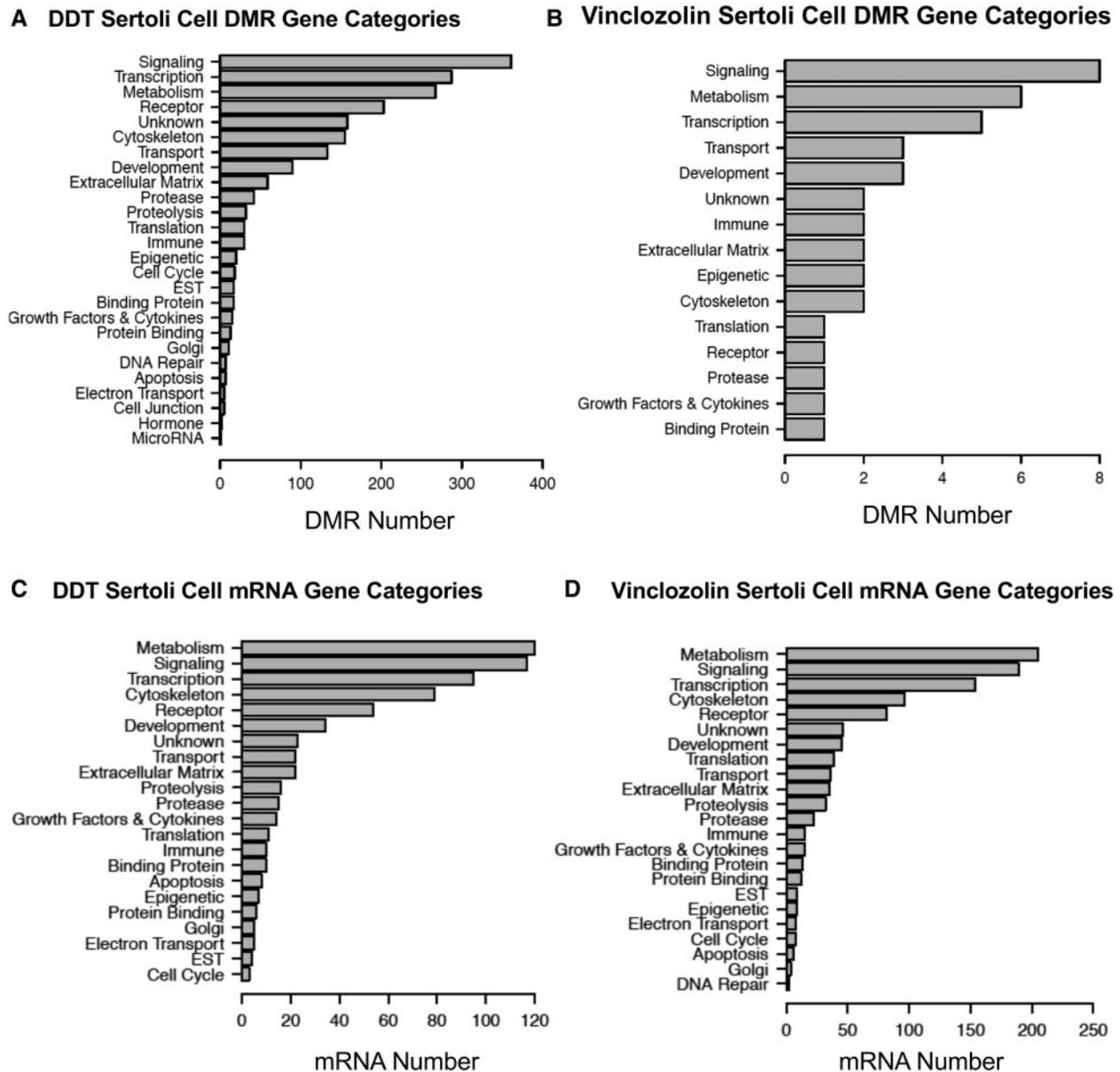


Figure 7: gene category frequency plots. (a) DDT Sertoli cell DMR and (b) vinclozolin Sertoli cell DMR gene categories. (c) DDT Sertoli cell mRNA and (d) vinclozolin Sertoli cell mRNA gene categories. The categories and numbers of DMRs or mRNAs are presented

aspects of the F3 generation. The 18–22 days old F3 generation males were sacrificed, and Sertoli cells were isolated for preparation of DNA and RNA for further study by MeDIP-Seq and RNA-Seq.

The experimental design used an intercross within the lineage to allow both maternal and paternal allelic contributions for pathology and epigenetics to be considered in the transgenerational animals. Since epigenetics involves a parent of origin allelic transmission, an outcross to wild type animals can eliminate the paternal or maternal contribution. Previous studies have demonstrated the outcross of the F3 generation to the F4 generation will promote the loss or reduction of a specific pathology [25, 26]. For example, pesticides induced pathology in an outcrossed F4 generation demonstrated that the paternal allelic outcross promoted female obesity, while the maternal outcross promoted male obesity [25, 26]. Since biological

populations are generally an intercross within a specific exposed population and not a true outcross, the intercross also is considered a more normal biology. Therefore, the current study focused on the F3 generation from an intercross. However, future studies need to investigate the impacts of an outcross to assess sex specific allelic transmission.

Transgenerationally Affected Sertoli Cell DMR Analysis

DMRs were found for both the F3 generation DDT and vinclozolin lineages through read depth comparisons between control and DDT or vinclozolin sequencing data. Although a potential limitation is the use of three pools of 6–11 animals each versus individual animal analysis, the statistical analysis with an edgeR P -value $<1e-06$ and FDR <0.05 suggests there is a low variability in the data and that analysis of pooled samples yields

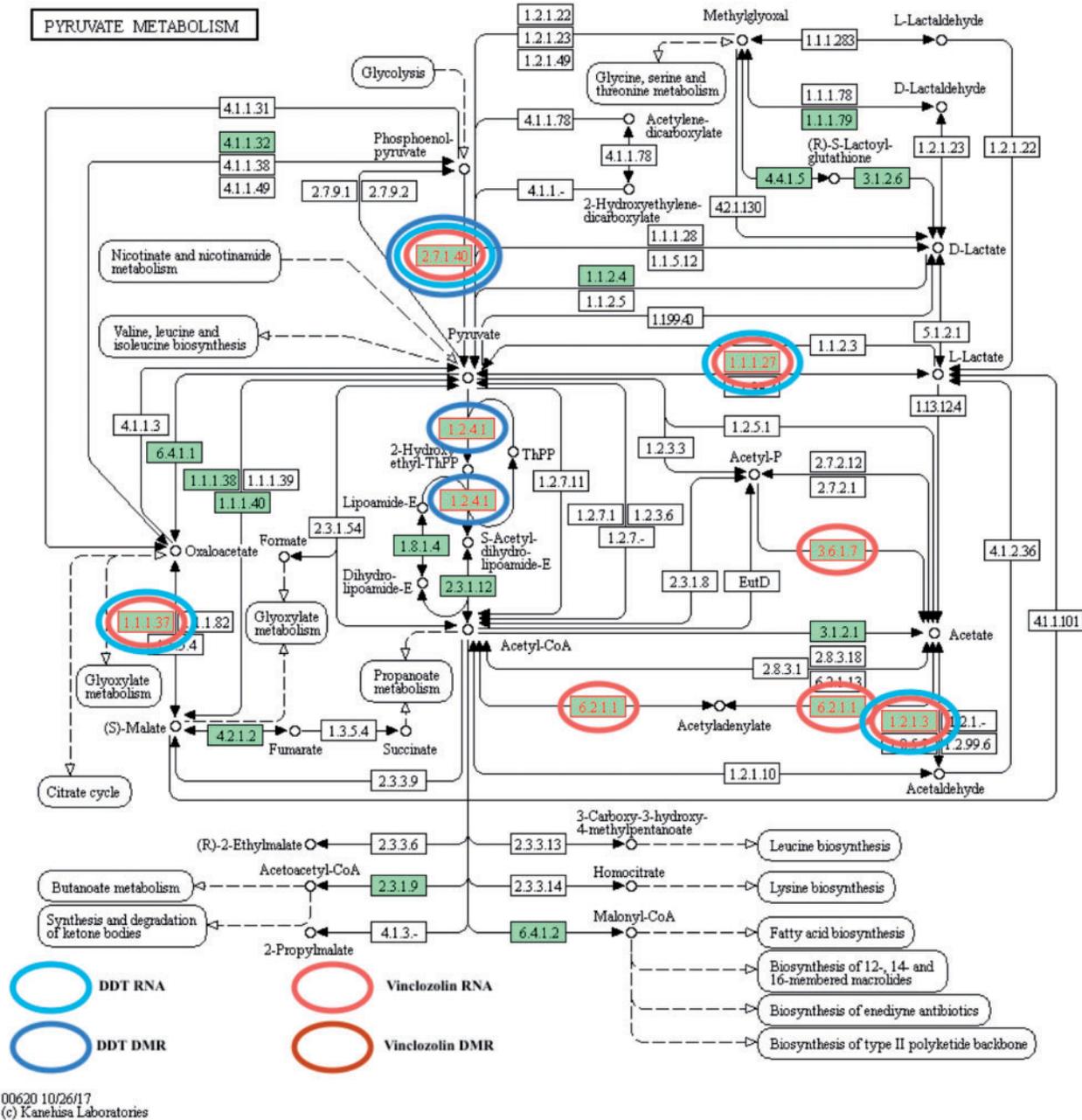


Figure 8: KEGG pathway pyruvate metabolism [69, 70]. The genes correlated with altered DMR and mRNA are indicated in the insert legend with the colored circled genes

adequate statistical power. The number of DMRs found in the DDT lineage F3 generation Sertoli cells is much higher than the number of DMRs found in the vinclozolin lineage at the same P -value $< 1e-06$. The numbers are 5359 for DDT versus 122 for vinclozolin. Although it is uncertain why DDT DMRs are higher in number, the estrogenic actions of DDT versus the anti-androgenic actions of vinclozolin may be a factor to consider. The developing male fetus may be particularly sensitive to DDT promoting a more profound effect on the sperm epigenome and male somatic cells like Sertoli cells.

The chromosomal distribution for both exposures is quite even with DMRs on all chromosomes for the DDT lineage, and on all except Y for the vinclozolin lineage. The Y chromosome contains large regions of repeat elements that are highly

methyated as heterochromatin, so they are anticipated to not be sensitive to DNA methylation alteration. Previous studies have demonstrated that the CpG density of the DMRs is 1–2 CpG per 100bp within the entire 1–2 kb DMR, which is considered a CpG desert [68]. Very few DMRs have a density of 3 and 4 per 100 bps with close to zero for even higher CpG densities. This is consistent with the observation that genes subject to epigenetic transgenerational inheritance are mainly regulated via “CpG deserts,” whereas high density CpG regions “CpG islands” have a housekeeping or maintenance role in tissue-specific gene regulation. Gene associations for the DMRs found in the F3 generation DDT and vinclozolin lineage Sertoli cells were determined, and the genes were sorted into categories. Signaling, transcription and metabolism were the main gene categories observed.

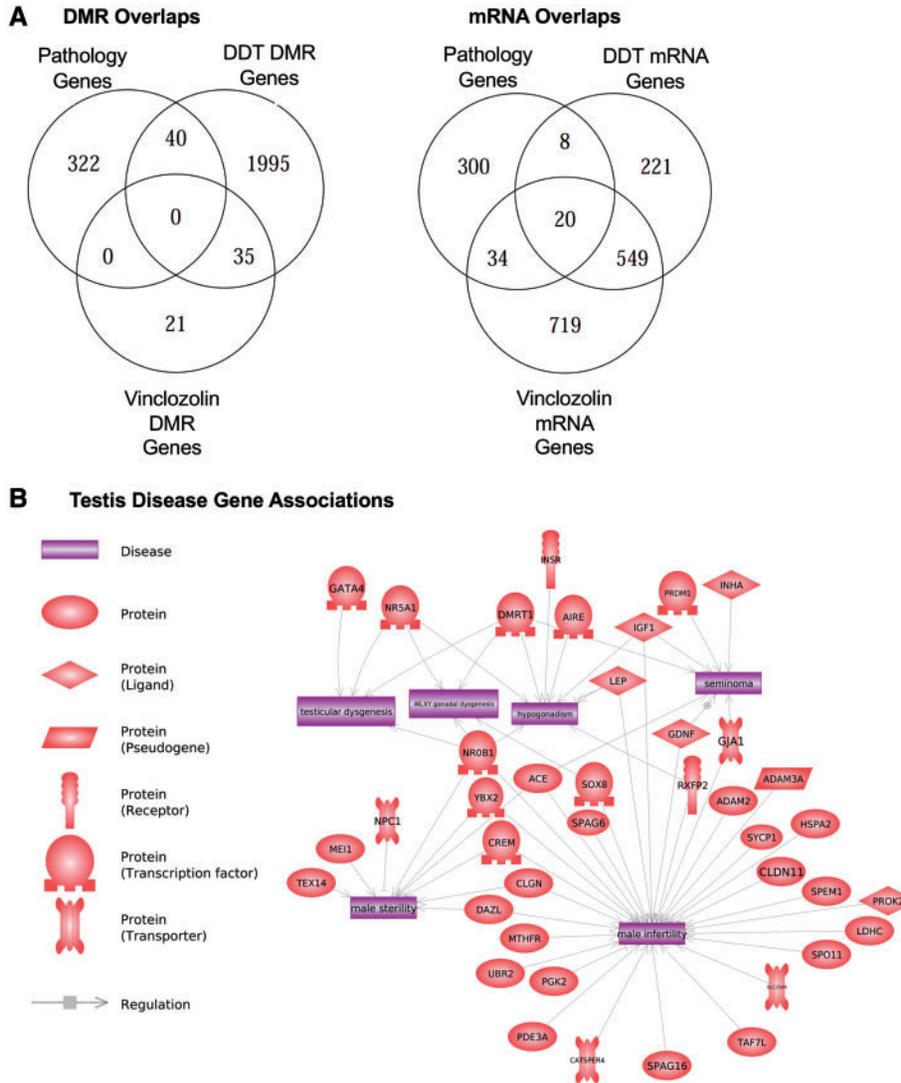


Figure 9: Sertoli cell testis and infertility-associated genes. The previously identified pathology-associated genes [71–74] in [Supplementary Table S13](#) were compared to the Sertoli cell DMR and mRNA-associated genes. (a) DMR-associated gene overlap with pathology genes. (b) mRNA overlap with pathology genes. (c) Testis disease associated genes within the gene lists in [Supplementary Fig. S4](#) in the overlapping associated genes, $P < 1e-05$. The various testis disease parameters have the associated genes linked

Transgenerationally Affected Sertoli Cell ncRNA Analysis

Being derived from the noncoding portion of the genome, non-coding RNAs were once considered to be “junk RNA.” These RNAs are transcribed, but are not protein coding, and can be divided into two categories of long (>200 nt) and small (<200 nt) ncRNA. It has since been observed that long noncoding RNAs are differentially expressed in certain diseases such as cancer [77], and after ancestral exposure to the environmental toxicant DDT [55]. This led to the proposal that ncRNAs may actually be functional, and may maintain epigenetic memory using post-transcriptional mechanisms. In addition, lncRNAs can regulate DNA methylation, remodel chromatin, and regulate histone modifications [61]. Small noncoding RNAs have been shown to be differentially expressed throughout spermatogenesis [78], such that their expression is regulated. At estimates of over 20 000 long and small ncRNAs in a single spermatozoon [79, 80], the impact of these noncoding RNAs on any offspring is potentially very significant. Observations indicate differential

expression of ncRNA is a component of transgenerationally affected Sertoli cell functions.

Transgenerationally Affected Sertoli Cell Transcriptome Analysis

The vinclozolin and DDT lineages both displayed differential coding and noncoding RNA expression profiles. Interestingly, the vinclozolin lineage had more differentially expressed RNAs in all categories, including sncRNAs, lncRNAs, and mRNAs. The most significant difference was found in the sncRNAs category with the vinclozolin lineage having 2920 altered sncRNAs, and DDT having only 489 sncRNAs. When the sncRNAs were separated by small ncRNA categories, the pattern between the vinclozolin and DDT lineages was surprisingly similar. Both overwhelmingly had piRNAs as the most differentially altered class, followed by miRNAs. Chromosomal locations of the differentially expressed RNAs indicated that although the majority of the genome was affected by differential expression, some of

the clusters were in similar chromosomal locations by type of differentially expressed large RNAs between the different lineages. The ncRNAs are anticipated to play a critical role in the epigenetic transgenerational inheritance of testis pathologies. The similarities of the DDT and vinclozolin transgenerational differentially expressed ncRNAs and mRNAs were dramatic, and future studies should further investigate the similarities of the exposure lineage transgenerational effects on the Sertoli cells.

An analysis of overlapping differential epigenetic modifications between the vinclozolin and DDT lineages revealed similarities in some RNA expression. For the vinclozolin lineage, the small noncoding RNAs had a substantial overlap with both the mRNAs with 77 and the lncRNAs with 347. In addition, out of the 122 DMRs in the vinclozolin Sertoli cells, only 3 overlapped with any RNAs, specifically the mRNA. In contrast, the DDT lineage Sertoli cells had a greater overlap of 84 of the DMRs with the mRNAs. In addition, the DMRs had a significant overlap with the lncRNAs with 26. The sncRNAs also overlapped with the lncRNAs and the mRNAs, indicating that although the DMRs may be an effector of altered RNA expression, the sncRNAs may also play a part. Observations suggest all differentially expressed epimutations may affect gene expression in an integrated manner.

Analysis of the vinclozolin and DDT actions on the transgenerational mRNA expression demonstrates a significant overlap of the altered gene expression, and nearly all the major gene pathways identified for both were the same (Supplementary Fig. S3). Interestingly, similar KEGG pathways were identified in all the epimutation data sets with metabolism, pathways in cancer, cAMP signaling pathway, P13K-Act signaling pathway, and MAPK pathway in common. The pyruvate metabolism pathway that is essential for spermatogenesis through the production of pyruvate and lactate by Sertoli cells also appears to be transgenerationally impacted (Fig. 8) as previously described [8].

Transgenerational Epimutation Associations with Sertoli Cell, Testis and Infertility Pathology

As previously described for vinclozolin lineage animals [8], the pyruvate metabolism pathway (Fig. 8) was affected in the DDT and vinclozolin lineage animals with both the DMR and mRNA-associated genes. This suggests a deficiency in an energy source for the developing germ cells, and correlates with the spermatogenic cell apoptosis and testis pathology observed (Fig. 1) [8]. Since the primary energy source (metabolite) for developing spermatogenic cells is pyruvate or lactate produced by Sertoli cells, alterations would directly impact germ cell survival.

Previous studies have identified a large number of genes associated with Sertoli cell, testis and male pathology, and infertility (Supplementary Table S13). This list of genes was obtained from several exhaustive reviews [71–74]. The previously identified pathology-associated genes were compared to the current study epimutation-associated genes for DMR and differentially expressed mRNA. A number of interesting overlaps were observed for the DDT- and vinclozolin-associated genes (Fig. 9 and Supplementary Fig. S4). Interestingly, 20 of the genes that overlapped with the pathology genes were the same between the DDT and vinclozolin altered mRNA. These included *Gata4*, *Sox8*, *Crem*, *Dmrt1*, and *Inha*. This supports the conclusion that the transgenerational epigenetic impacts on the Sertoli cells may be a component of the testis pathology observed.

Conclusions

A number of environmental toxicants have been shown to promote the epigenetic transgenerational inheritance of testis disease and male infertility [5, 8, 37, 81]. This involves epigenetic alterations in the germline (e.g. sperm) to impact the early embryonic stem cells epigenome and transcriptome. Subsequently, all cell types and tissues will have altered epigenomes and transcriptomes. The current study demonstrates that the transgenerationally affected Sertoli cells following ancestral vinclozolin or DDT exposure have alterations in DMRs, ncRNA, and mRNA. The correlations in the DMR, ncRNA, and mRNA support abnormal Sertoli cell functions that can impact spermatogenic cell development and subsequent male infertility. Since the current human male population has a dramatic increase in infertility and a decrease in sperm numbers [44, 50–53], observations suggest ancestral exposures promoting the epigenetic transgenerational inheritance of testis disease may be an important component of the etiology of male infertility. Therefore, ancestral exposures and generational impacts on male infertility need to be seriously considered in testis disease etiology.

Methods

Animal Studies and Breeding

Female and male rats of an outbred strain Hsd: Sprague Dawley SD[®]TM (Harlan) at about 70 and 100 days of age were fed ad lib with a standard rat diet and received 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 body weight. On days 8 through 14 of gestation [82], the females received daily intraperitoneal injections of vinclozolin (100 mg/kg BW/day), DDT (25 mg/kg BW/day), or DMSO. The doses of DDT and vinclozolin used are anticipated environmental exposure [83, 84]. The vinclozolin and DDT were obtained from Chem Service Inc. (West Chester, PA) and were injected in a 20 µl DMSO vehicle, as previously described [24, 81]. Treatment lineages are designated “control,” “DDT,” or “vinclozolin” lineages. The treated gestating female rats were designated as the F0 generation. The offspring of the F0 generation rats were the F1 generation. Non-littermate females and males aged 70–90 days from the F1 generation of control, DDT or vinclozolin lineages were bred to obtain F2 generation offspring. The F2 generation rats were bred to obtain F3 generation offspring. F3 generation males were euthanized at 18–22 days for testis collection and Sertoli cell isolation, or at 1 year of age for testis pathology analysis. The F1–F3 generation offspring were not themselves treated directly with vinclozolin or DDT. The control, DDT, and vinclozolin lineages were housed in the same room and racks with lighting, food and water as previously described [24, 59, 81]. The diet consisted of free choice Teklad 2020X rodent chow (Envigo Inc.). All experimental protocols for the procedures with rats were pre-approved by the Washington State University Animal Care and Use Committee (IACUC approval # 6252).

Testis Pathology Analysis

The Washington Animal Disease Diagnostic Laboratory (WADDL) at the Washington State University College of Veterinary Medicine has board certified veterinary pathologists that assisted in initially establishing the criteria for the pathology analyses and identifying testis parameters to assess [9].

WADDL performed full necropsies as required on animals that died prior to the time of scheduled sacrifice at one year, and performed tumor classifications in the current study.

Testis were evaluated for pathologies, as previously described [24]. Testis histopathology criteria included the presence of: (i) Atrophic seminiferous tubules showing reduced Sertoli and germ cells. (ii) Round, smooth-edged, basally located vacuoles in the seminiferous epithelium. (iii) Sloughed spermatogenic cells in the tubule lumen. Representative pathologies are presented in [Supplementary Fig. S1](#). Testis sections were also examined by Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (In situ cell death detection kit, Fluorescein, Sigma, St. Louis, MO). A cut-off was established to declare a tissue “diseased” based on the mean number of histopathological abnormalities of each type (atrophy, vacuoles, sloughed cells in lumen, or TUNEL positive) plus two standard deviations from the mean of control tissues by each of the three individual evaluators blinded to the treatment groups. This number was used to classify rats into those with and without testis disease in each lineage. Two of three evaluators would have to score an animal’s testes as having an above-cutoff quantity of a specific histopathological abnormality (atrophy, vacuoles, or sloughed cells in lumen) in order for a rat tissue section to finally be declared “diseased”.

Testis Tissue Collection and Isolation of Sertoli Cells

The 20-day-old rats were sacrificed and the testes were dissected. Sertoli cells were prepared as described in [Tung et al. \[85\]](#). Briefly, testes were washed two times with HBSS. The tunica albuginea was removed from each testis and discarded. The tissue was then chopped with a razor blade until it appeared homogeneous, and then underwent sequential enzymatic digestion and wash steps to isolate the purified Sertoli cells. The first step uses trypsin (0.25%) and DNase (0.3 mg/ml) followed by a trypsin inhibitor (0.3 mg/ml) incubation and washes. The next digestion step involves collagenase (0.5 mg/ml) and DNase followed by the final digestion with hyaluronidase (1 mg/ml) and DNase. The final pellet will contain the purified Sertoli cells, which can be used for isolation of DNA and RNA. Enzymes used were DNase I (Sigma DN-25), Collagenase (Sigma C1639), Hyaluronidase (Sigma #H3757), and Trypsin Inhibitor Type I-S (Sigma T-6522). The purity of the Sertoli cell populations are routinely monitored with microscopy and has been previously established with fibronectin immunocytochemistry to be >98% pure, as described [8, 63–65].

DNA and RNA Isolation

Sertoli cell pellets were resuspended in 100 μ l 1 \times PBS, then combined with 820 μ l DNA extraction buffer (50 mM Tris HCl, 10 mM EDTA, pH 8.0, 0.5% SDS) and 80 μ l proteinase K (20 mg/ml). The sample was incubated at 55°C for at least 2 hours under constant rotation. Then 300 μ l of protein precipitation solution (Promega, A7953) was added, the sample mixed thoroughly and incubated for 15 minutes on ice. The sample was centrifuged at 14 000 rpm for 30 minutes at 4°C. One ml of the supernatant was transferred to a 2 ml tube and 2 μ l of glycoblue 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 14 000 rpm for 20 minutes at 4°C. The supernatant was taken off and discarded without disturbing the (blue) pellet. The pellet was washed with 70% cold ethanol and centrifuged for

10 minutes at 4°C at 14 000 rpm and the supernatant was discarded. The tube was spun again briefly to collect residual ethanol to bottom of tube, and then as much liquid as possible was removed with gel loading tip. Pellet was air-dried at RT until it looked dry (about 5 minutes). Pellet was then resuspended in 100 μ l of nuclease free water.

Total RNA Isolation

Trizol reagent (Thermo Fisher) or mirVana miRNA isolation kit (Life Technologies) was used to extract total RNA from purified Sertoli cells with some modifications to the manufacturer’s protocol. The mirVana kit was used to extract control lineage Sertoli cells stored as pellets at –80°C until use. Lysis buffer was added to the cell pellets, which were heated to 65°C for 10 minutes and manually homogenized. The default protocol was used for the remainder of the extraction. The vinclozolin and DDT lineage cells were stored in 1.2 ml Trizol at –80°C until use. To recover small RNA at the RNA precipitation step, the amount of isopropanol was increased to 1 ml, then the default protocol was resumed. All RNA was eluted in 50 μ l of water with 0.5 μ l murine RNase inhibitor (NEB). RNA concentration was determined using the Qubit RNA HS Assay Kit (Thermo Fisher). An RNA 6000 Pico chip was run on the Agilent 2100 Bioanalyzer for quality control analysis.

Methylated DNA Immunoprecipitation (MeDIP)

MeDIP with genomic DNA was performed as follows: DNA isolated from Sertoli cell pools (three pools each of control, DDT, and vinclozolin lineage animals) was sonicated using the Covaris M220 the following way: the genomic DNA was diluted to 130 μ l with TE buffer into the appropriate Covaris tube. Covaris was set to 300 bp program, and the program was run for each tube in the experiment. About 10 μ l of each sonicated DNA was run on 1.5% agarose gel to verify fragment size. The sonicated DNA was transferred from the Covaris tube to a 1.7 ml microfuge tube and the volume was measured. The sonicated DNA was then diluted with TE buffer (10 mM Tris HCl, pH 7.5; 1 mM EDTA) to 400 μ l, heat-denatured for 10 minutes at 95°C, then immediately cooled on ice for 10 minutes. Then 100 μ l of 5 \times IP buffer and 5 μ g of antibody (monoclonal mouse anti 5-methyl cytidine; Diagenode #C15200006) were added to the denatured sonicated DNA. The DNA-antibody mixture was incubated overnight on a rotator at 4°C. The following day, magnetic beads (Dynabeads M-280 Sheep anti-Mouse IgG; 11201 D) were pre-washed as follows: The beads were resuspended in the vial, then the appropriate volume (50 μ l per sample) was transferred to a microfuge tube. The same volume of Washing Buffer (at least 1 ml 1 \times PBS with 0.1% BSA and 2 mM EDTA) was added and the bead sample was resuspended. Tube was then placed into a magnetic rack for 1–2 minutes and the supernatant was discarded. The tube was removed from the magnetic rack and the beads were washed once. The washed beads were resuspended in the same volume of 1 \times IP buffer (50 mM sodium phosphate pH 7.0, 700 mM NaCl, 0.25% TritonX-100) as the initial volume of beads. 50 μ l of beads were added to the 500 μ l of DNA-antibody mixture from the overnight incubation, then incubated for 2 hours on a rotator at 4°C. After the incubation the bead-antibody-DNA complex was washed three times with 1 \times IP buffer as follows: The tube was placed into magnetic rack for 1–2 minutes and the supernatant discarded, then washed with 1 \times IP buffer three times. The washed bead-DNA solution is then resuspended in 250 μ l digestion buffer with 3.5 μ l Proteinase K

(20 mg/ml). The sample was then incubated for 2–3 hours on a rotator at 55°C and then 250 µl of buffered phenol–chloroform–isoamylalcohol solution was added to the supe and the tube was vortexed for 30 seconds then centrifuged at 14 000 rpm for 5 minutes 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 seconds, and centrifuged at 14 000 rpm for 5 minutes 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), 20 µl of 5 M NaCl and 500 µl ethanol were added and mixed well, then precipitated in –20°C freezer for 1 hour to overnight. The precipitate was centrifuged at 14 000 rpm for 20 minutes at 4°C and the supernatant was removed, while not disturbing the pellet. The pellet was washed with 500 µl cold 70% ethanol in –20°C freezer for 15 minutes, then centrifuged again at 14 000 rpm for 5 minutes at 4°C and the supernatant was discarded. The tube was spun again briefly to collect residual ethanol to bottom of tube and as much liquid as possible was removed with gel loading tip. Pellet was air-dried at RT until it looked dry (about 5 minutes) then resuspended in 20 µl H₂O or TE. DNA concentration was measured in Qubit (Life Technologies) with ssDNA kit (Molecular Probes Q10212).

MeDIP-Seq Analysis

The MeDIP DNA samples were used to create libraries for NGS using the NEBNext[®] Ultra[™] RNA Library Prep Kit for Illumina[®] (NEB, 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 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 100 million reads per pool. Five to six libraries were run in one lane. MeDIP analysis data validation has previously been shown with mass-spectrometry sequencing and bisulfite sequencing [84, 86].

Statistics and Bioinformatics

The basic read quality was verified using summaries produced by the FastQC [Simon Andrews, <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>] program. The new data was cleaned and filtered to remove adapters and low quality bases using Trimmomatic [87]. The reads for each MeDIP sample were mapped to the Rnor 6.0 rat genome using Bowtie2 [88] with default parameter options. The mapped read files were then converted to sorted BAM files using SAMtools [89]. To identify DMRs, the reference genome was broken into 100 bp windows. The MEDIPS R package [90] was used to calculate differential coverage between control and exposure sample groups. The edgeR *P*-value [91] 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. Since sequencing reads overlap for the entire DMR length some DMR with CpGs outside the DMR length are listed as 0 CpG, but when flanking regions are considered contain CpG.

This allowed the DMR to be precipitated with the antibody in the MeDIP procedure.

DMRs were annotated using the biomaRt R package [92] to access the Ensembl database [93]. 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 manually then sorted into functional groups by consulting information provided by the DAVID [94], Panther [95], and Uniprot databases incorporated into an internal curated database (www.skinner.wsu.edu “under genomic data”). DMR containing repeat elements were identified using the RepeatMasker output file provided by NCBI for the Rnor 6.0 reference genome. All molecular data have been deposited into the public database at NCBI (GEO # GSE118306; SRA PRJNA480508) and R code computational tools available at GitHub (<https://github.com/skinnerlab/MeDIP-seq>) and www.skinner.wsu.edu.

mRNA and ncRNA Analysis

KAPA RNA HyperPrep Kit with RiboErase was used to construct mRNA and lncRNA libraries following the manufacturer's protocol with the following modifications: the adaptor and barcodes used were from NEBNext Multiplex Oligos for Illumina. Before the final amplifications, libraries were incubated with the USER enzyme (NEB) for 15 minutes at 37°C. PCR cycle number was determined using qPCR with the KAPA RealTime Library Amplification Kit. KAPA Pure beads were used for size selection (200–700 bp). Agilent DNA High Sensitivity chips were used for quality control analysis and the Qubit dsDNA high sensitivity assay (Thermo Fisher) was used to determine concentration. Libraries were pooled and sequenced with the Illumina HiSeq 4000 sequencer (PE100 sequencing).

The NEBNext Multiplex Small RNA Library Prep Set for Illumina was used to construct small RNA libraries with the NEBNext Multiplex Oligos for Illumina as barcodes. KAPA Pure beads were used at 1.3× and 3.7× ratios for purification and size selection, followed by the Pippin Prep 3% gel with marker P (Sage Science) for final size selection (115–160 bp). Qubit dsDNA high sensitivity assay (Thermo Fisher) was used to determine concentration and the Agilent DNA High Sensitivity Chips were used for quality control analysis. Following pooling and concentration with 2.2× KAPA Pure beads, libraries were sequenced with the Illumina HiSeq 4000 sequencer (single-end 50 bp) with a customized sequencing primer: 5'-ACA CGT TCA GAG TTC TAG AGT CCG A-3'.

ncRNA Bioinformatics and Statistics

The small ncRNA data were annotated as follows: low-quality reads and reads shorter than 15 nt were discarded by Trimmomatic (v0.33). The remaining reads were matched to known rat sncRNA, consisting of mature miRNA (miR-Base, release 21), precursor miRNA (miRBase, release 21), tRNA (Genomic tRNA Database, rn5), piRNA (piRBase), rRNA (Ensembl, release 76), and mitochondrial RNA (Ensembl, release 76) using AASRA pipeline with default parameters. Read counts generated by AASRA were statistically normalized by DESeq2.

The long ncRNA and mRNA data were annotated as follows: trimmomatic (v0.33) was used to remove adaptor sequences and the low-quality reads from the RNA sequencing data of the large RNA libraries. To identify all the transcripts, we used HiSAT2 (v2.1.0) and StringTie (v1.3.4d) to assemble the sequencing reads based on the Ensembl_Rnor_6.0. The differential expression analyses were performed by Cuffdiff. The coding and

the noncoding genes were primarily annotated through rat CDS data ensembl_Rnor_6.0. The non-annotated genes were extracted through our in-house script and then analysed by CPAT, indicating the true noncoding RNAs.

Ethics Statement

All experimental protocols for the procedures with rats were pre-approved by the Washington State University Animal Care and Use Committee (IACUC approval # 6252).

Authors Roles

MKS conceived the study and obtained funding. MKS & WY supervised the study. ISR, RK, EN, DB, YX performed technical analysis and obtained the data. DB & YX performed bioinformatics and statistical analysis. ISR and RK wrote the first draft of the manuscript. All authors edited and revised the manuscript.

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Data Availability

All molecular data has been deposited into the public database at NCBI (GEO # GSE118306; SRA PRJNA480508) and R code computational tools available at GitHub (<https://github.com/skinnerlab/MeDIP-seq>) and www.skinner.wsu.edu.

Supplementary Data

Supplementary data are available at *EnvEpig* online.

Conflict of interest statement. None declared.

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