Spring 2019 – Epigenetics and Systems Biology
Discussion Session (Epigenetics and Disease Etiology)
Michael K. Skinner – Biol 476/576
Week 14 (April 11)

Epigenetics and Disease Etiology

Primary Papers

1. Anway et al. (2006) Endocrinology. 147(12):5515-5523
2. Nilsson et al. (2018) Epigenetics. 13(8): 875-895
3. Skinner et al. (2013) BMC Medicine 11:228
4. Kabasenche, et al. (2014) Environmental Health 13:62

Discussion

Student 39 – Ref #1 above

- What is the experimental design?
- What is the epigenetic mechanism involved?
- What diseases exist that could have epigenetic origins?

Student 40 – Ref #2 above

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

Student 41 – Ref #3 & 4 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?
- Should transgenerational environmental justice be an issue?

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Endocrine Disruptor Vinclozolin Induced Epigenetic Transgenerational Adult-Onset Disease

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The fetal basis of adult disease is poorly understood on a molecular level and cannot be solely attributed to genetic mutations or a single etiology. Embryonic exposure to environmental compounds has been shown to promote various disease states or lesions in the first generation (F1). The current study used the endocrine disruptor vinclozolin (antiandrogenic compound) in a transient embryonic exposure at the time of gonadal sex determination in rats. Adult animals from the F1 generation and all subsequent generations examined (F1–F4) developed a number of disease states or tissue abnormalities including prostate disease, kidney disease, immune system abnormalities, testis abnormalities, and tumor development (*e.g.* breast). In addition, a number of blood abnormalities developed including hypercholesterolemia. The incidence or prevalence of these transgenerational disease states was high and consistent across all generations (F1–F4) and, based on data from a previous study, appears to be due in part to epigenetic alterations in the male germ line. The observations demonstrate that an environmental compound, endocrine disruptor, can induce transgenerational disease states or abnormalities, and this suggests a potential epigenetic etiology and molecular basis of adult onset disease. (*Endocrinology* 147: 5515–5523, 2006)

THE ETIOLOGY OF disease involves genetic, nutritional, and environmental factors. The fetal basis of adult onset disease has been demonstrated (1), but the molecular mechanisms involved are poorly understood. Epigenetic mechanisms involving DNA methylation have been shown to influence several disease states (2, 3), including cardiovascular and intestinal abnormalities (4, 5). Endocrine disruptors are an example of a class of environmental toxicants that interfere with specific endocrine signaling and, after fetal or postnatal exposure, promote disease states in the adult (6, 7). Recently, we have made the observation that a transient embryonic exposure to endocrine disruptors at the time of gonadal sex determination can cause an epigenetic transgenerational disease state of subfertility and spermatogenic defects in F1 through F4 generations (8). Altered DNA methylation was observed in two different genes in F1–F4 generations after endocrine disruptor exposure (8). Several recent observations suggest that abnormal fetal conditions (*e.g.* caloric restriction) (9) and fetal exposure to therapies (*e.g.* diethylstilbesterol) (10) can cause abnormalities in the F2 generation. This includes the development of an abnormal reproductive tract (11) and a diabetes-like condition (9, 12). The previous observations (8) led to the hypothesis tested in the current study that a transient embryonic exposure to an endocrine disruptor at the time of gonadal sex determination leads to transgenerational disease states in adults.

The endocrine disruptor used in the current study was vinclozolin, which is a fungicide used in agricultural crops such as grapes grown for the wine industry (13, 14). Vinclozolin is an antiandrogenic compound (13) that is metabolized into more active (*i.e.* higher affinity binding to the androgen receptor) compounds (13). Embryonic exposure to vinclozolin can influence sexual differentiation, gondal formation, and reproductive functions in the F1 generation (13, 15–18). Vinclozolin also promotes a transgenerational phenotype in the testis that affects male reproduction (8). Although steroid production in the developing fetal gonad is negligible, androstenedione is produced by the fetal adrenal. The androgen and estrogen receptors appear to be expressed in germ cells, Sertoli cells, and precursor peritubular cells in the embryo, such that the fetal gonad may be responsive to endocrine disruptors (19, 20). However, potential toxicology of these environmental compounds also needs to be considered. Vinclozolin has been shown to promote an epigenetic alteration in the germ line that appears to transmit a transgenerational disease state (8). The previous study euthanized the majority of animals at less than 120 d of age to assess tissue abnormalities and the primary lesion identified was a spermatogenic defect and male infertility phenotype (8). In the current study, the progeny of previously treated F0 mothers (8) were analyzed together with progeny from new sets of treated F0 mothers. All progeny (F1–F4) were maintained for 6–12 months of age. The current study confirms the original observations but also documents a more extensive disease phenotype in the older adult animals. The ability of an environmental factor to promote a variety of different disease states or abnormalities at high frequency for multiple generations suggests a novel mechanism for disease etiology involving epigenetic transmission through the germ line.

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Abbreviations: BUN, Blood-urea nitrogen; E, embryonic day; RVOC, reverse VOC; VOC, vinclozolin outcross.

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Materials and Methods

In vivo procedures

Gestating outbred Sprague Dawley rats from timed pregnant colonies housed at the Washington State University Vivarium were given ip daily morning injections of vinclozolin (100 mg/kg·d) from embryonic d 8–14 (E8–E14) of gestation (F0 generation) as previously described (21). A previous study has demonstrated that 100 *vs.* 200 mg/kg·d doses induced a similar phenotype, but a 50 mg/kg·d dose was more variable (18), such that 100 mg/kg·d was selected for the current study. The sperm-positive vaginal smear date was designated embryonic d 0. Gestating control mothers received vehicle alone (*i.e.* dimethylsulfoxide and sesame oil). At least six different lines (individual F0 injected gestating females) were generated for both controls and vinclozolin generation groups for these analyses. The majority of animals from a previous study (8) were used by 120 d of age, and all by 180 d of age. The current study used the progeny from four F0 mother lines from the previous study (8) and two completely new sets of F0 mothers progeny for a total of six F0 control and six F0 vinclozolin-treated mothers. Male and female rats from control and vinclozolin generations were collected at 6–14 months of age for analyses. Some animals were euthanized at earlier ages (*e.g.* 6–14 months) due to the development of a clinical disease or abnormality requiring euthanasia. F1 vinclozolin generation males at postnatal d 60 (P60) were bred to P60 F1 vinclozolin generation females to generate the F2 vinclozolin generation; F2 vinclozolin males were bred to F2 vinclozolin females to generate the F3 generation; and the F3 generation rats were bred in the same manner to generate the F4 generation. Rats for the control groups were bred in the same manner for all the generations. No inbreeding or sibling crosses were generated. A vinclozolin outcross (VOC) experiment involved breeding an F2 vinclozolin generation male with a wild-type female to generate an F3 generation VOC. Wild-type is defined as the same Sprague Dawley strain but not from the control generation population. A reverse VOC (RVOC) experiment involved breeding an F2 vinclozolin generation female with a wild-type male to generate an F3 generation RVOC. All procedures were approved by the Washington State University Animal Care and Use Committee. The number of male animals used for replicates in the experiments (*i.e.* n value) for vinclozolin treatment are as follows: F1 (control 6, vinclozolin 9); F2 (control 5, vinclozolin 6); F3 (control 4, vinclozolin 16); and F4 (control 13, vinclozolin 10 and VOC 19). The total number of female animals used was as follows: RVOC, 6; F1–F4 (control, 12; and vinclozolin, 13).

Histology

Tissues were fixed in 10% neutral buffered formalin or Bouin's (Sigma, St. Louis, MO), embedded in paraffin, sectioned, and then stained with hematoxylin and eosin according to standard procedures. Multiple sections were obtained for each tissue for comparison to allow a representative histology to be selected. The Center for Reproductive Biology, Histology Core Laboratory, and the Washington Animal Disease Diagnostic Laboratory assisted with these procedures.

Pathology

Disease diagnoses were identified by the Washington Animal Disease Diagnostic Laboratory (WADDL) located at Washington State University. All animals submitted to WADDL had a complete necropsy with histopathology and bacteriological analyses. Animal identification and treatment group were blinded to all pathologists for analysis. Data were tabulated for each abnormality based on the percentage of tissue with pathological changes per total tissue per cross-section in two to five tissue cross-sections. Rats developing tumors were submitted as whole animals or excised formalin-fixed tissue for tumor identification. All tissue cross-sections were stained with hematoxylin and eosin for analyses. The testis cross-sections were determined to be abnormal if the number of tubules with atrophy, vacuoles or germ cell agenesis was greater than 20% of the total tubules present in the testis cross section, examining a minimum of 100 tubules. Renal lesions were diagnosed by an increase in morphologically identified tubular damage. The kidney was considered abnormal if more than 30% of the tissue contained tubular lesions. Kidney tubular changes involved extreme dilation with protein-rich fluids, fluid-filled cystic tubules, thickening of the Bowman's capsule surrounding the glomerulus, as well as reduced glomerular area. Ventral prostate tissue was considered abnormal if more than 30% of the prostatic ducts were atrophic and contained no columnar secretory epithelial cells. Cross-sectional views of the ventral prostate samples were sectioned so that distal, intermediate, and proximal regions were visible. Ventral prostate lesions were not region specific in the samples analyzed as previously described (23). Lateral and dorsal prostatic lobes were analyzed as well, but no gross morphological changes between control and vinclozolin generations were found in the animals analyzed in this study. Immune-related abnormalities were defined as rats having excessive macrophage and lymphocyte invasion into multiple organs and was generally accompanied by bacterial infection. The immune-related abnormalities involved several types of inflammation of the inner ear (otitis), inflammation in the lower limbs, inflammation in the lower respiratory tract (pneumonia), and development of subdermal abscesses, which grew in size and caused septicemia (widespread infection). Immune-related abnormalities were defined as increased macrophage and lymphocyte invasions into multiple tissues and at least one form of inflammation. Occasionally, vinclozolin generation rats did not have widespread inflammation but did have increased macrophage and lymphocyte invasion into a single organ such as the lung, spleen, seminal vesicles, or ventral prostate. These rats were not included as an immune-related abnormality due to variability between animals. Premature aging-related abnormalities were defined as rats developing poor grooming behavior, causing hair to become discolored and coarse, along with reduced mobility and some weight loss. All blood analyses were performed by the Clinical Pathology Laboratory at Washington State University, Veterinary Teaching Hospital with standard procedures previously described. For the blood counts (red and white) data were collected on a Horiba ABX Hematology Analyzer System 910 + CP, with all differentials analysis done manually. The metabolic panel/profile was done on a Cobras MIRA Plus Analyzer (Roche, Indianapolis, IN). The testosterone serum concentrations were determined by the Center for Reproductive Biology Assay Core Laboratory. Body and tissue (*i.e.* prostate, kidney, spleen, and testis) weights were monitored in age-matched adults.

Statistical analysis

When indicated, the values were expressed as the mean ± SEM and data were analyzed using a SAS program (JMP version 3.1.6; SAS Institute Inc., Cary, NC). Statistical analysis was performed and the difference between the means of treatments and respective controls was determined using a Student's *t* test. Statistical analysis of the disease prevalence in the total population of control *vs.* vinclozolin F1–F4 generation animals used a Fisher's exact test analysis for a 2×2 tables using Minitab (Minitab Inc., State College, PA) and was performed by the Statistics Consulting Service of the Department of Statistics at Washington State University. *In vivo* experiments were repeated with six to 16 individuals for each data point. A statistically significant difference was defined at $P < 0.05$.

Results

Gestating Sprague Dawley rats at the time of gonadal sex determination, E8–E14, were transiently exposed to vinclozolin (100 mg/kg·d) and compared with a vehicle (dimethylsulfoxide buffer)-treated control animals. The F0 generation gestating mothers were the only animals exposed, although this implies that the F1 generation embryo and germ cells generating the F2 generation are also exposed. F1 generation progeny at 60 d of age were bred to generate an F2 generation and breeding continued out to the F4 generation. No sibling breeding was used to avoid any inbreeding issues. In addition, littermates were used for control and treated F0 mothers to reduce any genetic variation. As previously reported (8, 18), no effects were observed on litter size, pup weights, or gross developmental defects of any of the F1–F4 generation progeny. Both males and females examined between 20 and 120 d of age showed no weight differences or gross abnormalities in any tissue (*i.e.*

prostate, kidney, spleen) examined except the testis (8). In animals older than 6 months of age, no differences were observed in body weights (data not shown), and tissue weights were only different if disease was detected (data not shown). As previously reported, the testis had increased spermatogenic cell apoptosis and subfertility (8). The F1–F4 generation progeny from control and vinclozolin-treated F0 mothers were aged 6–14 months to assess effects on aging adults. In the event, a clinical disease state developed (*i.e.* tumor or infection), the animal was euthanized and a complete necropsy and blood analysis were performed. All remaining animals were euthanized by 10–14 months of age according to the Washington State University Animal Care and Use Committee guidelines, followed by complete necropsies for both control and vinclozolin generation F1–F4 animals. A number of disease states or tissue abnormalities developed, as discussed below, for all generations.

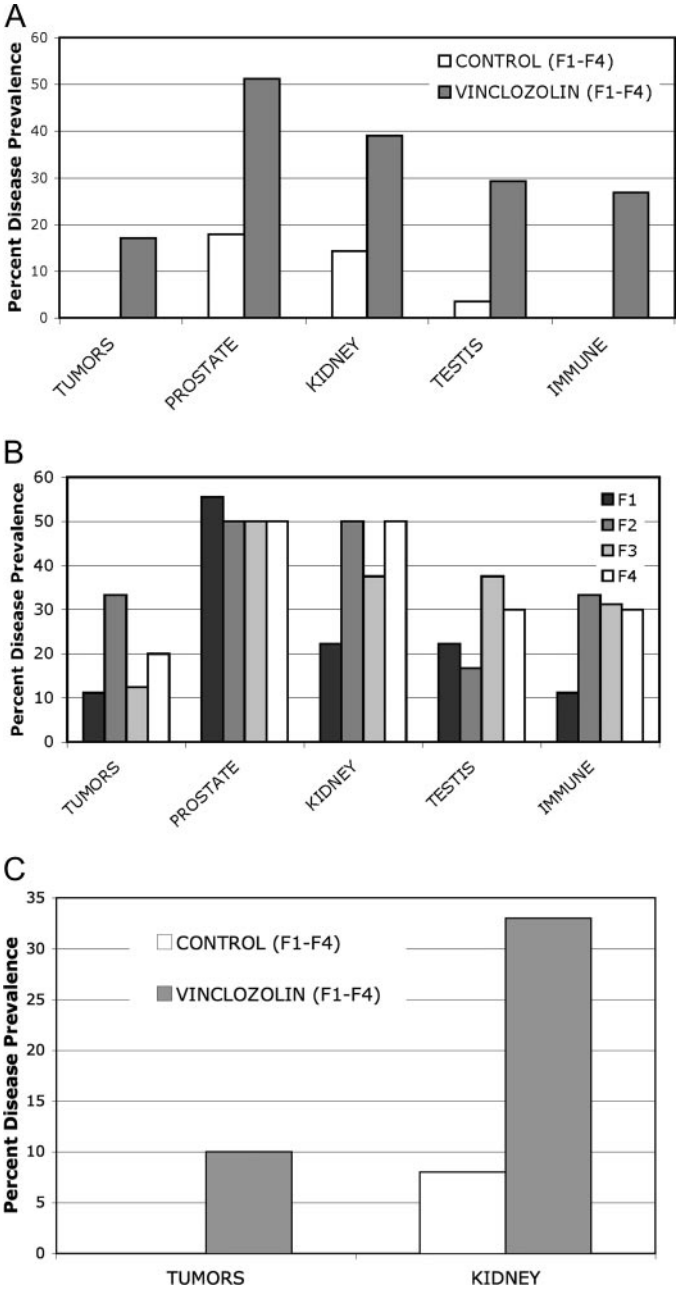

FIG. 1. Disease states or abnormalities in vinclozolin and control generation (F1–F4) animals. A, Males from combined (F1–F4) generation animals. B, Males from individual (F1–F4) generation animals. C, Females from combined (F1–F4) generation animals. The percentage disease prevalence is presented for tumors, prostate disease, kidney disease, testis (*i.e.* spermatogenesis) abnormalities and immune abnormalities. The total number of animals for each generation (F1–F4) is listed in *Materials and Methods*. The absence of a control bar indicates zero in the control population of animals. All the comparative (A) vinclozolin generation animal disease and abnormality prevalence data provided were statistically different from controls with $P < 0.05$ using a Fisher's exact test analysis as described in *Materials and Methods*.

Tumors developed in 12–33% of the animals (Fig. 1), in only the vinclozolin F1–F4 generation animals and not the controls. From the total tumors identified, four were breast adenomas, one was a breast carcinoma, one was a lung sarcoma, and one was a skin (*i.e.* Merkel cell) melanoma, with the breast adenoma presented (Fig. 2, A and B). Only the lung sarcoma, Merkel cell tumor and breast carcinoma were malignant. The numbers of animals at each generation are shown in Tables 1 and 2. Animals euthanized due to tumor development are indicated in Table 1. The frequency of tumor development in the males was similar among the F1–F4 vinclozolin generation animals (Fig. 1B). Tumors developed in females (*i.e.* breast) at approximately a 10% frequency for vinclozolin generations (Fig. 1C). No tumors developed in control F1–F4 generation animals.

Prostatic lesions were detected in 45–55% of F1–F4 vinclozolin generation males (Fig. 1). A regression of prostatic secretory epithelium involving atrophic glands or ducts were observed (51%), along with prostatitis in selected animals (10%) (Fig. 3, A and B). Abnormal prostate histology was observed in the distal, intermediate, and proximal regions of the ventral prostate, but no morphological effects were observed in the lateral or dorsal prostatic lobes (data not shown). The range of ventral prostate pathology was from atrophic defects to cystic hyperplasia and focal prostatitis, with cystic changes as the most severe pathology. A similar percentage of affected animals in all the F1–F4 vinclozolin generations were observed with prostate abnormalities (Fig. 1B). The numbers of animals at each generation are shown in Tables 1 and 2. Ventral prostatic hyperplasia was observed in three rats from vinclozolin generations and one control animal. Serum testosterone concentrations in the male F1–F4 vinclozolin generation progeny, 1.20 ± 0.71 ng/ml, were similar to controls, 1.57 ± 0.86 ng/ml, such that the prostatic abnormalities cannot be attributed to low serum testosterone.

Renal lesions appeared in 20–50% of males of all F1–F4 vinclozolin generations (Fig. 1B). These kidney abnormalities also

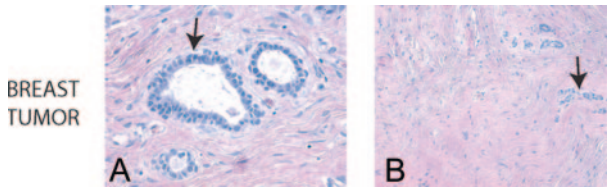

FIG. 2. Histology of representative vinclozolin generation breast fibroadenoma. A representative ×400 magnification (A) and ×200 magnification (B) micrograph is presented from a minimum of five different animals with breast adenoma analyzed. A *closed arrow* indicates a neoplastic glandular epithelial cell population.

TABLE 1. Individual male animal disease states (F1 and F2)

F1 generation	Tumor	Prostate	Kidney	Testis	Immune	Pre-aging	Euthanized (months)
Control generation							
1	−	+	+	−	−	−	14
2	−	+	−	−	−	−	14
3	−	−	−	−	−	−	14
4	−	−	−	−	−	−	14
5	−	−	−	−	−	−	14
6	−	−	−	−	−	−	14
Vinclozolin generation							
1	−	+	+	+	+	+	10(I)
2	+	+	−	−	−	−	6(T)
3	−	+	+	+	−	+	14
4	−	+	−	−	−	−	14
5	−	+	−	−	−	−	14
6	−	−	−	−	−	+	14
7	−	−	−	−	−	−	14
8	−	−	−	−	−	−	14
9	−	−	−	−	−	−	14
F2 generation							
1	−	−	−	−	−	−	14
2	−	−	−	−	−	−	14
3	−	−	−	−	−	−	14
4	−	−	+	−	−	−	14
5	−	−	−	−	−	−	14
Vinclozolin generation							
1	−	−	−	−	−	−	12
2	−	+	+	−	−	+	12
3	−	+	+	+	−	+	12
4	+	−	−	−	−	+	10(T)
5	−	+	+	−	+	+	12(I)
6	+	−	−	−	+	+	12(T)

The absence (−) or presence (+) of a disease or lesion is indicated for individual male animals for F1–F2 control and vinclozolin generation animals. The age in months the specific animal was euthanized is indicated. If the animal was euthanized due to a clinical condition [*i.e.* tumor (T) or infection (I)] is presented respectively with the age of euthanization. Pathology criteria are described in *Materials and Methods*.

were observed in female F1–F4 vinclozolin generation animals (Fig. 1C). Tubular nephropathy with protein casts, degenerated ductal epithelium, and sclerotic glomeruli were the principal histologic changes observed in 39% of the vinclozolin generation animals with 20% being severe (Fig. 3, C and D). The

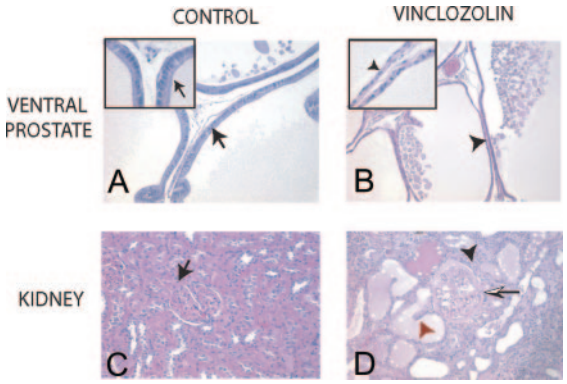

FIG. 3. Histology of representative control (A and C) and vincozolin (B and D) F2 or F3 generation tissues are presented, ventral prostate (A and B), and kidney (C and D). A representative ×200 magnification micrograph is presented from a minimum of five animals analyzed. *Insets* in A and B are ×1000 magnification. A *closed arrow* indicates a normal epithelial cell and a *red arrowhead* an abnormal epithelial cell, and a *closed arrowhead* an abnormal tubule and a *half-arrow* an increased width of Bowman's capsule.

numbers of animals at each generation are shown in Tables 1 and 2. Blood analysis demonstrated an increased blood-urea nitrogen (BUN) and creatinine in several animals with renal lesions (Fig. 4). These increased blood markers for renal lesions correlated to the animals with kidney abnormalities as shown in the *boxed* values in Fig. 4.

As previously reported (8), abnormal testis function and abnormalities were observed in 15–38% of the F1–F4 generation animals (Fig. 1B). The numbers of animals affected at each generation are shown in Tables 1 and 2. The pathology includes an increased spermatogenic cell apoptosis, gross morphological defects in spermatogenesis, and complete lack of spermatogenesis (8). The testis abnormalities were compiled from animals with histologic defects in spermatogenesis in greater than 20% of the seminiferous tubules and those with a complete lack of spermatogenesis (Fig. 5).

Liver histology was normal in vinclozolin generation animals (Fig. 5), and serum concentrations of liver markers, alanine transferase, and alkaline phosphatase, were similar to the control (Fig. 6). Observations suggest no major hepatic defects. In addition, no significant lesions were seen in other tissues including the adrenal glands, epididymis, seminal vesicles, lungs, heart, spleen, ovary, and brain of F1–F4 control or vinclozolin generation animals (data not shown).

Complete blood cell counts (*i.e.* white blood cell and red

TABLE 2. Individual male animal disease states (F3 and F4)

F3 generation	Tumor	Prostate	Kidney	Testis	Immune	Pre-aging	Euthanized (months)
Control generation							
1	−	+	−	−	−	−	14
2	−	−	−	−	−	−	14
3	−	−	−	−	−	−	14
4	−	−	+	−	−	−	14
Vinclozolin Generation							
1	−	+	+	+	−	+	14
2	−	+	+	+	−	−	14
3	−	−	−	−	−	−	14
4	−	−	−	−	−	−	14
5	−	+	−	+	−	+	14
6	−	+	−	−	−	+	14
7	−	−	−	−	−	−	14
8	−	+	−	+	+	−	14(I)
9	−	+	+	−	−	−	14
10	−	−	−	−	−	−	14
11	−	−	+	−	+	+	10(I)
12	−	+	+	−	+	+	12(I)
13	−	+	+	+	+	+	12(I)
14	+	−	−	−	−	+	12(T)
15	−	−	−	+	+	+	10(I)
16	+	−	−	−	−	+	14(T)
F4 generation							
1	−	−	−	−	−	−	14
2	−	−	−	−	−	−	14
3	−	−	−	−	−	−	14
4	−	−	+	−	−	−	14
5	−	−	−	−	−	−	14
6	−	+	−	−	−	+	14
7	−	+	−	+	−	−	14
8	−	−	−	−	−	−	14
9	−	−	−	−	−	−	14
10	−	−	−	−	−	−	12
11	−	−	−	−	−	−	12
12	−	−	−	−	−	−	12
13	−	−	−	−	−	−	12
Vinclozolin generation							
1	−	+	+	+	−	−	14
2	+	+	+	−	−	+	14(T)
3	+	−	−	−	+	+	12(T)
4	−	−	−	−	+	−	10(I)
5	−	+	−	−	−	+	14
6	−	+	+	−	−	−	14
7	−	−	−	−	−	−	14
8	−	+	+	−	−	+	14
9	−	−	−	+	−	+	14
10	−	−	+	+	+	−	14(I)

The absence (−) or presence (+) of a disease or lesion is indicated for individual male animals for F3-F4 control and vinclozolin generation animals. The age in months the specific anima was euthanized is indicated. If the animal was euthanized due to a clinical condition [*i.e.* tumor (T) or infection (I)] is presented respectively with the age of euthanization. Pathology criteria are described in *Materials and Methods*.

blood cell counts) were not different between vinclozolin and control generation animals (data not shown). Inflammation including inner ear (otitis), subdermal abscesses and bacterial infection (*e.g.* respiratory infection) were observed in 12–33% of F1–F4 vinclozolin generation animals, but no inflammation was present in control animals (Fig. 1B). The numbers of animals affected at each generation are shown in Tables 1 and 2. Animals euthanized due to infection are indicated in Tables 1 and 2. Although immune abnormalities are observed, the immune status of vinclozolin generation animals requires further characterization on a molecular level.

Several blood analyses (*e.g.* glucose, serum protein, sodium, potassium) were not different between F1–F4 control and vinclozolin generation animals (Table 3). The total samples analyzed were combined and had a composition of 5% F1, 26% F2, 59% F3, and 10% F4 from each generation and showed no difference. Although serum chloride and magnesium concentrations were statistically different between aged control and vinclozolin generations, the slight changes are likely not physiologically relevant. No major metabolic or physiological effects were observed from the blood analysis. Interestingly, a significant increase in serum cholesterol was observed in approxi-

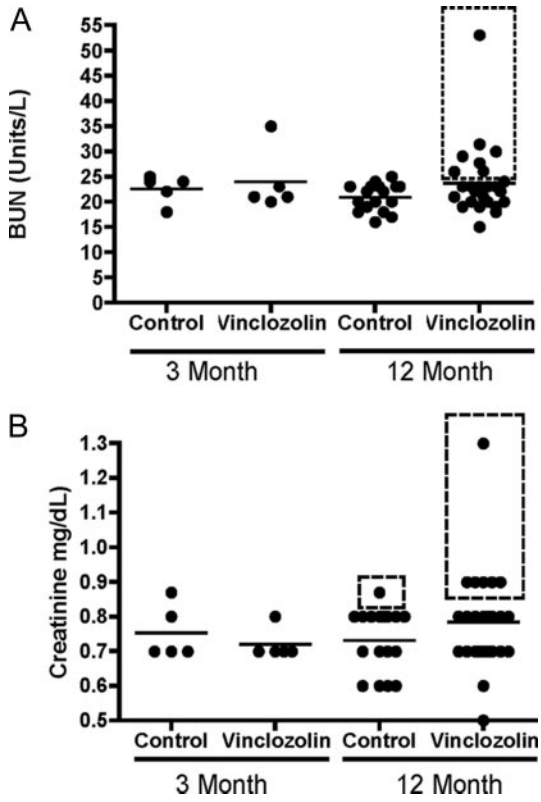

FIG. 4. Blood analysis for BUN (A) and creatinine (B) from control and vinclozolin (F1–F4) generation animals. Scatter plots are presented with a mean line indicated. Samples in the *dashed boxes* correlate with animals with diagnosed morphological kidney abnormalities and corresponding renal blood marker increases.

mately 35% of 6- to 14-month-old F1–F4 vinclozolin generation animals, compared with controls (Fig. 6). None of these changes were observed at 3 months of age and only developed in older animals. Basal serum cholesterol in the control animals did rise between 3 and 12 months but increased more dramatically in vinclozolin generation animals (Fig. 6). Fasting state of the animals did not alter the cholesterol differences between control

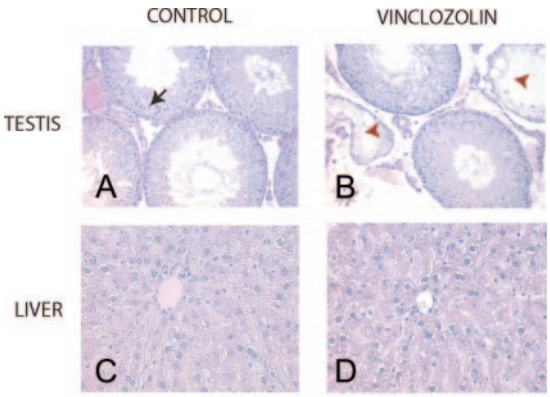

FIG. 5. Histology of representative control (A, C) and vincozolin (B and D) F2 or F3 generation tissues are presented for normal testis (A), abnormal testis (B), and liver (C and D). A representative ×200 (A and B) or ×400 (C and D) magnification micrograph is presented from a minimum of five animals analyzed. A *closed arrow* indicates a normal epithelial cell and tubule, whereas a *red arrowhead* an abnormal epithelial cell and tubule. No histologic abnormalities were observed in the liver.

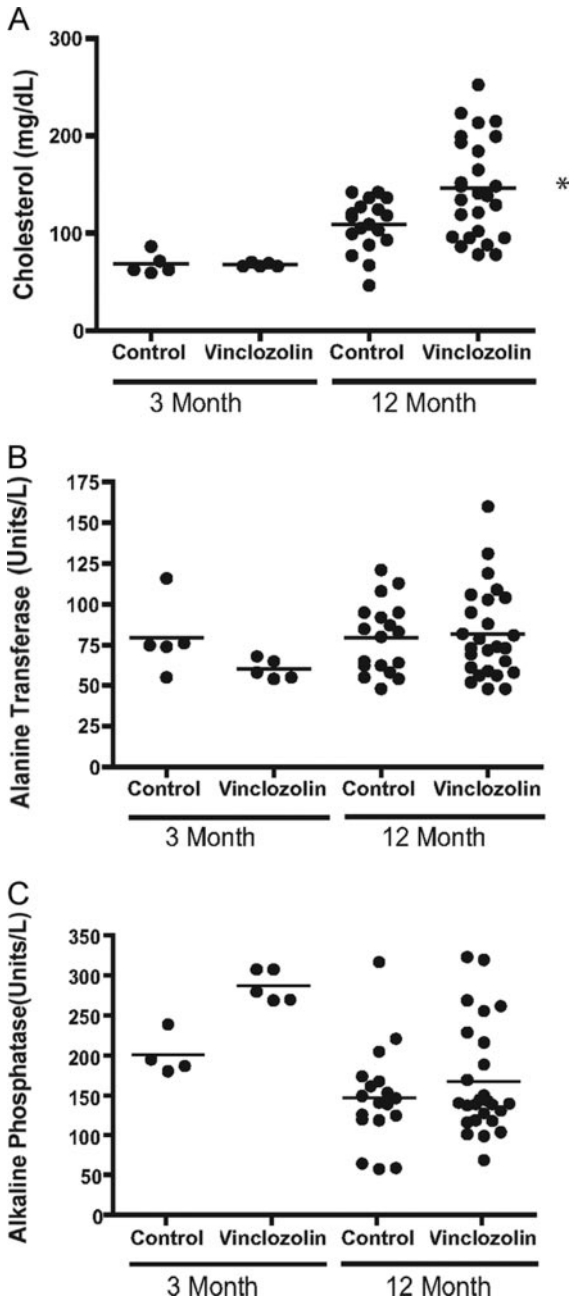

FIG. 6. Blood analysis for combined F1–F4 control and vinclozolin generation animals. A, Cholesterol concentrations and liver serum markers of (B) alanine transferase and (C) alkaline phosphatase are presented (units/liter). Scatter plots are indicated for 3-month-old and 6- to 12-month-old animals with the *bar* representing the mean. *, Mean values are statistically different ($P < 0.05$) from control values for cholesterol. No statistical differences were detected in B or C means.

and vinclozolin generation animals (data not shown). Serum cholesterol levels were also elevated in the aged female vinclozolin generation animals compared with control female animals (data not shown).

Animals from F1–F4 vinclozolin generations also developed an apparent premature aging phenotype. This was characterized as decreased grooming behavior resulting in color distortion, decreased mobility and movement, increased skin abnor-

TABLE 3. Blood chemistry and analysis

	3 Months		12 Months	
	Control (n = 5)	Vinclozolin (n = 5)	Control (n = 12)	Vinclozolin (n = 19)
Glucose	157 ± 9	192 ± 25	124 ± 4	131 ± 9
Total protein	6.5 ± 0.2	7.0 ± 0.2	6.8 ± 0.2	6.9 ± 0.1
Abumin	3.6 ± 0.1	3.7 ± 0.1	3.3 ± 0.1	3.2 ± 0.1
Globulin	2.9 ± 0.1	3.2 ± 0.1	3.5 ± 0.1	3.7 ± 0.1
Calcium	10.2 ± 0.1	10.6 ± 0.2	10.5 ± 0.3	10.8 ± 0.2
Phosphorous	6.0 ± 0.6	8.9 ± 0.5	7.2 ± 0.4	7.5 ± 0.3
Magnesium	2.9 ± 0.1	3.2 ± 0.1	2.80 ± 0.09	3.00 ± 0.08[a]
Sodium	152 ± 1	153 ± 2	153 ± 0.1	152 ± 0.8
Potassium	7.7 ± 0.5	7.5 ± 0.4	7.0 ± 0.2	7.4 ± 0.2
Chloride	105 ± 1	103 ± 1	107.0 ± 0.8	104.0 ± 0.6[a]

[a] Statistical difference with $P < 0.05$ between control and vinclozolin generation animals. The number of F1–F4 generation animal combined for the analysis (n value) is indicated.

malities, and periodic weight loss. These same characteristics are observed in control animals greater than 18 months old (23), but none of the 6- to 14-month-old control animals had any of these characteristics (data not shown). In contrast, 50% of the F1–F4 vinclozolin generation animals developed this premature aging phenotype. Although scoring the presence or absence was done, these premature aging characteristics were difficult to measure in degrees or quantitate. These conditions also are present in diseased animals (Tables 1 and 2). Therefore, the premature aging phenotype could not exclude the possibility that it was simply associated with diseased animals. Therefore, the premature aging phenotype is only presented as a subjective measure requiring further investigation. Because neoplasms, renal lesions, and prostate lesions have been observed in aged (24 months) rats (23, 24), one interpretation of the data is that the endocrine disruptor induced a potential transgenerational premature aging phenomena.

The data presented demonstrate that *in utero* exposure to an environmental compound, the endocrine disruptor vinclozolin, has the ability to induce multiple transgenerational disease states. The prevalence of the disease states or abnormalities described are consistent among F1–F4 generations with no apparent decline (Fig. 1). However, the F1 generation disease prevalence was often less than subsequent generations. Most other organs examined had no gross abnormalities or lesions such that the effects observed appear to be specific to the prostate, kidney, breast, testis, and skin. Analysis of individual animals and specific disease states demonstrated that many animals had multiple abnormalities and 85% of all F1–F4 vinclozolin generation animals developed a transgenerational disease state (Tables 1 and 2). The females did have a high prevalence of tumors and kidney disease in the F1–F4 vincozolin generations (Fig. 1). Vinclozolin generation females also had increased serum cholesterol concentrations and when renal defects were present, increased BUN (data not shown).

The previous study (8) demonstrated that the transgenerational disease state (*e.g.* testis abnormality) was transmitted only through the male germ line. In the current study, a vinclozolin outcross (VOC) experiment was performed with an F2 vinclozolin generation male bred to a wild-type female, along with a reverse vinclozolin outcross (RVOC) experiment involving an F2 vinclozolin female bred to a wild-type male. The RVOC demonstrated no significant increase in disease frequency over control, Fig. 7. As a comparison the F1–F4 vinclozolin and control generation-treated cross is presented. The VOC animals had an increase in disease prevalence over control in ventral prostate, testis, and kidney disease, but at a reduced incidence to the treated F3 generation cross (Fig. 7). No tumors or immune abnormalities were detected in the VOC animals. Therefore, the transgenerational disease phenotype was primarily transmitted through the male germ line, but the increased prevalence of disease when the vinclozolin generation female was used in a treated cross suggests a potential contribution of the female germ line. The influence of the female germ line now needs to be investigated more thoroughly.

Discussion

The frequencies of the disease states or abnormalities observed in the current study are consistent for four generations. Although neoplasms, renal lesions, and prostate lesions are observed in aged (24 months) rats (23, 24), none of these pathologies were observed in the 6- to 14-month-old control rats. As a comparison, the frequencies observed are similar to that

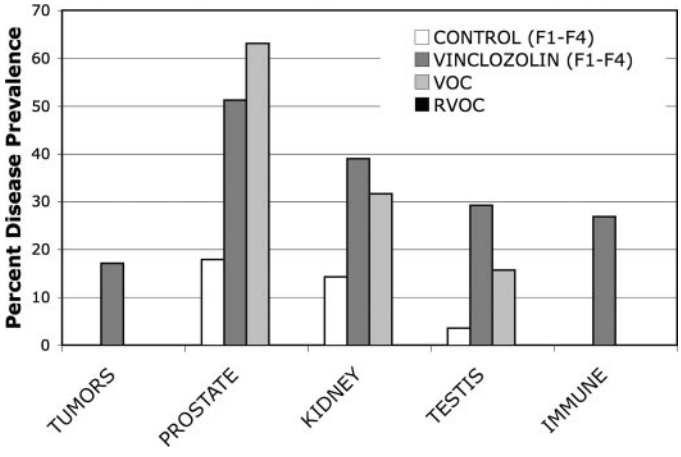

FIG. 7. Comparison of outcross disease states or abnormalities using F1–F4 control and vinclozolin generation-treated cross compared with VOC (F2 vinclozolin generation male and wild-type female) and a RVOC (F2 vinclozolin generation female and wild-type male). The percentage disease prevalence is presented for tumors, prostate disease, kidney disease, testis abnormalities, and immune abnormalities. The absence of a *bar* indicates zero in the population of animals. The total number of animals for VOC and RVOC were n = 19 and 6, respectively. The VOC was statistically different from control with $P < 0.05$.

seen in the human population. Prostatic lesions occur in 50% of men over the age of 50 yr, compared with the 51% observed in the current study. The progression of human prostatic disease has been suggested to involve an initial atrophy of epithelium and glands followed by prostititis, as observed in the current study (25). Renal lesions occur at frequencies in specific human subpopulations (26, 27), similar to the 30% observed in the current study. The abnormal kidney morphology observed corresponded to changes in serum BUN and creatinine levels, as is seen in the human population. Testis abnormalities occur in approximately 10–15% of the human male population (28) compared with the 30% prevalence observed in the current study. The morphological changes and spermatogenic cell defects are similar to the reported human defects (28). The tumor rates for breast cancer are approximately 15% in the human population, but less than 1% in males (27, 29, 30). In contrast, the male rats in the F1–F4 vinclozolin generations had approximately a 10% frequency. As with human tumors, rat tumors observed were primarily of epithelial cell origin with a low frequency of metastasis. Overall, several similarities in frequency and etiology were made with the abnormalities observed in the current study with those found in humans. Future studies are now required to allow a comparison of the rat observations to human disease. This transgenerational phenotype provides a useful experimental animal model to help elucidate the diseases of a variety of tissues with potential application to human disease.

The ability of the endocrine disruptor vinclozolin to induce an epigenetic transgenerational disease state or abnormality suggests fetal exposure to environmental toxicants may be a significant factor in the molecular basis of disease. Previously, both the antiandrogenic compound vinclozolin and the estrogenic compound methoxychlor were found to induce a transgenerational phenotype (8). The concentration of vinclozolin used in the current study is higher than anticipated in the environment. For vinclozolin, the lowest observed adverse effect level recommended is 11 mg/kg·d, but doses at the 1 mg/kg·d have biological effects (31). The environmental levels of vinclozolin have not been rigorously determined, such that no conclusions regarding the toxicology of this compound can currently be made. Toxicology studies to determine whether environmental levels of the compound can induce these disease states are now needed. The mechanism of vinclozolin actions could involve androgen receptor-mediated events and/or toxicity. The androgen receptor has been shown to be present in the embryonic testis at the time of gonadal sex determination in the germ cells, Sertoli cells, and precursor peritubular cells (19, 20). Although the embryonic gonadal steroid production is minimal at this time, androstenedione is produced by the fetal adrenal. Therefore, endocrine disruptors have the capacity to influence embryonic androgen receptor actions (32, 33). Alternatively, vinclozolin actions could involve toxicologic actions on the developing gonad to subsequently influence germ cell development (17, 34, 35). Further studies are needed to elucidate the endocrine *vs.* toxicologic actions of vinclozolin on the embryonic testis.

Previous studies have shown that an embryonic exposure during gonadal sex determination (E8–E14) can induce onset of disease in the F1 generation, but later embryonic exposure (E15–E20) had no effect (18, 21). The actual sex determination event for the testis occurs from E10–E13.5, with cord formation complete at E14 and initial transcriptional events likely at E8-E10, such that E8–E14 covers the entire period. The primordial germ cells undergo an erasure (*i.e.* demethylation) of DNA methylation during migration down the genital ridge before colonizing the gonad (36, 37). During sex determination, the germ cells undergo a remethylation in a sex-specific manner (38). Endocrine disruptor exposure during this period appears to cause an epigenetic reprogramming of the germ line that is permanent and is transferred transgenerationally to subsequent generations (8). The male germ line is critical in the transmission of the transgenerational disease phenotype; however, the female germ line appears to influence the phenotype and remains to be investigated. The current study describes the ability of a variety of disease states to be induced through this apparent epigenetic transgenerational effect on the germ line. Correlation of specific changes in DNA methylation of imprinted-like genes (39) with specific tissue abnormalities will be important to elucidate in the future and may identify valuable diagnostic and therapeutic markers.

A previous report demonstrated that transient embryonic exposure to vinclozolin at the time of gonadal sex determination induces an apparent epigenetic effect on the programming of the male germ line (8). An epigenetic transgenerational phenotype is likely responsible for the disease states or abnormalities observed in the current study. The frequency of the abnormal phenotypes observed ranges from 12–50%, as shown in Fig. 1. The frequency of a hot spot DNA sequence mutational event has been shown to be approximately 5% at its highest and generally is less than 1% (22, 40). A genetic DNA sequence mutation also involves segregation with reduced frequency in subsequent generations. Therefore, the high frequency of the disease states and absence of normal Mendelian transmission observed in the current study suggests the transgenerational nature of the phenotype appears to be epigenetic through the germ line. The previous study demonstrated the presence of two genes with altered methylation in the germ line (8), and preliminary studies have revealed the presence of over 15 new imprinted-like genes/DNA sequences with alterations in methylation involving reprogramming of the male germ line (39). Therefore, the molecular basis for the transgenerational disease states observed appears to be epigenetic and due in part to a permanent reprogramming of the germ line.

The potential that an epigenetic (*i.e.* DNA methylation) transgenerational background may influence disease susceptibility, premature disease onset, and/or development of disease, is a factor in disease etiology not previously appreciated (1). This transgenerational phenomenon could explain how some subpopulations may have differences in the frequency of disease. Due to the ability of an environmental factor to alter this epigenetic transgenerational background, variability in environmental exposures could explain alterations in disease prevalence in different populations and regions. The most sensitive exposure period is at the time of gonadal sex determination, which is early to midgestation in humans. Therefore, the fetal basis of disease will likely in part involve the epigenetic transgenerational mechanism described. The specific genes that have altered methylation states and are transmitted through the germ line (39) remain to be fully elucidated. These genes may provide diagnostic and/or therapeutic markers to better un-

derstand specific diseases. Risk assessment for environmental toxicant exposure could consider the use of these genes as biomarkers. The current study describes the phenomena that an environmental compound, the endocrine disruptor vinclozolin, can induce epigenetic transgenerational diseases. This is a novel molecular mechanism to consider for disease etiology.

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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

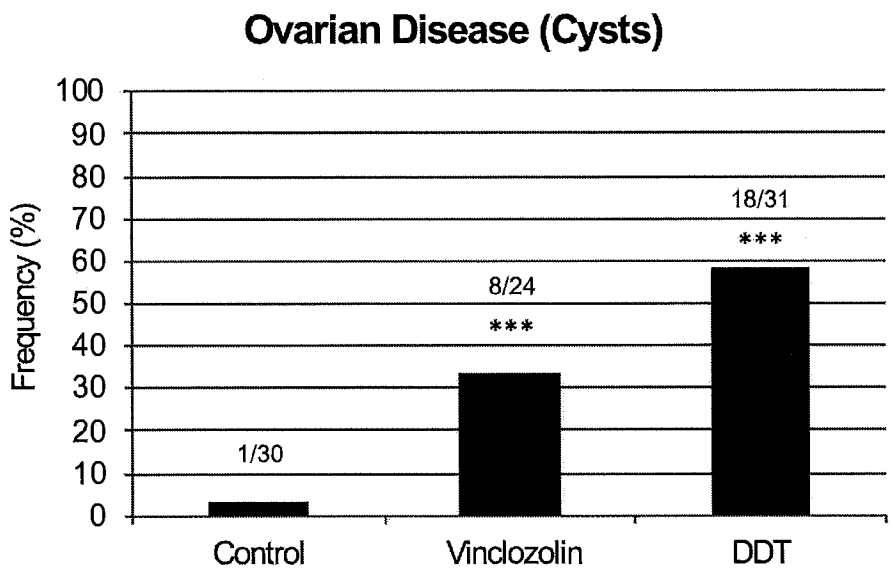

Figure 1. Ovarian pathology frequency. Transgenerational ovarian disease in F3 generation control, vinclozolin and DDT lineage rats at 1 y of age. Numbers for diseased individuals versus the total number of individuals analyzed is shown and (***) indicates statistical significance of $P < 7 \times 10^{-3}$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

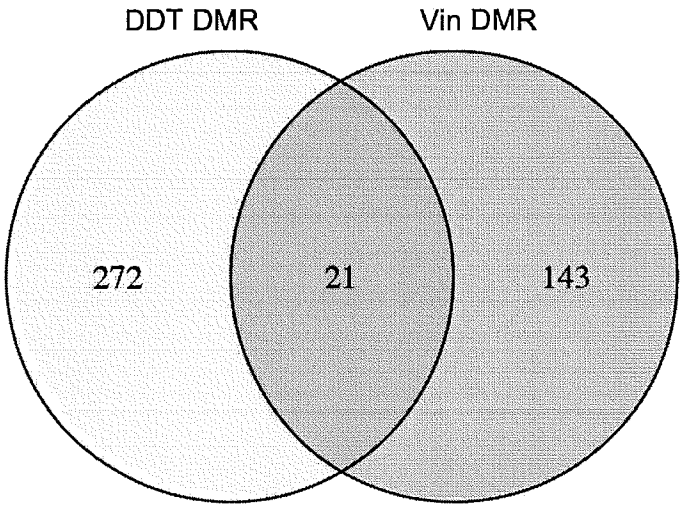

Figure 2. DMR identification. The number of DMRs found using different *P* value cutoff thresholds. The all window column shows all DMRs. The multiple window column shows the number of DMRs containing at least two significant windows. Lower table of each set shows the number of DMR having each specific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

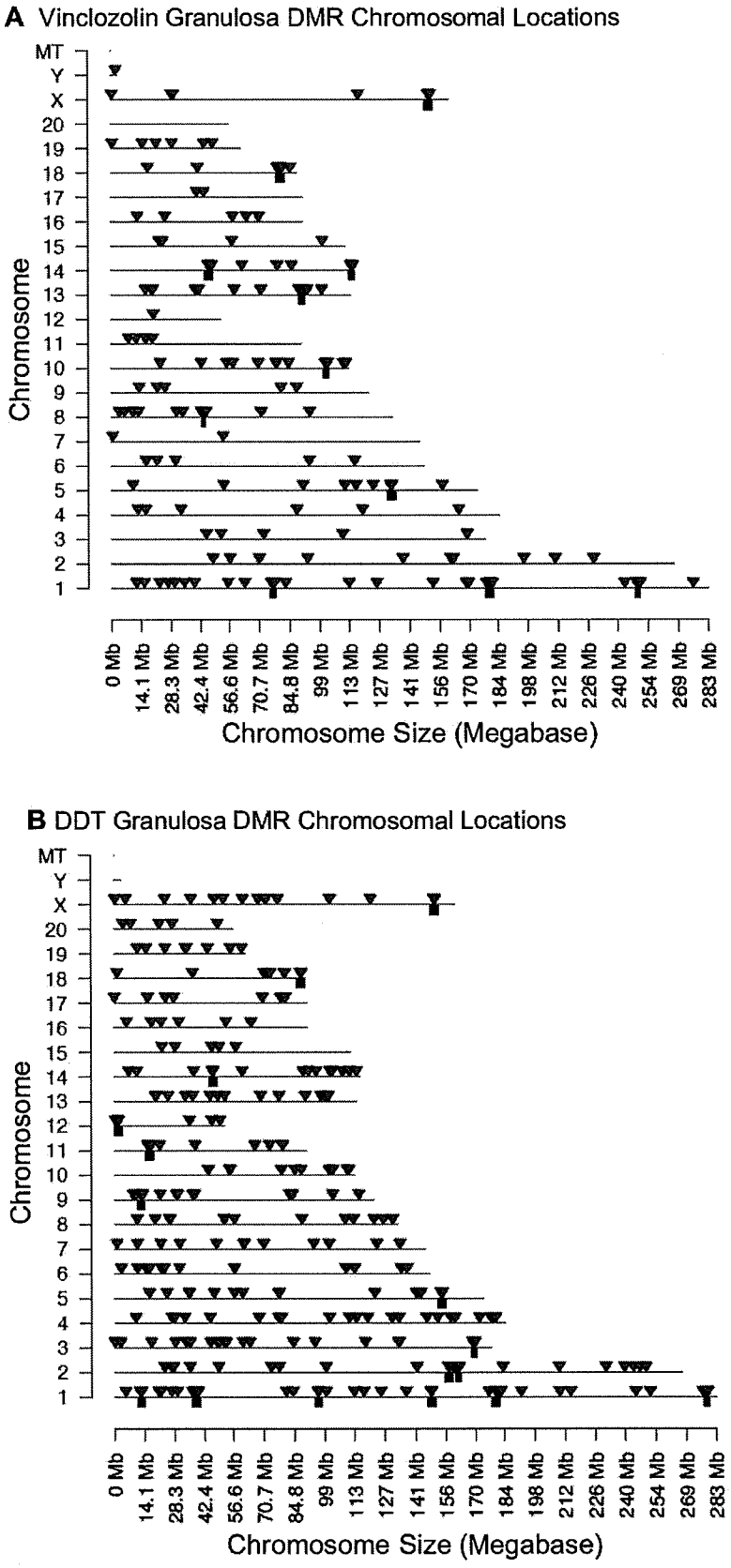

Figure 3. DMR chromosomal locations. The DMR locations on the individual chromosomes for all DMRs at a *P* value threshold of $< 1x10^{-6}$. (a) Vinclozolin F3 generation. (b) DDT F3 generation. Red arrowheads indicate positions of DMRs and black boxes indicate clusters of DMRs.

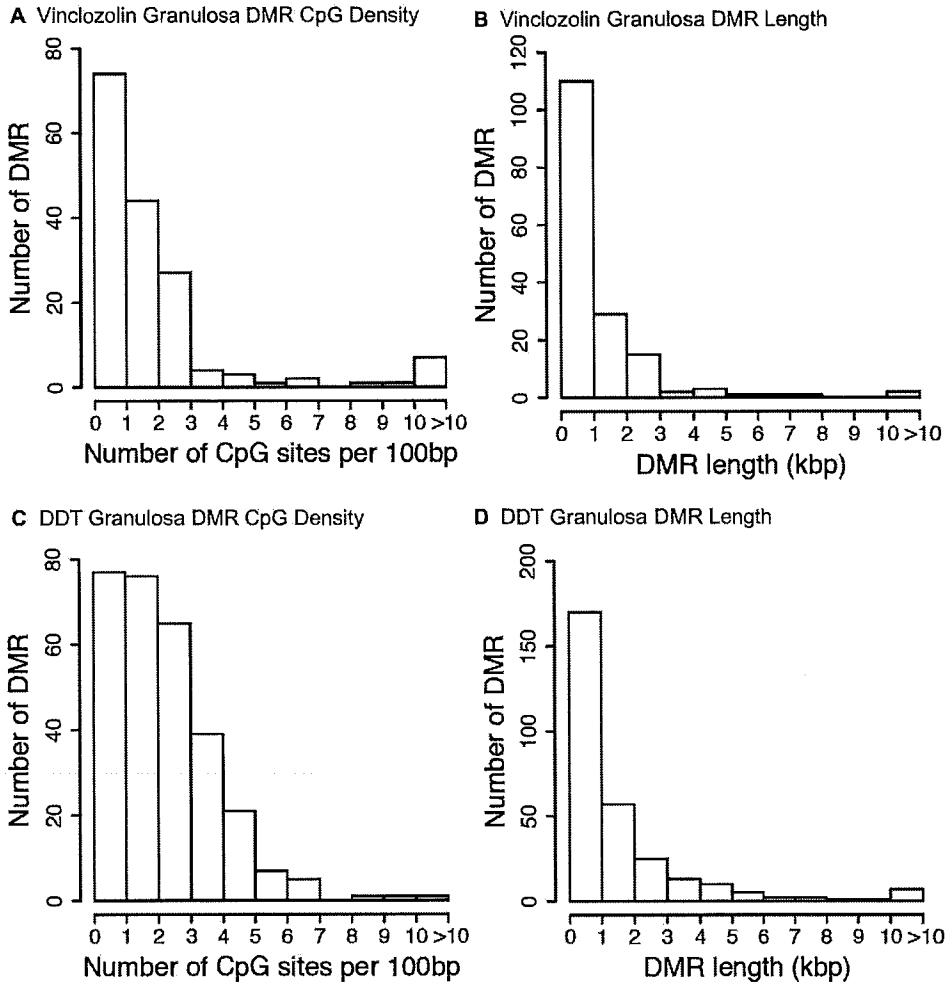

Figure 4. DMR genomic features. (a & c) The number of DMRs at different CpG densities for all DMRs at a p-value threshold of $P < 1x10^{-6}$. (b & d) The DMR lengths for all DMRs are at a P value threshold of $< 1x10^{-6}$. (a & b) Vinclozolin F3 generation. (c & d) DDT F3 generation.

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

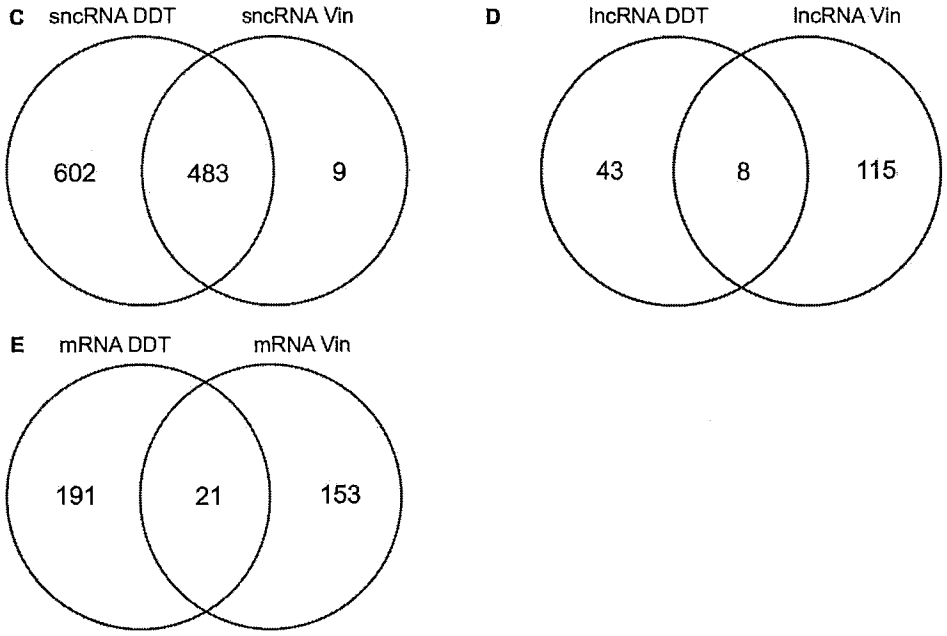

Figure 5. Differential RNA expression at different p-value thresholds for vinclozolin (a) and DDT (b). *P* value < $1x10^{-4}$ was used for subsequent analysis. Venn diagrams show overlap of RNA categories between the two lineages for (c) sncRNA, (d) lncRNA, and (e) mRNA.

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

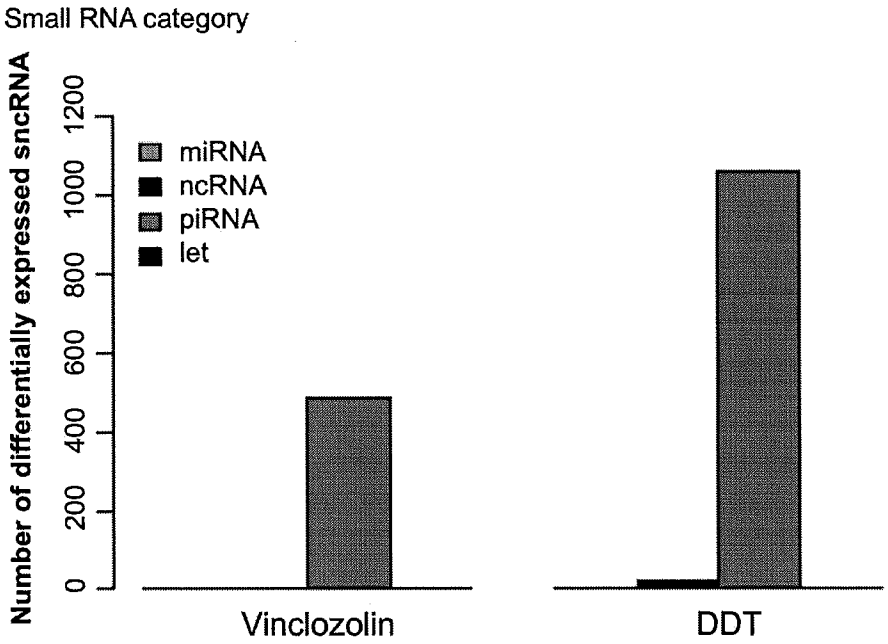

Figure 6. Differentially expressed small, noncoding RNA broken down by class and lineage. *P* value < 1 x 10^{-4} was used for analysis.

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

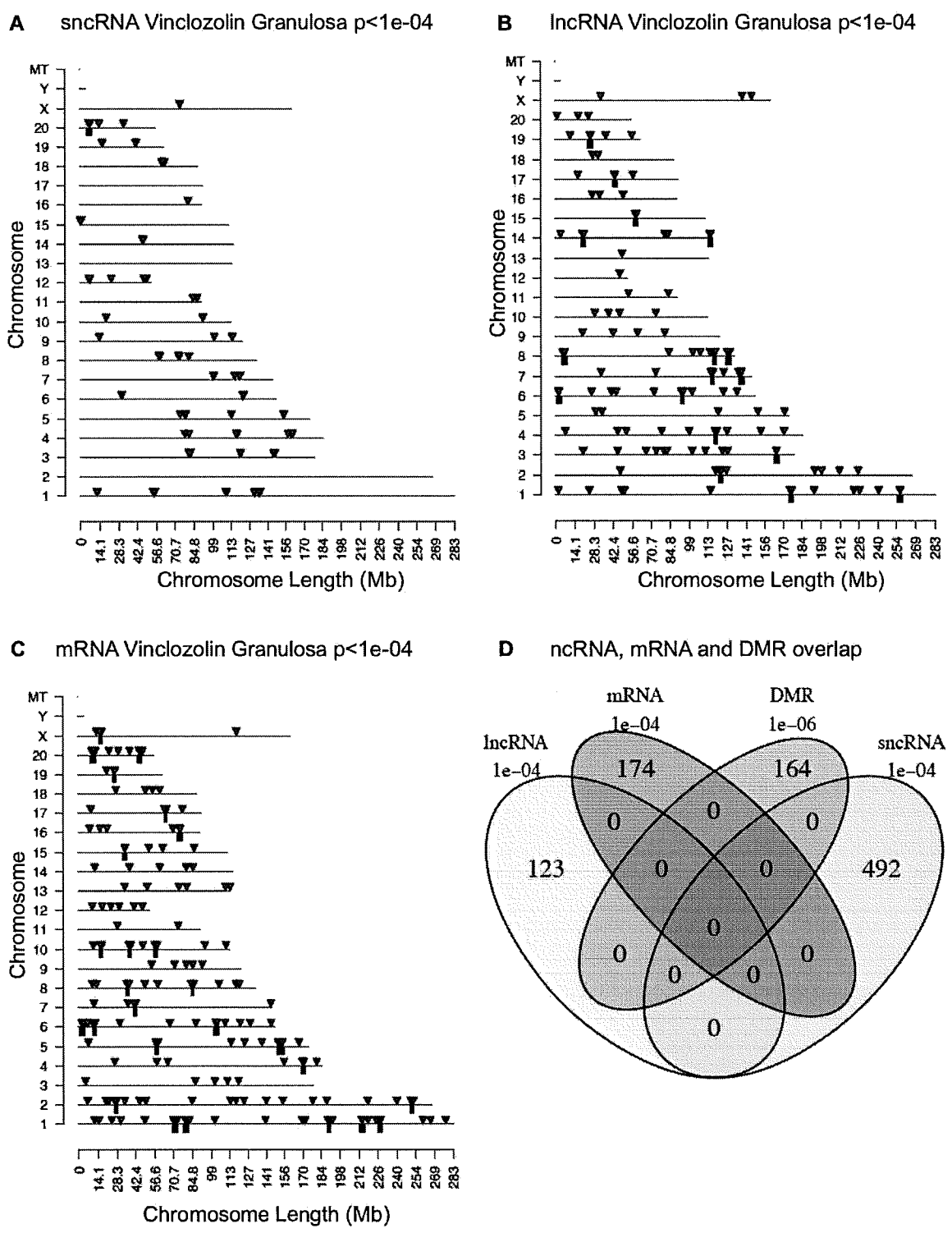

Figure 7. Differentially expressed RNAs from the vinclozolin lineage. Chromosomal locations of differentially expressed sncRNA (a), lncRNA (b), or mRNA (c). Individual RNAs are shown as red arrows and clusters are shown as black boxes. RNAs with unknown locations are not shown. (d) Venn diagram showing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

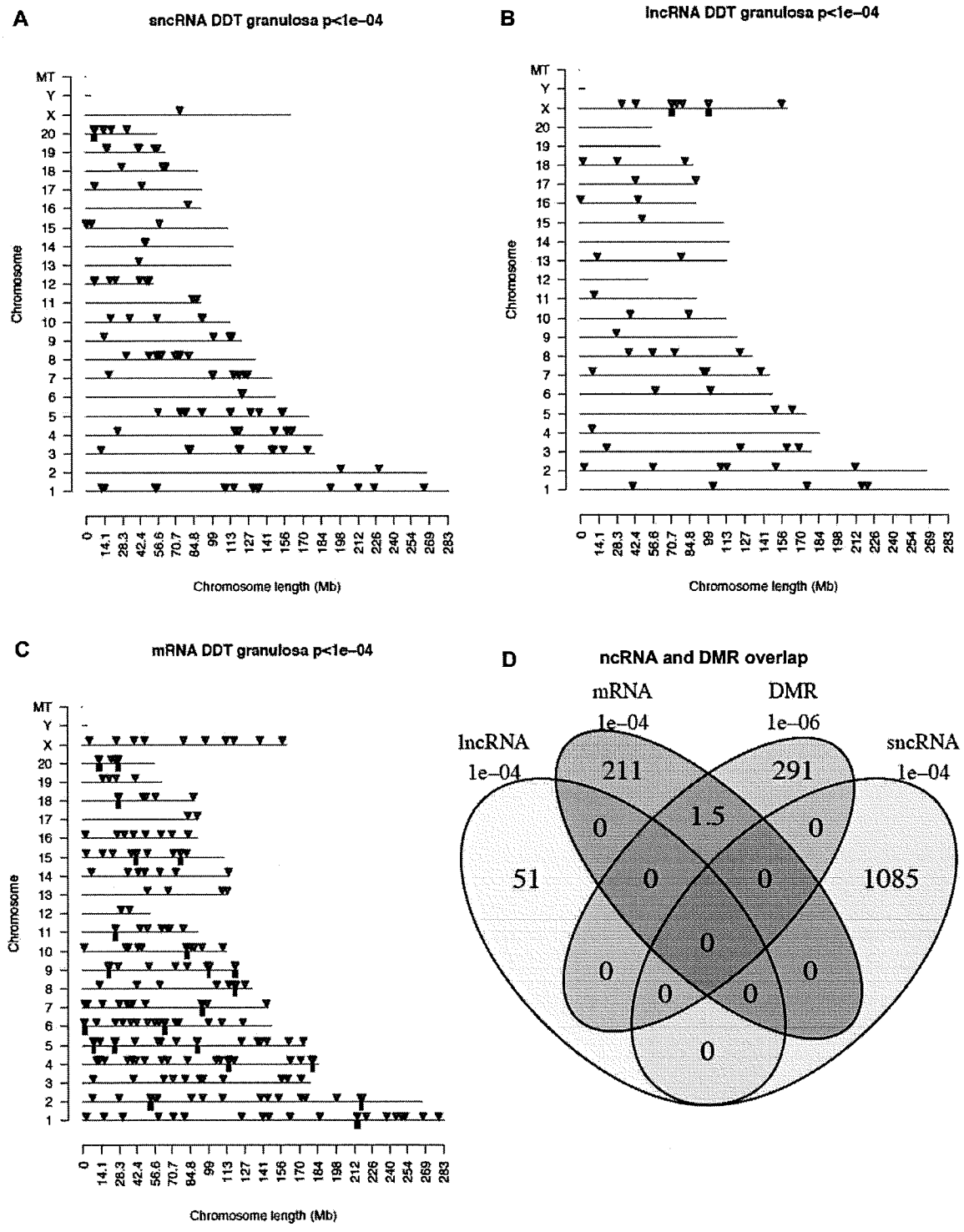

Figure 8. Differentially expressed RNAs from the DDT lineage. Chromosomal locations of differentially expressed sncRNA (a), lncRNA (b), or mRNA (c). Individual RNAs are shown as red arrows and clusters are shown as black boxes. RNAs with unknown locations are not shown. (d) Venn diagram showing overlap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

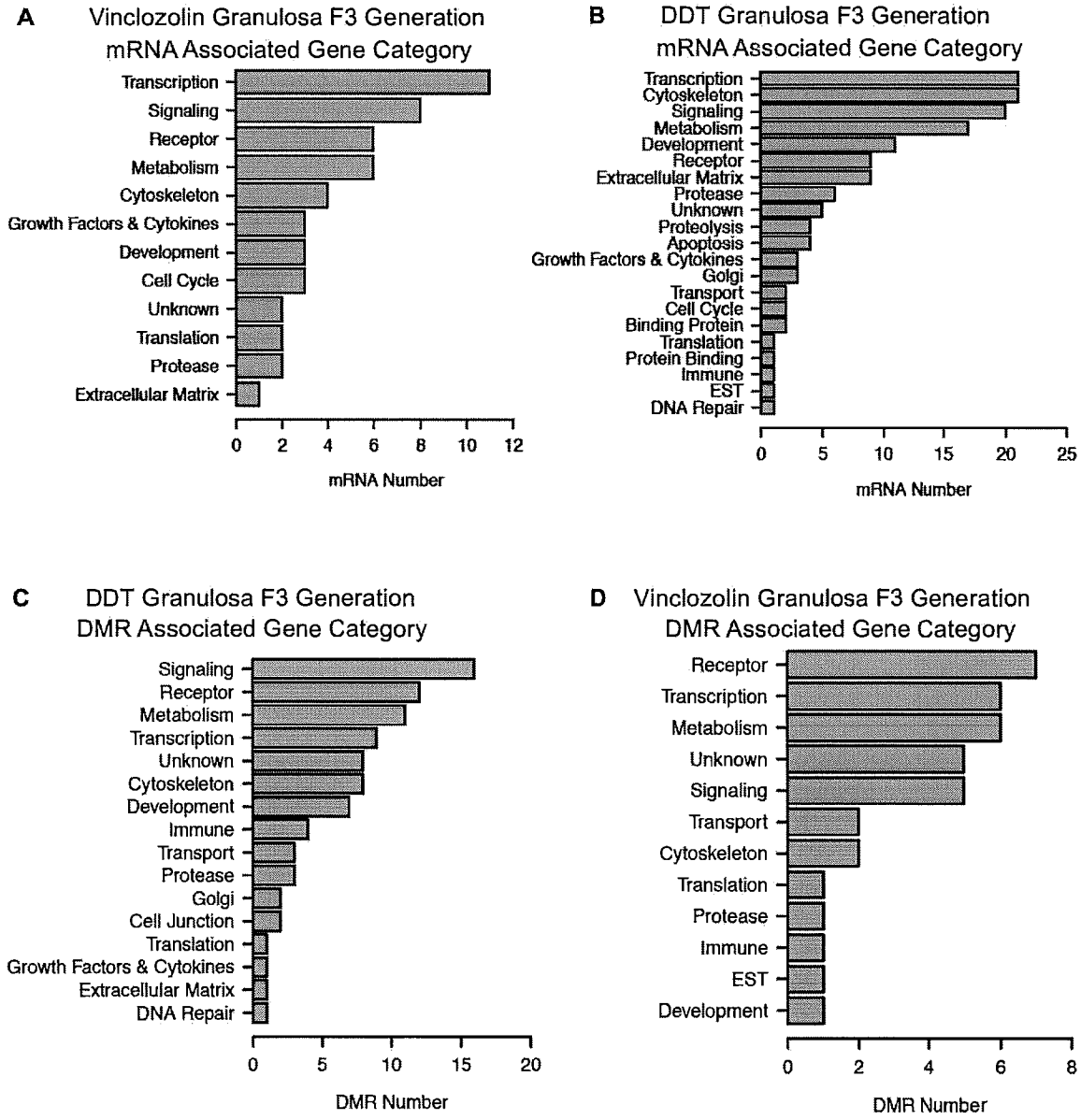

Figure 9. Differentially expressed epigenetic modifications were broken down by predicted associated gene category. Top row: mRNAs from vinclozolin (a) and DDT (b) lineages. Bottom row: DMRs from DDT (c) and vinclozolin (d). Genes or DMRs that could not be assigned a category are not shown.

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

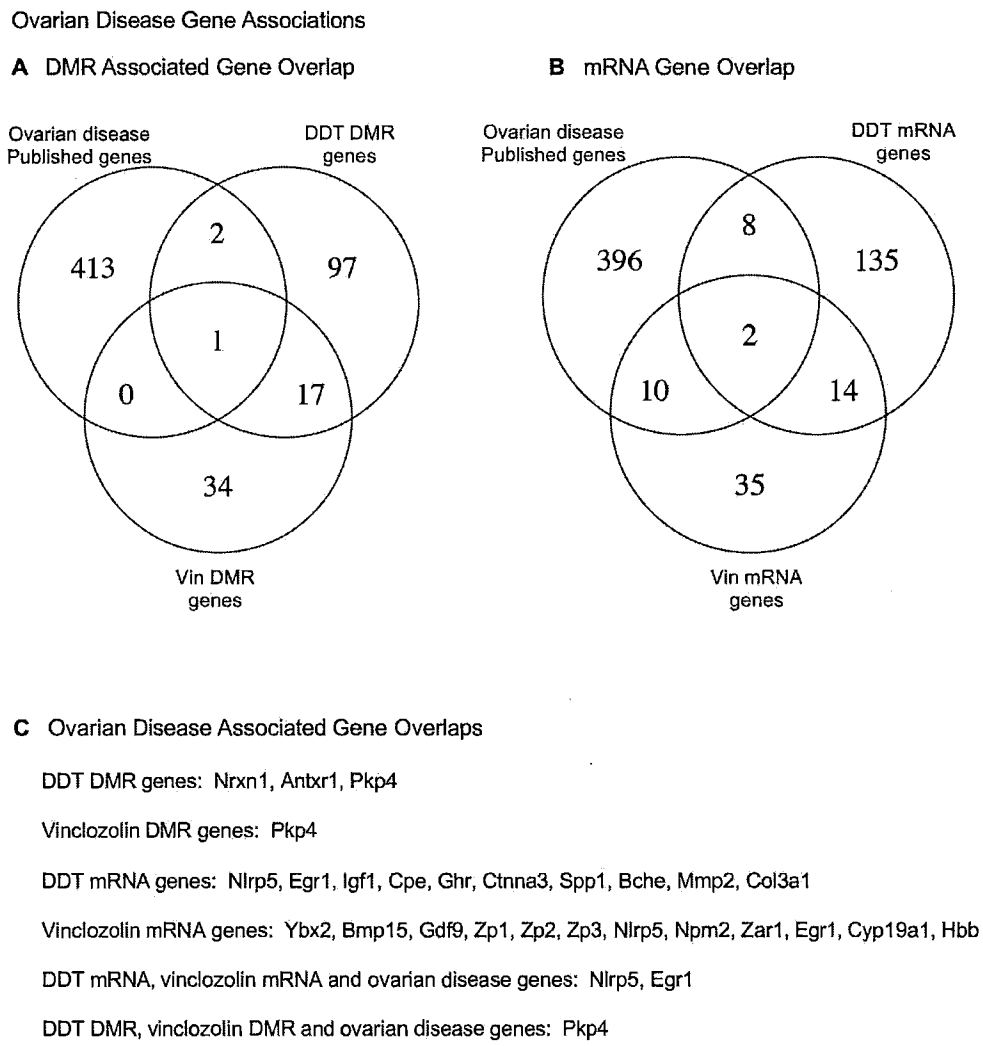

Figure 11. Ovarian disease gene associations. (a) DMR associated gene overlap with published ovarian disease genes. (b) mRNA gene overlaps with published ovarian disease genes. (c) Specific ovarian disease associated gene overlap with DMR associated genes and mRNA genes.

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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RESEARCH ARTICLE Open Access

Ancestral dichlorodiphenyltrichloroethane (DDT) exposure promotes epigenetic transgenerational inheritance of obesity

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Abstract

Background: Ancestral environmental exposures to a variety of environmental factors and toxicants have been shown to promote the epigenetic transgenerational inheritance of adult onset disease. The present work examined the potential transgenerational actions of the insecticide dichlorodiphenyltrichloroethane (DDT) on obesity and associated disease.

Methods: Outbred gestating female rats were transiently exposed to a vehicle control or DDT and the F1 generation offspring bred to generate the F2 generation and F2 generation bred to generate the F3 generation. The F1 and F3 generation control and DDT lineage rats were aged and various pathologies investigated. The F3 generation male sperm were collected to investigate methylation between the control and DDT lineage male sperm.

Results: The F1 generation offspring (directly exposed as a fetus) derived from the F0 generation exposed gestating female rats were not found to develop obesity. The F1 generation DDT lineage animals did develop kidney disease, prostate disease, ovary disease and tumor development as adults. Interestingly, the F3 generation (great grand-offspring) had over 50% of males and females develop obesity. Several transgenerational diseases previously shown to be associated with metabolic syndrome and obesity were observed in the testis, ovary and kidney. The transgenerational transmission of disease was through both female (egg) and male (sperm) germlines. F3 generation sperm epimutations, differential DNA methylation regions (DMR), induced by DDT were identified. A number of the genes associated with the DMR have previously been shown to be associated with obesity.

Conclusions: Observations indicate ancestral exposure to DDT can promote obesity and associated disease transgenerationally. The etiology of disease such as obesity may be in part due to environmentally induced epigenetic transgenerational inheritance.

Keywords: Environmental epigenetics, Metabolic syndrome, Obesity associated disease, Epimutations, Disease etiology, Maternal transmission

Background

A number of environmental factors such as toxicants and nutrition have been shown to promote the epigenetic transgenerational inheritance of adult onset disease and phenotypic variation [1-3]. Examples of environmental compounds include the fungicide vinclozolin [4-6], plasticizers bisphenol-A (BPA) and phthalates [7], dioxin [7-9], hydrocarbons [7,8], and pesticides [4,7,8]. Nutritional abnormalities such as caloric restriction and high-fat diets can also promote transgenerational phenotypes [10]. Epigenetic transgenerational inheritance involves the germline (sperm or egg) transgenerational transmission of epigenetic marks that influence physiological parameters and disease, in the absence of direct environmental exposures [1,3]. This phenomenon has been observed in plants [11], flies [12], worms [13], rodents [4], and humans [14]. Therefore, your ancestors' environmental exposures may influence your disease development, even though you have never had a direct

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exposure. Environmentally induced epigenetic transgenerational inheritance of disease appears to be a factor in disease etiology that needs to be considered and elucidated.

The present study was designed to examine the potential transgenerational actions of the most common historically used insecticide dichlorodiphenyltrichloroethane (DDT) [15,16]. DDT has been banned from the USA, but is used globally as an insecticide for control of vectors for malaria and visceral leishmaniasis. DDT is 1 of the 12 chemicals proposed for elimination by the 2001 Stockholm Convention of United Nations Environmental Program [17]. However, DDT use in Africa has increased since the Stockholm Convention due to the recent Gates Foundation Malaria Control Program [18]. The reported global use of DDT for disease vector control is 4,000 to 5,000 metric tons per year, with India being by far the largest consumer [19]. In 2006, the World Health Organization issued a position statement promoting the use of indoor residual spraying with DDT for malaria vector control. Although DDT is a low-cost antimalarial tool, the possible adverse human health and environmental effects of exposure must be carefully weighed against the benefits to malaria control [16]. Recent evidence indicates that indoor spraying causes high levels of human exposure to DDT [20]. The direct exposure toxic effects of DDT in humans have been reviewed [15] and include reproductive disease [21], neurological disease [22], developmental abnormalities [23], and cancer [24]. Studies have also shown DDT's potential to cause birth defects in wildlife [25]. Exposure to DDT and its breakdown product dichlorodiphenyldichloroethylene (DDE) may be associated with adverse health outcomes such as diabetes and obesity in children [26,27].

The dramatic increase in obesity over the past 50 years has suggested environmental factors are important in the disease etiology. The prevalence of obesity has increased substantially since the mid-20th century with an accelerated rate of increase in the 1980s [28]. The US Centers for Disease Control in 2010 reported that 33% of adults in the US are obese and 17% of children between ages 2 to 19 are obese. Obesity has not only increased in the US, but also increased in virtually every country where detailed data are available [29]. The primary causal factor suggested is overnutrition [28], however, recent studies have suggested environmental toxicants [28] such as plastics [30,31], hydrocarbons [32], and tributyltin [33] can promote obesity in rodents. Although overnutrition and reduced physical activity are critical elements of the disease, other contributing factors include maternal age, endocrine disruptors, sleep deprivation, pharmaceutical introgenesis, ambient temperature, and intrauterine and intergenerational effects [28]. All these contributing factors have been shown to be involved in obesity, but the underlying molecular mechanisms are unclear. Genetic abnormalities have been identified in a number of the genes associated with obesity [34,35], however, no significant genome-wide associations have been shown to correlate with the majority of obese individuals [35]. In addition, no known genetic mechanism could explain the rapid increase in the incidence of obesity in the last 30 years. Clearly, genetics will be a critical aspect of any disease, including obesity, but it simply cannot explain many of the elements of the disease etiology. An alternate consideration is the role of environmental epigenetics in obesity [28] and in the developmental origins of disease [36]. The present study further investigates the role of epigenetics in the etiology of obesity.

Obesity is now known to be associated with a number of different clinical conditions in a complex disease trait known as metabolic syndrome [37]. Although a number of diseases have associations, the functional link and correlation remains to be elucidated. Predominant associated conditions are insulin resistance [38] and polycystic ovarian disease [39]. Other obesity-associated conditions include type 2 diabetes [40], non-alcoholic fatty liver disease [41], obstructive sleep apnea [42], kidney/renal disease [43] and testis disease and male infertility [44]. The scientific literature suggests over 50% of females with polycystic ovarian disease are obese [45,46]. Therefore, polycystic ovarian disease and several other diseases that are associated with obesity are also investigated in the present study.

Although the direct exposure toxicity of DDT is documented [47], no previous transgenerational studies involving DDT exposure have been reported. The present study tests the hypothesis that DDT promotes the epigenetic transgenerational inheritance of obesity and associated disease. In the event DDT ancestral exposures promote obesity in subsequent generations, in the absence of any direct exposures, the biohazards of DDT are significantly greater than anticipated. It may be that ancestral exposures to environmental toxicants such as DDT have a significant role in the etiology of the obesity observed in the current human population. Interestingly, the F3 generation of the majority of the gestating women exposed in the 1950s in the USA are adults today. The elucidation of the epigenetic biomarkers and molecular mechanisms involved in this environmentally induced epigenetic transgenerational inheritance is anticipated to lead to new diagnostics and therapeutics for obesity and associated diseases.

Methods

Animal studies and breeding

Female and male rats of an outbred strain Hsd:Sprague Dawley®™SD®™ Harlan (Indianapolis, IN) at about 70 and

100 days of age were fed *ad libitum* 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 body weight. On days 8 to 14 of gestation [48], the females were administered daily intraperitoneal injections of DDT (either 50 or 25 mg/kg BW/day) or dimethyl sulfoxide (vehicle). The p,p'-DDT was obtained from Sigma (St Louis, MO, USA) (no. PS699) and was injected in a 20 μl dimethylsulfoxide (DMSO)/oil vehicle as previously described [7]. Treatment lineages are designated 'control', 'DDT' or 'lower' dose DDT lineages. This is not meant to represent a 'low' dose analysis. The gestating female rats treated were designated as the F0 generation. The offspring of the F0 generation rats were the F1 generation. Non-littermate females and males aged 70 to 90 days from F1 generation of control, DDT or low dose DDT lineages were bred to obtain F2 generation offspring. The F2 generation rats were bred to obtain F3 generation offspring. Outcross F4 generation offspring (n = 8 litters per lineage) were obtained by breeding the F3 generation males from control and low dose DDT lineages with wild type females. Reverse outcross F4 generation progeny (n = 8 litters per lineage) were obtained by breeding the F3 generation females from control and low dose DDT lineages with wild type males. The outcross and the reverse outcross individuals were maintained until 10 months of age and then euthanized for tissue collection and disease evaluation. The F1 to F4 generation offspring were not themselves treated directly with DDT. The control and DDT lineages were housed in the same room and racks with lighting, food and water as previously described [1,5,7]. All experimental protocols for the procedures with rats were preapproved by the Washington State University Animal Care and Use Committee (IACUC approval no. 02568–029).

Tissue harvest and histology processing

Rats at 10 to 12 months of age were euthanized by CO_2 inhalation for tissue harvest. Body and organ weights were measured at dissection time. No significant changes in body weight were observed within this 2-month period and statistical analysis did not identify this as a confounder in the analysis. Testis, epididymis, prostate, seminal vesicle, ovaries, uterus and kidney were fixed in Bouin's solution (Sigma) and 70% ethanol, then processed for paraffin embedding by standard procedures for histopathology examination. Tissue sections of 5 μm were made and were either unstained and used for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) analysis or stained with hematoxylin and eosin (H&E) stain and examined for histopathology. Blood samples were collected at the time of dissection, allowed to clot, centrifuged and serum samples stored for steroid hormone assays.

Histopathology examination and disease classification

Obesity was assessed with an increase in body weight and marked abdominal adiposity. The obesity classification has been defined as these abnormalities and the presence of associated pathologies [28,36,49-51]. Testis histopathology criteria included the presence of a vacuole, azoospermic atretic seminiferous tubule and 'other' abnormalities including sloughed spermatogenic cells in center of the tubule and a lack of a tubule lumen. Testis sections were examined by TUNEL assay (*in situ* cell death detection kit, Fluorescein, Roche Diagnostics, Mannheim, Germany). Prostate histopathology criteria included the presence of vacuoles, atrophic epithelial layer of ducts and hyperplasia of prostatic duct epithelium as previously described [52,53]. No prostatic intraepithelial neoplasia (PIN) lesions were observed in the prostates. Kidney histopathology criteria included reduced size of glomerulus, thickened Bowman's capsule and the presence of proteinaceous fluid-filled cysts. A cut-off was established to declare a tissue 'diseased' based on the mean number of histopathological abnormalities plus 2 standard deviations from the mean of control tissues by each of the three individual observers. This number was used to classify rats into those with and without testis, prostate or kidney disease in each lineage. A rat tissue section was finally declared 'diseased' only when at least two of the three observers marked the same tissue section 'diseased'. The proportion of rats with obesity or tumor development was obtained by accounting those that had these conditions out of all the animals evaluated.

Ovary sections were stained with H&E stain and three stained sections (150 μm apart) through the central portion of the ovary with the largest cross section were evaluated. Ovary sections were assessed for two diseases, primordial follicle loss and polycystic ovary disease. Primordial follicle loss was determined by counting the number of primordial follicles per ovary section and averaging across three sections. An animal was scored as having primordial follicle loss if the primordial follicle number was less than that of the control mean minus 2 standard deviations. Primordial follicles had an oocyte surrounded by a single layer of either squamous or both squamous and cuboidal granulosa cells [8,54]. Follicles had to be non-atretic and showing an oocyte nucleus in order to be counted. Polycystic ovary was determined by microscopically counting the number of small cystic structures per section averaged across three sections. A polycystic ovary was defined as having a number of small cysts that was more than the control mean plus 2 standard deviations. 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 to 250 μm in diameter measured from the inner cellular boundary across the longest axis. Percentages of females with primordial follicle loss or polycystic ovarian disease were computed.

Overall disease incidence

A table of the incidence of individual diseases in rats from each lineage was created and the proportion of individual disease, total disease and multiple disease incidences was computed. For the individual diseases, only those rats that showed a presence of disease (plus) or absence of disease (minus) are included in the computation. For the total diseases, a column with total number of diseases for each rat was created and the number of plus signs were added up for each of the rats and the proportion was computed as the number of rats with total disease out of all the listed rats. For the multiple diseases, the proportion was computed as the number of rats with multiple diseases out of all the listed rats.

Epididymal sperm collection and DNA isolation and methylated DNA immunoprecipitation

The epididymis was dissected free of connective tissue, a small cut made to the cauda and placed in 5 ml of F12 culture medium containing 0.1% bovine serum albumin for 10 minutes at 37°C and then kept at 4°C to immobilize the sperm. The epididymal tissue was minced and the released sperm centrifuged at 13,000 *g* and stored in fresh nuclear isolation medium (NIM) buffer at −20°C until processed further. Sperm heads were separated from tails through sonication following previously described protocol (without protease inhibitors) [55] and then purified using a series of washes and centrifugations [56] from a total of nine F3 generation rats per lineage (control or DDT) that were 120 days of age. DNA extraction on the purified sperm heads was performed as described [57]. Equal concentrations of DNA from three individual sperm samples were used to produce three DNA pools per lineage and employed for chromatin immunoprecipitation of methylated DNA fragments (MeDIP). MeDIP was performed as previously described [7,57].

MeDIP-chip analysis

The comparative MeDIP-chip was performed with Roche Nimblegen's Rat DNA Methylation 3 × 720 K CpG Island Plus RefSeq Promoter Array which contains 3 identical subarrays, with 720,000 probes per subarray, scanning a total of 15,287 promoters (3,880 bp upstream and 970 bp downstream from transcription start site). Probe sizes range from 50 to 75 bp in length with the median probe spacing of 100 bp. Three different comparative (MeDIP vs MeDIP) hybridization experiments were performed (three subarrays) for DDT lineage versus control, with each subarray encompassing DNA samples from six animals (three each from DDT and control). MeDIP DNA samples from experimental lineages were labeled with Cy3 and MeDIP DNA samples from the control lineage were labeled with Cy5. Selected differential DNA methylation regions (DMR) identified with the MeDIP-chip analysis were confirmed with a MeDIP-quantitative polymerase chain reaction (QPCR) analysis involving real-time PCR analysis of the MeDIP samples as previously described [31,32].

Bioinformatic and statistical analyses of MeDIP-chip data

The bioinformatic analysis was performed as previously described [7,57]. The statistical analysis was performed in pairs of comparative immunoprecipitation hybridizations between DDT (D) and controls (C) (for example, D1-C1 and D2-C2, D1-C1 and D3-C3, D2-C2 and D3-C3). In order to assure the reproducibility of the candidates obtained, only the candidates showing significant changes in all of the single paired comparisons (intersection) were chosen as a having a significant change in DNA methylation between DDT lineage and control lineage. This is a very stringent approach to select for changes, since it only considers repeated changes in all paired analysis. The statistically significant differential DNA methylated regions were identified and *P* value associated with each region presented. Each region of interest was then annotated for gene and CpG content. This list was further reduced to those regions with an average intensity value exceeding 9.5 (log scale) and a CpG density ≥1 CpG/100 bp.

Statistical analysis of rat organ and disease data

Individual animals from different litters were used for analysis and n values presented for all experiments. For statistical analysis, all the continuous data on body and organ weights and apoptosis were used as input in the program GraphPad Prism 5 statistical analysis program and t tests were used to determine if the data from the DDT lineages differ from those of control lineages. For the number of rats with or without disease, logistic regression analysis was used to analyze the data (control or DDT and diseased or unaffected). A simple logistic regression was performed using an online calculator tool (http://vassarstats.net/logreg1.html). The binary outcome variable was diseased/not diseased (for example, obese/non-obese). The predictor variable was treatment (control vs DDT and control vs LD DDT) and each was performed separately for the analysis. Each treatment group was only compared to its own control using the numbers of affected/non-affected individuals evaluated for

each treatment group. All treatment differences were considered significant if the *P* value was less than 0.05.

Results

Transgenerational obesity and associated disease analysis

The transgenerational actions of control (vehicle DMSO), DDT (50 mg/kg body weight) and a lower dose (LD) DDT (25 mg/kg BW) administered female rats (F0 generation) during days 8 to 14 of gestation were investigated. The doses of DDT used are anticipated environmental exposures [22,58]. The F1 (direct exposure) and F3 (transgenerational) generation rats of control, DDT and lower dose DDT lineages were aged to 1 year and euthanized for analysis. The testis, prostate, kidney, ovary and uterus were collected and examined for histopathologies. To assess if there was any direct fetal exposure toxicity to DDT the F1 generation litter size, sex ratio, body weights and organ weights were measured (Additional file 1: Table S1A). No effect was observed on litter size or sex ratio ($P > 0.05$). The body weights of the F1 generation DDT and LD DDT were slightly reduced, while several organ weights were slightly increased. Therefore, no overt toxicity to DDT was observed in the direct *in utero* exposed F1 generation lineages.

The incidence of obesity in DDT and LD DDT lineages are presented in Figure 1a,b. The obesity was determined using an increase in body weight and abdominal adiposity (fat deposition) and presence of associated disease. The body weights of the non-obese (506.6 ± 8.2 g, male; 278.4 ± 2.7 g, female) compared to the obese (515 ± 5.8 g, male; 297.1 ± 4.2 g, female) DDT and LD DDT lineages indicated a statistically significant increase in body weight in the DDT and LD DDT lineage obese animals ($P < 0.05$). Although the mean body weight for all DDT and LD DDT lineage individuals was not increased (Additional file 1: Table S1), all the obese animals did have an increase in body weight. The magnitude of the statistically significant weight gain was not large, but we did observe larger weight gain as the animals aged, as previously described [59]. No weight gain effect was observed on younger 120-day-old animals, indicating the obesity weight gain was an adult onset condition. Analysis of the abdominal adiposity for a non-obese (Figure 1c) compared to an obese (Figure 1d) animal demonstrated a dramatic increase in abdominal fat deposition (adiposity) on nearly all organs in obese animals. An increased body weight and abdominal adiposity was observed in all the obese animals identified.

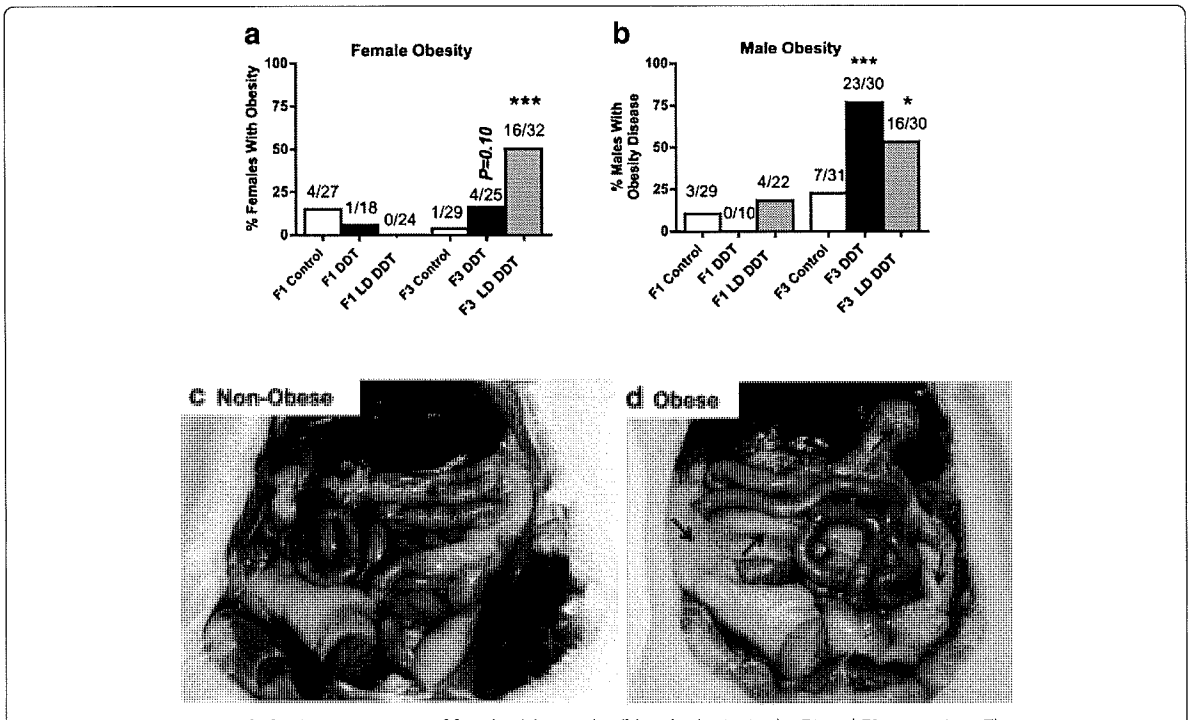

Figure 1 Transgenerational obesity. Percentages of females **(a)** or males **(b)** with obesity in the F1 and F3 generations. The dichlorodiphenyltrichloroethane (DDT) or lower dose (LD) DDT is indicated. The number of diseased rats/total number of rats (n value) is shown above the respective bar graphs. A logistic regression analysis was performed and *P* values indicate a significant difference from controls (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). The representative abdominal adiposity is shown for non-obese **(c)** and obese **(d)** rats, with the pink fat tissue deposition indicated (arrows) around the liver, intestines, subcutaneous and epididymis in the obese animal.

Interestingly, no increase in the incidence of obesity was observed in the F1 generation DDT or LD DDT lineages (Figure 1). In contrast, the F3 generation LD DDT lineage females and males had 50% of the animals develop obesity. In the F3 generation DDT lineage males 75% of the animals developed obesity. Although the DDT and LD DDT F1 generation animals did not have an altered incidence of obesity, the ancestral DDT and LD DDT exposures were found to promote transgenerational (F3 generation) obesity in the majority of males and females. Therefore, DDT was found to promote the transgenerational inheritance of obesity and as discussed below the molecular mechanisms of F1 and F3 generation disease are distinct.

Since obesity is a component of a complex disease trait (Figure 2), a number of the other obesity-associated diseases (for example, testis disease, polycystic ovarian disease, and kidney disease) were investigated. The causal correlation of these diseases remains to be determined, but the presence of associated disease is presented. The incidence of testis disease in DDT and LD DDT lineages is presented in Figure 3a. Testis disease was characterized by the presence of histopathology including azoospermia, atretic seminiferous tubules, presence of vacuoles in basal regions of seminiferous tubules, sloughed germ cells in the lumen of seminiferous tubules, and lack of seminiferous tubule lumen (Additional file 2: Figure S1A). DDT exposure had no influence on testis disease in the F1 generation males. Testis disease incidence increased significantly in the F3 generation DDT lineage with 47% of the males affected (Figure 3a). Further analysis of the testis examined the number of apoptotic spermatogenic cells in the testis and epididymal sperm counts (Figure 3b,c). Spermatogenic cell apoptosis was decreased in the F1 generation DDT and LD DDT lineages and increased in the F3 generation lineages compared to control. Sperm counts were decreased in the F3 generation DDT lineage males. Therefore, DDT was found to promote obesity-associated transgenerational testis disease.

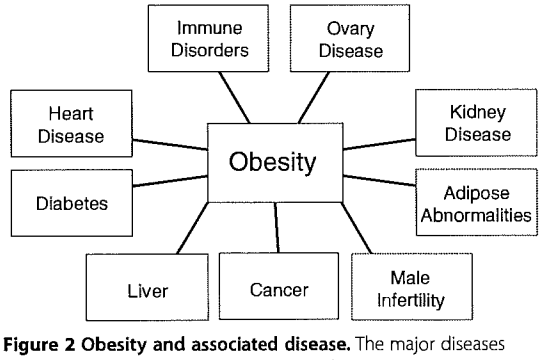

Figure 2 Obesity and associated disease. The major diseases previously shown to be associated with obesity are indicated with those identified in the present study shown with a red box.

The incidence of ovarian disease in DDT and LD DDT lineages is presented in Figure 3d. An increase in ovarian disease was observed in both the F1 and F3 generation DDT lineages. The primary ovarian disease detected was the development of polycystic ovaries with an increase in the number of small and large cysts as previously described [60] (Figure 3e). In contrast to previous environmental toxicants examined [60], no effect on primordial follicle numbers were detected in the DDT or LD DDT lineages (Figure 3f). The increase in ovarian disease in the F3 generation DDT and LD DDT lineages observed was primarily characterized by the development of ovarian cysts. Analysis of estradiol levels in the proestrous or diestrous F3 generation females revealed this was not changed in the DDT or LD DDT lineages (Additional file 3: Figure S2C,D). Therefore, DDT and LD DDT were found to promote the obesity-associated transgenerational polycystic ovarian disease.

The incidence of kidney disease in DDT and LD DDT lineages is presented in Figure 3g and 3h. Kidney disease was characterized by the presence of an increased number of proteinaceous fluid filled cysts, reduction in size of glomeruli and thickening of Bowman's capsules (Additional file 2: Figure S1B). There was an increase in female kidney disease in F1 and F3 generation LD DDT lineage, but only in the F1 generation in the DDT lineage (Figure 3g). The males showed a dramatic increase with over 60% of the F1 and F3 generation animals in the LD DDT lineage affected (Figure 3h). Therefore, DDT was found to promote the obesity-associated transgenerational kidney disease in both males and females.

Additional potential obesity-associated disease and abnormalities (Figure 2) investigated were prostate disease [61], pubertal abnormalities [62], tumor development [63] and immune abnormalities [64]. Prostate disease was examined in the F1 and F3 generation males (Additional file 2: Figure S1 and Table S2A). Prostate disease was characterized by atrophic prostate duct epithelium and hyperplasia (Additional file 2: Figure S1A) as previously described [52]. The incidence of prostate disease increased in F1 generation males of DDT and LD DDT lineages, but no effect was observed in the F3 generation males. Therefore, no transgenerational prostate disease was observed. In addition, no influence on F3 generation male testosterone levels was observed (Additional file 3: Figure S2E). Analysis of the onset of puberty, as previously described [7], was found not to be altered in either females (Additional file 3: Figure S2F) or males (Additional file 3: Figure S2G) in the F1 or F3 generations. The incidence of tumor development was monitored and the primary tumors observed were mammary tumors, as previously described [5]. No altered

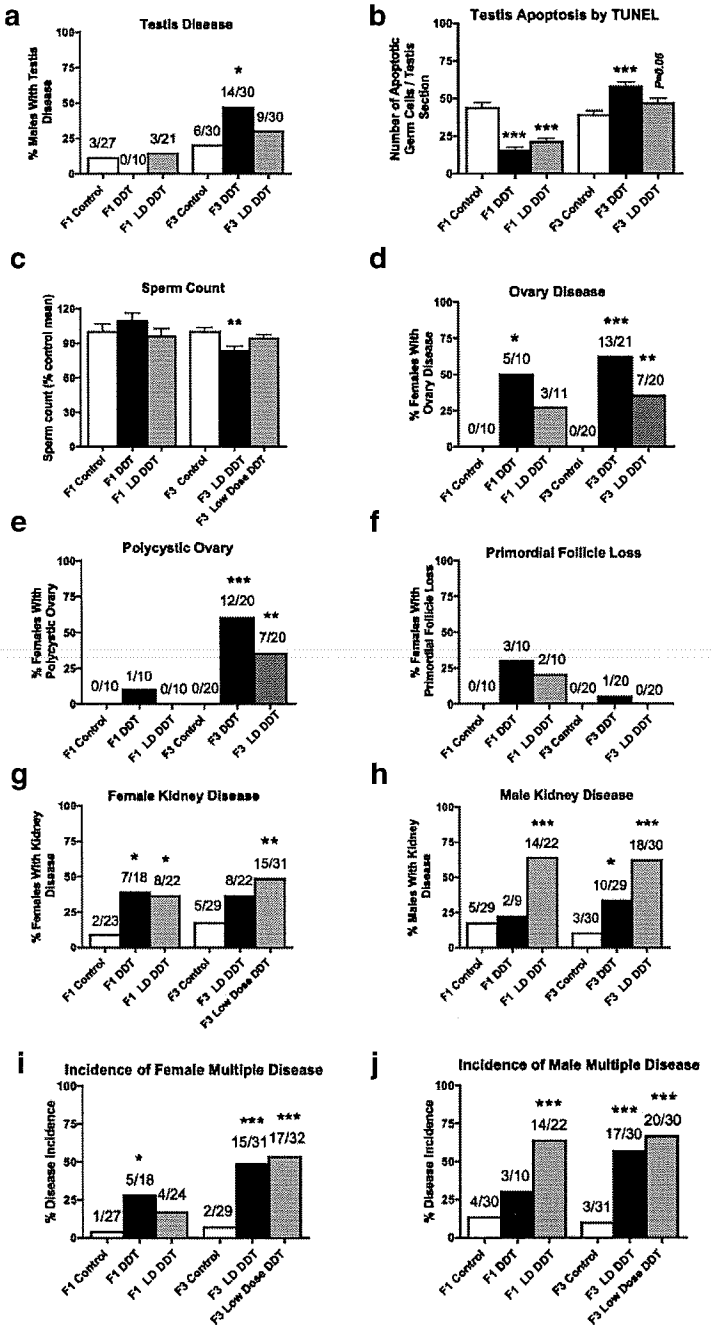

Figure 3 Transgenerational obesity and associated disease. Percentages of the F1 and F3 generation disease/abnormalities from control (open bars), dichlorodiphenyltrichloroethane (DDT) (black bars) and lower dose (LD) DDT lineages. Testis disease **(a)**, spermatogenic cell apoptosis **(b)** and sperm counts **(c)**, ovarian disease **(d)**, polycystic ovarian disease **(e)** and primordial follicle pool loss **(f)** are presented. Percentages of females **(g)** and males **(h)** with kidney diseases and percentages of females (I) and males (J) with incidence of multiple disease. The number of diseased rats/total number of rats (n value) is shown above the respective bar graphs. Those showing numbers above the bars were analyzed with a logistic regression analysis and those with a mean ± SEM indicated were analyzed with a *t* test with the *P* value indicated (**P* <0.05; ***P* <0.01; ****P* <0.001) (Additional file 4: Table S2 and Additional file 5: Table S3).

tumor development was observed in the F1 or F3 generation females (Additional file 3: Figure S2H). An increase in tumor development was observed in the F1 generation DDT lineage males, but no effect was observed in the F3 generation lineages (Additional file 3: Figure S2I). Therefore, no transgenerational influence was observed for prostate disease, pubertal abnormalities or tumor development. Analysis of the uterus identified a significant increase in uterine infection in the F3 generation DDT lineage females, with over 70% of the animals affected (Additional file 3: Figure S2B). Uterine infection was determined by the enlargement of uterus, accumulation of foul-smelling dark discolored purulent material and presence of inflammation within the uterine horns. Therefore, potential transgenerational immune abnormalities were detected in the females.

The incidence of multiple disease and abnormalities (≥2) per rat was determined (Figure 3i,j). The incidence of diseases in individual rats from control, DDT and LD DDT lineages are presented in Additional file 4: Table S2 for F1 generation females (S2A) and males (S2B), and in Additional file 5: Table S3 for F3 generation females (S3A) and males (S3B). The incidence of multiple diseases in F1 generation females increased in the DDT lineage, while the incidence in the F3 generation females increased in approximately 50% of the animals in the DDT and LD DDT lineages (Figure 3i). The incidence of multiple disease in the F1 generation males increased in the LD DDT lineage, while the incidence in the F3 generation males increased to affect approximately 60% of the animals in the DDT and LD DDT lineages (Figure 3j). Therefore, exposure of F0 generation females to two different doses of DDT increased the incidence of obesity and associated multiple diseases in the F1 and F3 generation male and female progeny.

Parental germline transmission of DDT-induced transgenerational obesity and associated disease

An experiment was designed to determine if the epigenetic transgenerational inheritance of adult onset obesity and associated disease is transmitted through the male (sperm) and/or female (egg) germline. The F3 generation control and LD DDT lineage animals were outcrossed to wild-type animals to generate the F4 generation. The outcross (OC) involved an F3 generation male being crossed with a wild-type female and the reverse outcross (ROC) involved an F3 generation female being crossed with a wild-type male. The F4 generation animals were aged to 10 months and then sacrificed to assess transgenerational obesity and associated disease incidence as previously described (Additional file 6: Tables S4 and Additional file 7: Table S5).

The F3 generation LD DDT-induced transgenerational obesity in the female was transmitted with a trend to the F4 generation outcross LD DDT lineage, but was not statistically different (Figure 4a). In contrast, the F3 generation LD DDT-induced transgenerational obesity in the male was transmitted to the F4 generation reverse outcross LD DDT lineage (Figure 4b). Therefore, the obesity in the female appears to be transmitted through the male germline or require both parental germline contributions, while the obesity in the male is transmitted through the female germline. The LD DDT-induced obesity-associated transgenerational testis disease was transmitted to the F4 generation reverse outcross, LD DDT lineage (P <0.06) (Figure 4c). Therefore, the testis disease was transmitted through the female germline. The LD DDT-induced obesity-associated transgenerational polycystic ovarian disease was transmitted to the F4 generation reverse outcross LD DDT lineage (Figure 4d). Therefore, the polycystic ovarian disease was also transmitted through the female germline. The LD DDT-induced obesity-associated transgenerational kidney disease in the female was transmitted to the F4 generation reverse outcross LD DDT lineage with a strong trend (P <0.09) (Figure 4e). The LD DDT-induced transgenerational kidney disease in the male was transmitted to both the F4 generation outcross and reverse outcross LD DDT lineages (Figure 4f). Therefore, the female kidney disease was transmitted through the female germline, but the male kidney disease was transmitted by both the male and female germlines. Combined observations indicate that the LD DDT-induced transgenerational obesity and associated disease is predominately transmitted through the female (egg) germline, but specific diseases (for example, kidney) are also transmitted through the male (sperm) germline. This is one of the first observations that the female germline can also transmit transgenerational disease. Observations suggest the parental germline origins for the transgenerational disease may be exposure specific and also disease or organ specific.

Epigenetic transgenerational transmission of sperm epimutations

The DDT-induced epigenetic transgenerational inheritance of obesity and associated disease requires the germline transmission of epimutations [1-3]. Previously, F3 generation sperm have been shown to have differential DMR induced by vinclozolin [4,57] and a variety of other environmental toxicants [7]. Interestingly, the sperm epimutations induced appear to be unique to the specific environmental exposure [7]. The present study investigated the sperm epimutations induced by DDT and present in the F3 generation sperm. Three different experiments with each involving a different pool of three different animals from different litters were used. The F3 generation control and LD DDT lineage sperm were

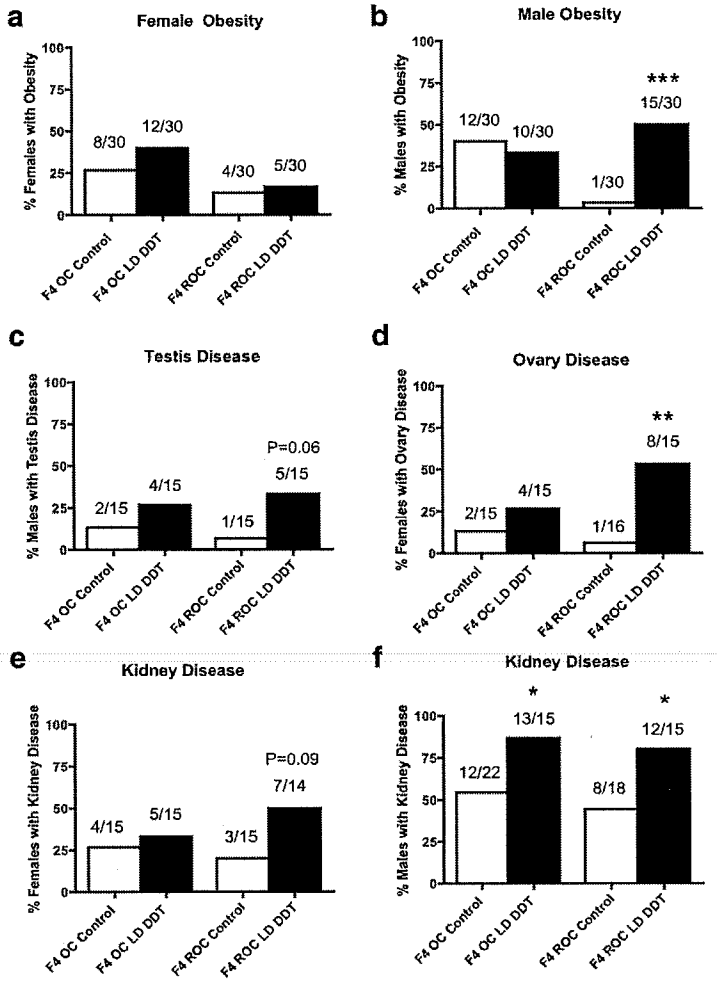

Figure 4 Transgenerational disease in F4 generation outcross or reverse outcross offspring for both male and female germline transmission. Incidences of obesity in females **(a)** and in males **(b)**, ovary disease **(c)**, testis disease **(d)**, kidney disease in females **(e)**, in males **(f)**, of the F4 generation outcross (OC) (F3 dichlorodiphenyltrichloroethane (DDT) lineage male cross with wild-type female) or reverse outcross (ROC) (F3 DDT lineage female cross with wild-type male) offspring of the control, DDT, and lower dose (LD) DDT F3 generation lineages. The number of diseased rats/total number of rats (n value) in each lineage are shown above the bar. A logistic regression analysis with *P* value compared to control is indicated (**P* <0.05; ***P* <0.01; ***P <0.001).

collected and analyzed using the MeDIP procedure followed by a promoter tiling array chip (MeDIP-chip) analysis as previously described [7,57]. Those DMR between the control and LD DDT lineage sperm samples that were statistically significant ($P < 10^{-5}$) were identified and termed epimutations. When the DMR were present in all three different experiments they were termed 'intersection' DMR. A total of 39 intersection DMR were identified and their chromosomal locations are indicated in Figure 5a and Additional file 8: Table S6. As previously described [7], the majority (28 DMR) of the intersection DMR were unique to the DDT exposure (Figure 5b) and not common with those epimutations previously identified in vinclozolin, plastics, dioxin, pesticide or hydrocarbon exposures [7,57] (Additional file 8: Table S6). A less stringent analysis uses the mean averages of the three different experiments to identify significantly different DMR termed 'average'. Using a $P < 10^{-5}$ cut-off a total of 231 average DMR were identified as shown in Figure 5a and Additional file 9: Table S7. Confirmation of the MeDIP-chip analysis DMR data used a quantitative PCR MeDIP-QPCR analysis. Three genes associated with the DMR from the intersection DMR list were selected due to having high interconnectivity in the gene network described below. The genes and statistically significant change ($P < 0.05$) between control versus

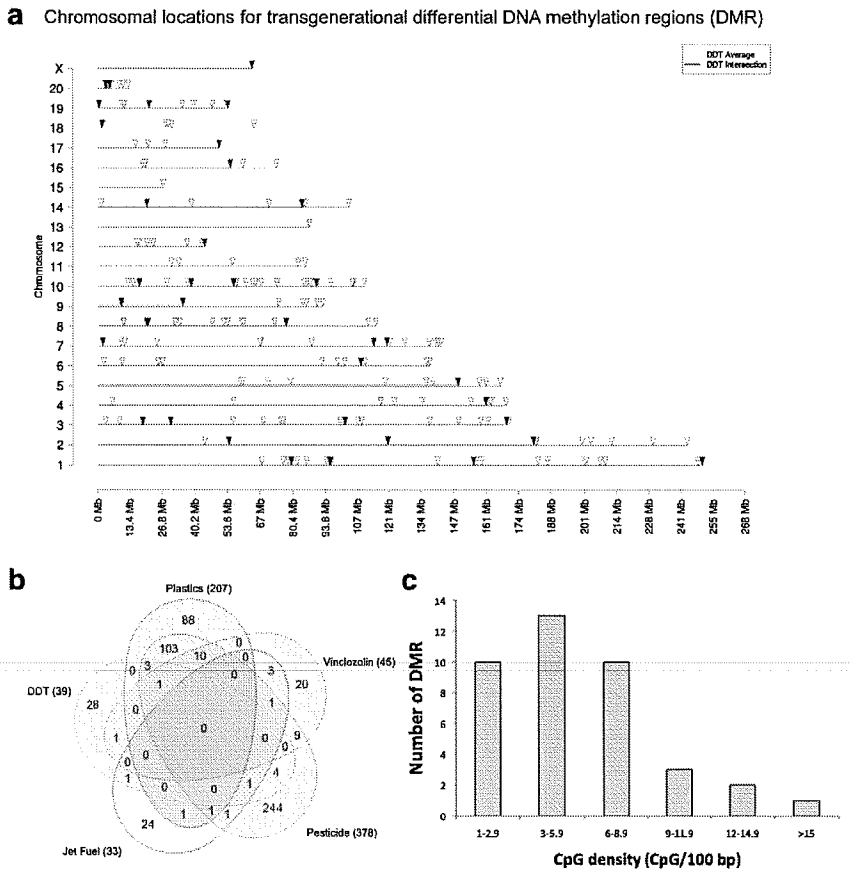

Figure 5 Transgenerational F3 generation sperm epimutations. (a) Chromosomal locations for differential DNA methylated regions (DMR) in sperm DNA from F3 generation dichlorodiphenyltrichloroethane (DDT) lineage rats compared to control lineage (arrowheads). The high stringency intersection epimutations are identified as red arrows, which is a subset of the less stringent 'average' DMR indicated with open arrows. The chromosomal size and number are listed (Additional file 8: Table S6 and Additional file: 9 Table S7). **(b)** A Venn diagram of DMR from various F3 generation exposure lineages including: vinclozolin, plastics, pesticides, hydrocarbons and DDT. The total number of DMR per exposure lineage in brackets is presented and unique and overlapping DMR identified. **(c)** The CpG/100 bp is presented and the corresponding number of DMR associated. The density is presented as the number of CpG for 100 bp of the DMR.

DDT lineage F3 generation sperm MeDIP samples were *Tubb3* (tubulin beta 3) (>13.8 fold change), *Carm1* (coactivator-associated arginine methyltransferase) (>50 fold change), and *Slc4a4* (solute carrier family 4 sodium bicarbonate cotransporter member 4) (>10 fold change) (Additional file 10: Figure S3). A profile of the MeDIP-chip data for these genes is presented and compared to the MeDIP-QPCR data (Additional file 10: Figure S3). Therefore, the MeDIP-QPCR confirmed the MeDIP-chip analysis for these DDT-induced sperm DMR. Observations demonstrate DDT induced a unique set of epimutations in the F3 generation sperm.

A previous study identified a unique genomic feature associated with the environmentally induced sperm DMR [7,57]. The genomic feature identified in all transgenerational sperm DMR previously identified is a low-density CpG of less than 10 CpG/100 bp [7,57]. These low-density regions are termed 'CpG deserts' that contain clusters of CpG [7]. The DDT transgenerational sperm DMR were found to contain less than 15 CpG/100 bp, with 3 to 6 CpG/100 bp being the most predominant density (Figure 5c). Therefore, the low-density 'CpG desert' genomic feature was a component of the DDT F3 generation sperm DMR.

Further bioinformatic analysis of the genes associated with the DMR in Table S7 identified a network of interconnected genes using a literature-based analysis (see Methods). A number of highly connected genes were identified that are involved in extracellular, signaling and transcriptional activities (Figure 6a). The potential role of this gene network or specific signaling processes in the DDT promoted epigenetic transgenerational

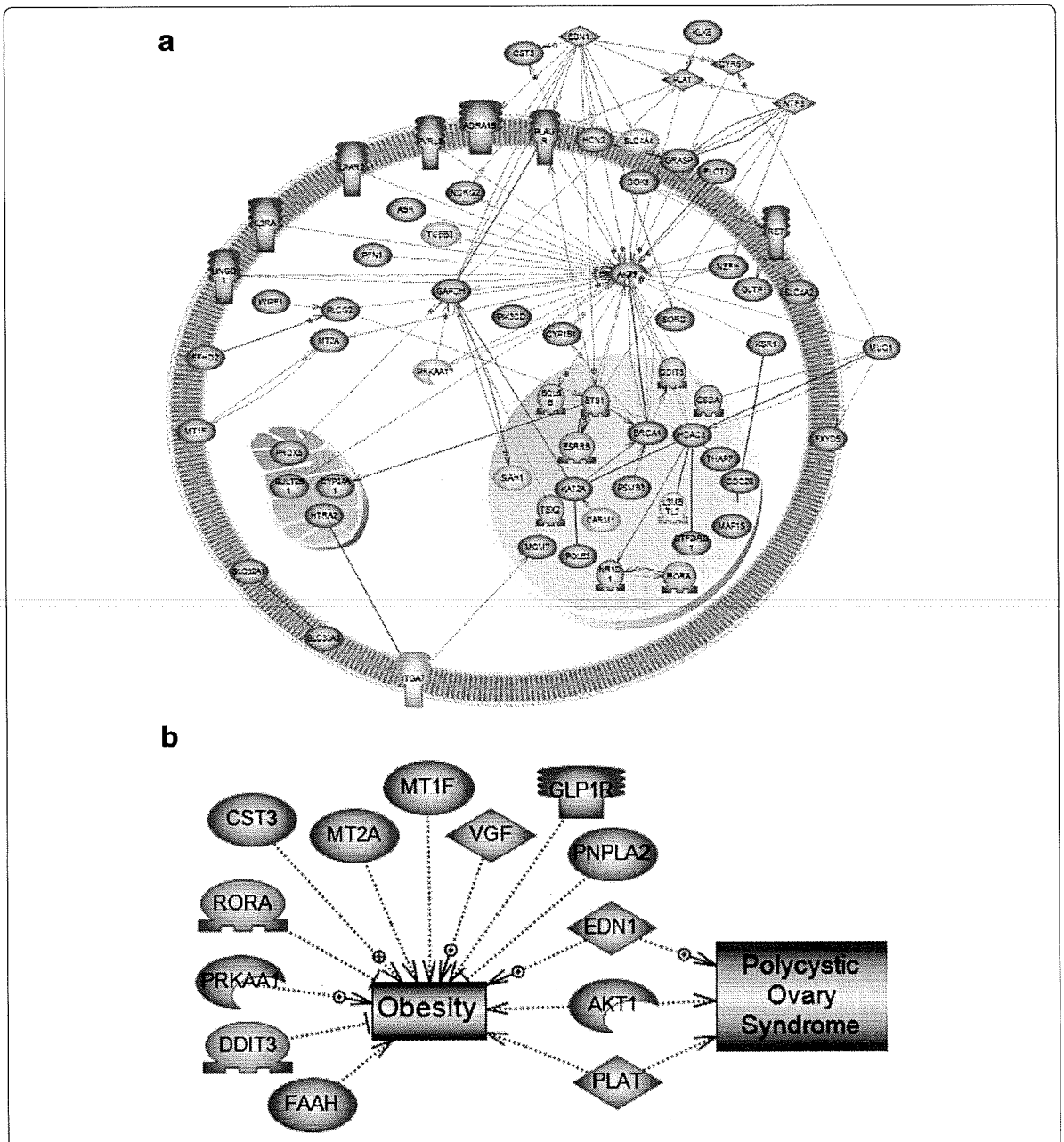

Figure 6 Transgenerational F3 generation sperm epimutation-associated gene network. (a) The gene network associated with the differential DNA methylation regions (epimutations) with cellular location indicated. The green labeled genes are those in the high stringency intersection set of 38 differential DNA methylated region (DMR) associated genes. **(b)** The genes that have a known link to obesity and polycystic ovarian disease that are associated with dichlorodiphenyltrichloroethane (DDT) induced sperm DMRgenes are presented.

inheritance of disease remains to be elucidated. The final analysis of the DMR-associated genes was to correlate specific genes known to be associated with obesity with the sperm DMR. The known obesity-associated genes had 13 correlated DMR (Figure 6b). The known obesity-associated genes also had three genes having correlation with genes known to be associated with polycystic ovarian disease. Therefore, a number of previously identified obesity-associated genes correlated with the epimutations identified.

Discussion

The present study demonstrates that ancestral DDT exposure during a critical window of germline development can promote the epigenetic transgenerational inheritance of obesity and a number of associated complex disease traits. The doses of DDT used have been shown to be wildlife and human environmental exposure levels [22,58]. The human DDT lowest observed adverse effect level LOAEL is 0.25 mg/kg/day which is lower than that used in the present study. The objective of the present study was to determine the potential transgenerational actions of DDT, such that a higher dose was used initially. We administered through an intraperitoneal injection to determine the potential to promote phenotypes. Previous studies have shown intraperitoneal injection is less stressful than oral gavage and less problematic to promote indirect effects from mode of administration [65]. Therefore, the present study was not designed as a risk assessment analysis for DDT exposures, but to determine the potential ability of DDT to promote transgenerational phenotypes. Future research can now use the information obtained to design risk assessment studies with more appropriate modes of administration for environmental doses. However, the observations do demonstrate the ability of ancestral DDT exposures to promote transgenerational adult onset obesity and associated disease.

The most dramatic increase in the incidence of adult onset disease observed was with obesity and was only in the F3 generation. Over 50% of the males and females with ancestral DDT exposure developed an obesity condition of increased body weight and abdominal adiposity. Previous reviews of the definition and cause for obesity suggest that weight gain, adiposity and presence of other obesity-associated disease is sufficient to refer to the pathology observed as obesity [28,36,49-51]. The US Centers for Disease Control in 2010 reported that 33% of adults in the US are obese and 17% of children between ages 2 to 19 are obese. Obesity is a contributing factor and/or precursor for many other diseases including polycystic ovarian disease, testis disease, kidney disease, liver disease, cardiovascular disease, type 2 diabetes, and diminished average life expectancy [66] (Figure 2). The dramatic increase in obesity over the past 50 years suggests that environmental factors are important in the disease etiology. The primary causal factor suggested is overnutrition [28,67,68]. However, recent studies have suggested environmental toxicants such as plastics [31], jet fuel hydrocarbons [32], and tributyltin [33] can promote transgenerational obesity in rodents. Waterland and colleagues suggested that epigenetic mechanisms are involved in transgenerational transmission of maternal obesity [10]. The present study demonstrated the epigenetic transgenerational inheritance of obesity in both males and females following an ancestral DDT exposure. The two obesity conditions of weight gain and abdominal adiposity are sufficient to identify obesity [28,49,50], however, future studies will need to assess the effects on other obesity parameters such as bone mineralization, body length and metabolic disease. Interestingly, several obesity-associated diseases were identified in the present study including polycystic ovarian disease, testis disease and kidney disease [9,27,44,45,69-72]. The obesity observed in humans and rodents have similar disease phenotypes and associations [28,36]. The functional correlation of these diseases needs to be elucidated. Interestingly, the DDT-induced obesity was only observed in the F3 generation and not the F1 direct exposure generation. The molecular mechanisms involved in the F1 generation and F3 generation are distinct [1]. The F1 generation pathology is due to direct exposure of the somatic cells of the fetus and does not involve the germline. The F3 generation pathology is due to a transgenerational germline mediated mechanism [1]. The epigenetic transgenerational inheritance of obesity in the F3 generation is distinct from the somatic exposure mechanism of the F1 generation [1,2]. Therefore, some germline mediated detrimental effects of DDT could be hidden for several generations before they become apparent. Observations suggest ancestral exposures to DDT may be a component of the rising incidence of obesity observed in the current human population. Since the primary human exposures to DDT in the US occurred in the 1950s, three generations have developed with a progressively increasing incidence of obesity in the population. Therefore, future studies are required to assess the importance of ancestral DDT exposures to the etiology of obesity.

The transgenerational obesity-associated diseases and abnormalities (Figure 2) observed include testis disease, polycystic ovarian disease, immune abnormalities and kidney disease. Direct DDT exposure has been shown to promote testis disease in rats (7.5 mg/kg/day for 36 weeks) [22], alligators [58], and fish [73]. Interestingly, male offspring of women exposed to DDT during pregnancy had an increase in testis disease observed 30 years following the exposure [74]. Polycystic ovarian disease is now the most common reproductive disease in women leading to infertility and endocrine abnormalities [75]. A number of environmental compounds have the ability to promote transgenerational ovarian disease in rats [60]. Kidney disease has been shown to be induced following direct DDT exposure in rats (10 mg/kg/day for 27 days) [22] and in humans [76]. A number of environmental toxicants can promote transgenerational kidney disease in rats [7]. All these diseases (that is, testis, ovary, and kidney) have increased dramatically over the past decades suggesting a potential environmental component. The presence of these associated diseases in the present study supports the conclusion that DDT exposure of a

gestating female promotes the transgenerational inheritance of obesity and associated complex disease traits.

The environmentally induced epigenetic transgenerational inheritance of disease requires the germline transmission of epimutations to subsequent generations [1-4]. The present study identified the DDT-induced transgenerational epimutations in the sperm of the F3 generation males. A comparison of these differential DMR with DMR induced by other environmental toxicants [7] demonstrated a unique DDT DMR signature that may be used to assess ancestral DDT exposure. The DDT transgenerational DMR had a low-density CpG 'CpG desert' genomic feature, as previously described with other exposures [7,57]. These low-density CpG deserts containing clusters of CpG are speculated to be a regulatory genomic feature associated with the DDT DMR. Further studies are needed to elucidate the functional significance of this DMR genomic feature. The presence of the F3 generation DMR demonstrates a transgenerational transmission of an epigenetic alteration in the germline. Future studies will need to compare the F3 generation DMR with the F1 and F2 generations. The present study demonstrates the epigenetic transmission and inheritance of sperm epimutations.

Analysis of the genes associated with the DDT sperm DMR identified a number of genes previously shown to be involved in obesity or the associated polycystic ovarian disease (Figure 6). The role of the DMR identified in the regulation of these correlated genes remains to be elucidated, however, these genes have previously been shown to be associated with obesity. Observations provide potential mechanistic links with the pathologies observed. Previous studies have shown that over 50% of females with polycystic ovarian disease are over weight or obese [45,69,70]. Therefore, a strong link exists between obesity and ovarian disease [69]. Interestingly, the transmission of many of the DDT-induced disease states was found to be through the female germline. This is the first female-germline-transmitted transgenerational phenotype identified. The imprinted-like nature of the transgenerational phenotype [2] suggests a parent-of-origin allele specific transmission of disease, which has been shown to be either paternal or maternal. This molecular mechanism needs to be elucidated in future studies, but the present study demonstrates sex specific germline transmission of transgenerational phenotypes. Future studies are now needed to assess the DMR in the egg induced by DDT. Observations suggest a potential exposure and disease specificity to the parental origins of the transgenerational phenomena.

Conclusions

Over 50 years have passed since the publication of the book *Silent Spring* by Rachel Carson that argued the hazards of DDT to wildlife and human health [77]. Since the Gates Foundation and World Health Organization (WHO) have now promoted the use of DDT in Africa and other developing countries for malaria control, the potential hazards of current day exposures now need to be considered in light of the transgenerational actions of DDT. Although the number of lives saved from malaria is significant, the long-term health and economic effects on survivors [78] and subsequent generations also need to be considered. Since other options exist with less toxic shorter half-life pesticides, a more careful risk/benefit consideration of the use of DDT is now needed.

The degree that environmentally induced epigenetic transgenerational inheritance is involved in human obesity and disease etiology is not known. However, since the majority of chronic diseases have increased dramatically over the past decades, environmental exposures and transgenerational epigenetics will likely be a component of disease etiology to seriously consider in the future. A more thorough and mechanistic understanding of the molecular etiology of disease, including the role of environmental epigenetics, is anticipated to provide insights into new diagnostics and therapeutics for specific diseases.

Additional files

Additional file 1: Table S1. **(A)** Body weight and organ weights in F1 and F3 generation female rats of control, dichlorodiphenyltrichloroethane (DDT) and lower dose DDT lineages (mean ± standard error). **(B)** Body weight (g) and organ weights (% of body weight) in F1 and F3 generation male rats of control, DDT and lower dose DDT (mean ± standard error). Asterisks (*, **, ***), if present, indicate statistically significant differences using a *t* test between 3 means of control and DDT or low dose DDT lineages ($P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively); ND = not determined.

Additional file 2: Figure S1. Histopathology of transgenerational disease. **(A)** The testis and prostate histopathology. **(B)** The male and female kidney histopathology. The F3 generation control lineage **(A)**, dichlorodiphenyltrichloroethane (DDT) lineage **(B)**, and lower dose DDT lineage **(C)** for each tissue presented. The bar is 100 μm and insets of higher magnification show the various pathologies described.

Additional file 3: Figure S2. Transgenerational physiological and disease incidence in the F1 and F3 generation. Prostate disease **(A)** and uterine infection **(B)** are presented. Serum estradiol concentrations in proestrus-estrus in F3 generation control, dichlorodiphenyltrichloroethane (DDT) and lower dose DDT lineage females **(C)**. Serum estradiol concentrations in diestrus in F3 generation control, DDT and low dose DDT lineage females **(D)**. Serum testosterone concentrations in the F3 generation control, DDT and low dose DDT lineage males **(E)**. Pubertal abnormalities in female **(F)** and male **(G)** animals. Tumor development in female **(H)** and male **(I)** animals from the F1 and F3 generation control, DDT, and low DDT dose lineages. The number of disease rates/total numbers of rats (n value) in each lineage are shown above the bars. Those showing numbers above the bars were analyzed with logistic regression analysis and those with a mean ± SEM indicated were analyzed with a *t* test with the *P* value represented (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

Additional file 4: Table S2. **(A)** Individual disease incidence in F1 generation female rats of control, dichlorodiphenyltrichloroethane (DDT) and lower dose DDT lineages. **(B)** Individual disease incidence in F1 generation male rats of control, DDT and lower dose DDT lineages. '+' indicates the presence and '-' indicates the absence of disease; a blank

cell indicates 'not determined'. Animal IDs with a 'C' belong to the control group, those with a 'D' belong to the DDT group and those with 'LD' belong to the lower dose DDT group. See Methods section for disease assessment in rats. The number of animals per litter (litter representation) mean ± SEM used for each specific disease/abnormality assessment within the control, DDT or lower dose DDT lineages were not found to be statistically different ($P > 0.05$), so no litter bias was detected.

Additional file 5: Table S3. (A) Individual disease incidence in F3 generation female rats of control, dichlorodiphenyltrichloroethane (DDT) and lower dose DDT lineages. **(B)** Individual disease incidence in F3 generation male rats of control, DDT and lower dose DDT lineages. '+' indicates the presence and '-' indicates the absence of disease; a blank cell indicates 'not determined'. Animal IDs with a 'C' belong to the control group, those with a 'D' belong to the DDT group and those with 'LD' belong to the lower dose DDT group. See Methods section for disease assessment in rats. The number of animals per litter (litter representation) mean ± SEM used for each specific disease/abnormality assessment within the control, DDT or lower dose DDT lineages were not found to be statistically different ($P > 0.05$), so no litter bias was detected.

Additional file 6: Table S4. (A) Individual disease incidence in F4 generation outcross female rats of control and lower dose dichlorodiphenyltrichloroethane (DDT) lineages. **(B)** Individual disease incidence in F4 generation outcross male rats of control and lower dose DDT lineages. '+' indicates the presence and '-' indicates the absence of disease; a blank cell indicates 'not determined'. Animal IDs with a 'C' belong to the control group, those with a 'LD' belong to the lower dose DDT group. See Methods section for disease assessment in rats. The number of animals per litter (litter representation) mean ± SEM used for each specific disease/abnormality assessment within the control or lower dose DDT lineages were not found to be statistically different ($P > 0.05$), so no litter bias was detected.

Additional file 7: Table S5. (A) Individual disease incidence in F4 generation reverse outcross female rats of control and lower dose dichlorodiphenyltrichloroethane (DDT) lineages. **(B)** Individual disease incidence in F4 generation Reverse Outcross male rats of Control and Lower Dose DDT lineages. '+' indicates the presence and '-' indicates the absence of disease; a blank cell indicates 'not determined'. Animal IDs with a 'C' belong to the control group, those with a 'LD' belong to the lower dose DDT group. See Methods section for disease assessment in rats. The number of animals per litter (litter representation) mean ± SEM used for each specific disease/abnormality assessment within the control or lower dose DDT lineages were not found to be statistically different ($P > 0.05$), so no litter bias was detected.

Additional file 8: Table S6. Dichlorodiphenyltrichloroethane (DDT) induced F3 generation sperm differential DNA methylation regions (DMR) (intersection). Epimutations found in F3-generation sperm after exposure of F0 generation gestating females to DDT, obtained by intersection of the five results of three methylated DNA fragment immunoprecipitation (MeDIP)-chip comparative hybridizations. The genes in bold were found to be unique DMR associated only with DDT-induced DMR.

Additional file 9: Table S7. Dichlorodiphenyltrichloroethane (DDT) induced F3 generation sperm differential DNA methylation regions (DMR) (average). Average found in F3-generation rat sperm after exposure of F0 generation to DDT, obtained averaging the results of three comparative hybridizations.

Additional file 10: Figure S3. Immunoprecipitation of methylated DNA fragments-quantitative polymerase chain reaction (MeDIP-QPCR) confirmation of selected differentially methylated DNA regions (DMR). Confirmation of MeDIP-chip identified DMR with an MeDIP-QPCR analysis. **(A)** The DMR-associated genes *Tubb3*, *Carm1*, and *Slc4c4* were selected and QPCR with a real-time PCR analysis on MeDIP samples from control and DDT lineage sperm samples performed. The relative changes (DDT/control) are presented with the asterisks (*) indicating statistical differences $P < 0.05$. The MeDIP-chip profiles for **(B)** *Carm1* DMR, **(C)** *Slc4c4* DMR, and **(D)** *Tubb3* DMR are presented with the bars indicating individual oligonucleotides probes and chromosomal location. The top gray bar represents the DDT lineage F3 generation sperm MeDIP sample hybridization and the bottom black bar represents the control lineage F3 generation sperm MeDIP sample hybridization. The region with an asterisk (*) above the bar represents statistical ($P < 0.05$) alterations with an increase in DDT MeDIP sample hybridization versus control. The data represent the mean of three different experiments and associated samples and the MeDIP-chip profiles are a representative hybridization profile.

Abbreviations

BPA: Bisphenol-A; DDE: Dichlorodiphenyldichloroethylene; DDT: Dichlorodiphenyltrichloroethane; DMR: Differential DNA methylation regions; LD: Lower dose; MeDIP: Methylated DNA immunoprecipitation.

Competing interests

The authors declare they have no competing interests.

Authors' contributions

MKS conceived and designed the study. MM, RT, MMH, EEN and CG-B performed the experiments and acquired the data. All authors analyzed the data. MKS and MM wrote the manuscript. All authors edited the manuscript. All authors read and approved the final manuscript.

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ENVIRONMENTAL HEALTH

COMMENTARY Open Access

DDT, epigenetic harm, and transgenerational environmental justice

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Abstract

Although the environmentally harmful effects of widespread dichlorodiphenyltrichloroethane (DDT) use became well-known following Rachel Carson's Silent Spring (1962), its human health effects have more recently become clearer. A ban on the use of DDT has been in place for over 30 years, but recently DDT has been used for malaria control in areas such as Africa. Recent work shows that DDT has transgenerational effects in progeny and generations never directly exposed to DDT. These effects have health implications for individuals who are not able to have any voice in the decision to use the pesticide. The transgenerational effects of DDT are considered in light of some widely accepted ethical principles. We argue that this reframes the decision to use DDT, requiring us to incorporate new considerations, and new kinds of decision making, into the deliberative process that determines its ongoing use. Ethical considerations for intergenerational environmental justice are presented that include concern and respect for autonomy, nonmaleficence, and justice. Here, we offer a characterization of the kinds of ethical considerations that must be taken into account in any satisfactory decisions to use DDT.

Keywords: DDT, Malaria, Africa, Bioethics, Generation, Inheritance, Environmental health

Background

A variety of environmental factors that include toxicants, nutrition and stress have been shown to induce the epigenetic transgenerational inheritance of disease [1,2] (Figure 1). Examples of such environmental compounds include pesticides [3,4], fungicide vinclozolin [3], hydrocarbons (jet fuel) [5], dioxin [6], and the plasticizers phthalates and bisphenol A (BPA) [7]. Nutritional effects such as high fat diets and caloric restriction can also promote transgenerational abnormalities [8]. Epigenetic transgenerational inheritance requires the germline (sperm or egg) transmission of epigenetic information that alters disease or phenotype, in the absence of direct environmental exposures [2]. Transgenerational phenomenon have been demonstrated in humans [9], rodents [3], worms [10], flies [11], and plants [12]. Therefore even though you have never had a direct exposure, your ancestors' environmental exposures may influence your disease development (Figure 1). Environmentally induced epigenetic transgenerational inheritance of disease is a factor in disease etiology that needs to be considered in environmental policy.

A recent study examined the epigenetic transgenerational actions of the most common historically used insecticide DDT (dichlorodiphenyltoxichloroethane) [1]. Observations demonstrate that DDT has the ability to induce the epigenetic transgenerational inheritance of obesity, kidney, testis and ovary disease [1]. Although the United States and most developed countries have banned the use of DDT, recently it has been used globally as an insecticide for control of vectors for malaria. In 2001 the Stockholm Convention of United Nations Environmental Program proposed the elimination of 12 chemicals that induced DDT [13]. However, due to the recent Gates Foundation Malaria Control Program the use of DDT in Africa and other parts of the world has increased since the Stockholm Convention [14]. The World Health Organization (WHO) issued a position statement in 2006 promoting the use of indoor residual spraying with DDT for malaria vector control. The reported use of DDT globally for disease vector control is over 5,000 metric tons per year with India being the largest consumer [15]. Studies have indicated indoor spraying of DDT causes high levels of human exposure [16]. The direct DDT exposure toxic effects in humans include

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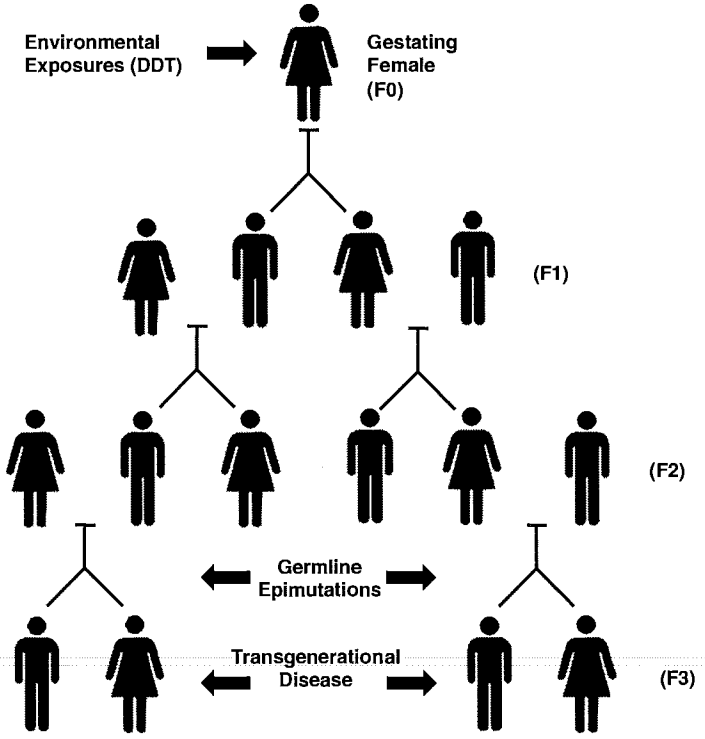

Figure 1 Scheme for DDT induced epigenetic transgenerational inheritance of disease.

developmental abnormalities [17], reproductive disease [18], neurological disease [19], and cancer [20]. The exposure DDT metabolite DDE (dichlorodiphenyldichloroehtane) also promotes abnormal human health effects such as childhood diabetes and obesity [21]. Therefore, DDT exposure directly impacts human health [22]. DDT exposure also influences the health and promotes birth defects in wildlife [23]. Despite DDT being a low-cost anti-malaria tool, the adverse human health and environmental effects (e.g. extremely long half-life) of DDT use must be carefully weighed against the benefits of malaria control [24].

The book 'Silent Spring' by Rachel Carson was published over 50 years ago and revealed the hazards of DDT to human and wildlife health [25]. Currently the World Health Organization (WHO) and the Gates Foundation promote the use of DDT in developing countries in Africa for malaria control. The current day potential hazards of DDT exposures need to now be considered in light of the transgenerational actions of DDT [1]. The various transgenerational diseases promoted by DDT include obesity, kidney disease and ovarian disease [1]. The long-term health and economic effects on survivors [26] and subsequent generations [1] now needs to be considered with respect to the number of lives saved from malaria. A more careful risk-benefit consideration of the use of DDT is needed since other options exist with less toxic shorter half-life pesticides. The primary objective of the following discussion is to incorporate the concept of transgenerational inheritance.

Discussion

The unique aspect of the emerging work on the epigenetic effects of DDT is that we now have good reason to believe that DDT will negatively affect future generations. This raises questions of *intergenerational environmental justice*. Environmental justice concerns the distribution of burdens and benefits on individuals via practices that affect our environment. In her work, Kristin Shrader-Frechette identifies the focus of *environmental* justice as being on the disproportionate burdens faced by socially disempowered individuals and groups (e.g., the poor and racial and ethnic minorities) [27,28]. There are now many accounts of these individuals and groups suffering the ill effects of environmental degradation. DDT use in the developing world looks set to be yet another case in that sad history. Some evidence suggests that the current generation is harmed by exposure to DDT. The recent work cited above indicates health hazards for descendants of those exposed now. Thus, the harm will only fully emerge over the course of a number of generations. This is why DDT use is also an issue of *intergenerational* justice.

Consideration of intergenerational justice invites us to examine how our practices and activities will impose burdens (and benefits) on those who will inhabit the world 50 or 100 or 500 years from now [29]. We now have good reason to believe, based on the evidence discussed above, that the use of DDT will impose burdens on individuals in the next two or four generations, at least, while the current generation enjoys the benefits of its use. As we discuss below, questions of intergenerational justice differ from other kinds of decisions. All the affected parties are not known in advance because some do not yet exist. Who comes to exist in the future, and what health deficits they might face, is determined by decisions, both individual and policy-level, made today. Of course, the question of what the present generation owes future generations is greatly complicated by the non-identity problem and related issues [30]. We will directly address these complications in future work, though here we hope to limit our claims so as to avoid the most difficult questions raised by that problem.

We characterize the ethical issues in terms of environmental injustice because those who might live in the future are the ultimate socially disempowered group. They can have no input into or control over environmental conditions that will affect their well-being. They are vulnerable to harms and have no clear opportunity to benefit from the current generation's use of DDT.

The provisional case that current DDT use's impacts on future generations is an instance of intergenerational environmental injustice can be developed in terms of three moral concerns. First, the offspring of those exposed to high levels of DDT today are harmed in that the offspring's health interests are set back by ancestral exposure. The principle of *nonmaleficence* concisely expresses the widely-held moral conviction that it is wrong to harm another, other things being equal. Assuming that *any* offspring of individuals exposed to DDT will be harmed by the ancestral exposure, the principle of nonmaleficence applies, even to future generations. Second, while many individuals might consent to undergo risk or actual harm, for some compensating benefit, the offspring cannot consent prior to the onset of the mechanism of injury. This violates *respect for autonomy*, which would otherwise be expressed, partially, in the ability to make an informed consent to assume risk or harm. Of course, those who do not exist yet do not have any autonomy to respect. Thus, they cannot consent to take on the epigenetic harm that will affect whoever comes to exist. Finally, the principle of *justice* calls for the distribution of benefits and burdens (including harms) in some kind of principled manner. DDT use affecting future generations through epigenetic harm seems to be a good provisional example of an unfair imposition of harm without corresponding benefit. At the very least, justice would seem to require that anyone likely to be harmed by action taken today be able to have a "place at the table" in discussion of whether to use substances like DDT. The three principles discussed here are elaborated and defended in Beauchamp & Childress [31] (Table 1).

One objection might say that if DDT had not been used in the current generation (F0), then members of a future generation (F3) who are the progeny of F0 might not have come to exist (Figure 1). Members of the F0 generation might have died of malaria before having children. Thus, the alleged cause of harm to the F3 generation, the use of DDT in F0, might actually also be part of what enables F3 to come to exist. How might this affect the provisional claim that current DDT use (in F0) is ethically suspect? First, that the objection exists does not immediately justify the status quo. The objection is based on quite a few conditional claims. For example, if members of F3 never came to exist, they would not be harmed by not existing [30]. The non-identity problem raises notorious complex questions of why it would be wrong to bring into existence a person who suffers health deficits, but who would not exist if not for the mechanism that also caused those deficits. Here we lack the space to fully address this concern, but in future work we hope to develop an agent-based account of wrong action that can be used to address the counterintuitive implications of the non-identity problem. Wasserman argues that an agent's reasons for acting can be the target of ethical evaluation [32]. Agents who act from moral vice or the absence of virtue might be ethically criticized even given the non-identity problem. We hope to develop this agent-based approach for dealing with actions that have transgenerational implications. While it is not clear to us that the current use of DDT is obviously wrong, it now requires, we think, a more elaborate justification given its epigenetic effects.

That deaths of members of F0 are avoidable, via malaria prevention, does raise an ethical concern in itself (again, the principle of nonmaleficence would be relevant here). But if there are alternative ways to prevent malaria deaths in F0, we should obviously consider them. A number of organochlorine pesticides with shorter half-lives (i.e. methoxychlor, aldrin, dieldrine and eldrin) have been used and shown not to be as persistent environmental

Table 1 Ethical considerations for intergenerational environmental justice

1 *Consent/Respect for Autonomy*: Members of future generations cannot consent to risks and harms imposed by earlier generations.

2 *Nonmaleficence*: Members of future generations are harmed, via health deficits associated with epigenetics, due to exposure of ancestors to DDT (and other toxicants).

3 *Justice*: Members of future generations bear a disproportionate balance of risks and harms, whereas members of the current generation, when DDT is being used, enjoy disproportionate benefits.

contaminants [33]. More recently developed pesticides such as bifenthrin [34], chlorfenapyr [35], and pirimiphos [36] have been shown to be effective as alternatives for DDT consideration. Although the alternatives like methoxychlor may promote transgenerational disease [37], more recently developed pesticides such as nicotinoids are also alternatives to consider [38,39]. Clearly factors such as cost and half-life which would require more frequent distribution are factors, this consideration would have to be part of the decision making process [15]. However, as our understanding of the health deficits to future generations due to the current generation's use of DDT become clearer, this can significantly shift the balance of burdens. The "true cost" of using the less expensive and long half-life pesticide is shifted to members of the F3 generation who experience that cost in terms of health deficits and in the money needed, if possible, to correct or ameliorate those health deficits. Their lives and well-being cannot be discounted in the same way that economists discount future commodities [40,41]. Thus, any future health care costs caused by actions taken today need to be incorporated into a cost-benefit assessment. We do not claim to have worked out that decision making process, but we do argue that new concerns about epigenetic harm and transgenerational inheritance should reframe that process. Policy makers need to incorporate these considerations of transgenerational justice into their deliberation.

Concern about the well-being of members of the F0 generation, as well as members of the F3 generation, appear to call for some sort of trade-off or balancing of benefits and burdens. While we do not have space here to fully consider all the ramifications of this trade-off, we believe it is important to recognize that the decision to use DDT in the current generation has this implication. Very briefly, we note that the F0 generation might benefit from DDT use by the preservation of life and health (freedom from malaria) in the current generation. The F0 generation might also experience some burdens associated with its exposure to DDT [42]. And, F0 might experience harms if DDT is not used. However, the F3 generation would not be harmed by not using DDT regardless of whether not using DDT harmed the F0 generation. In a scenario where members of the F3 generation never come to exist because their great-grandparents died prior to reproducing, there can be no harm to those who do not yet, or never, come to exist. Members of F3 would be harmed, again by appeal to the recent epigenetic findings, if DDT is used. Finally, it strikes us as important that a mechanism that might allow one to live (DDT use) would also be a mechanism that causes one's health deficits. The ethics of reproducing is surely complicated, but, again, it is not clear that ensuring F3's existence by means of inducing harm in those who come to exist is an obviously right action.

Conclusions

On this admittedly brief analysis of the trade-offs, it is certainly not clear that the F3 generation would benefit more from current use of DDT than from not using it. If there are alternatives for preventing malaria in the F0 generation that do not cause epigenetic harm, then the case for using them would seem to be ethically superior to any trade-off scenario involving continued use of DDT.

We draw two conclusions from this analysis. First, because recent empirical findings show that DDT is likely to cause intergenerational harm, policies involving its use should be re-considered to incorporate these new concerns into the decision procedure to use DDT. We have tried to highlight some of those new concerns in ethical terms (Table 1). Second, the provisional case against DDT use is fairly strong. This further strengthens the call for alternative means of preventing malaria and for discontinuing DDT use. But even if we lack a conclusive argument against current DDT use, we believe we have done enough to shift the burden of proof back to the advocates of its use. Perhaps our most important conclusion is that an unreflective continuation of the status quo with respect to DDT use is unacceptable. It needs to be defended against concerns about the intergenerational effects it will cause.

Abbreviations

BPA: Bisphenol A; DDE: Dichlorodiphenyldichloroehtane; DDT: Dichlorodiphenyltrichloroethane; F0: Generation pregnant female; F1: Generation fetus that becomes the offspring or children; F2: Generation (grandchildren); F3: Generation (great-grandchildren); WHO: The World Health Organization.

Competing interests

The authors declare no competing financial interests.

Authors' contributions

MKS conceived the study. MKS and WPK designed and wrote the study. Both authors edited and approved the manuscript.

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