Spring 2017 – Epigenetics and Systems Biology
Discussion Session (Epigenetics and Disease Etiology)
Michael K. Skinner – Biol 476/576
Week 14 (April 13)

Epigenetics and Disease Etiology

Primary Papers

1. Anway et al. (2006) Endocrinology. 147(12):5515-5523.
2. Nilsson et al. (2012) PLoS ONE 7:e36129.
3. Skinner et al. (2013) BMC Medicine 11:228
4. Kabasenche, et al. (2014) Environmental Health 13:62

Discussion

Student 37 – Ref #1 above

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

Student 38 – 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 39 – 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.

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

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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.

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.

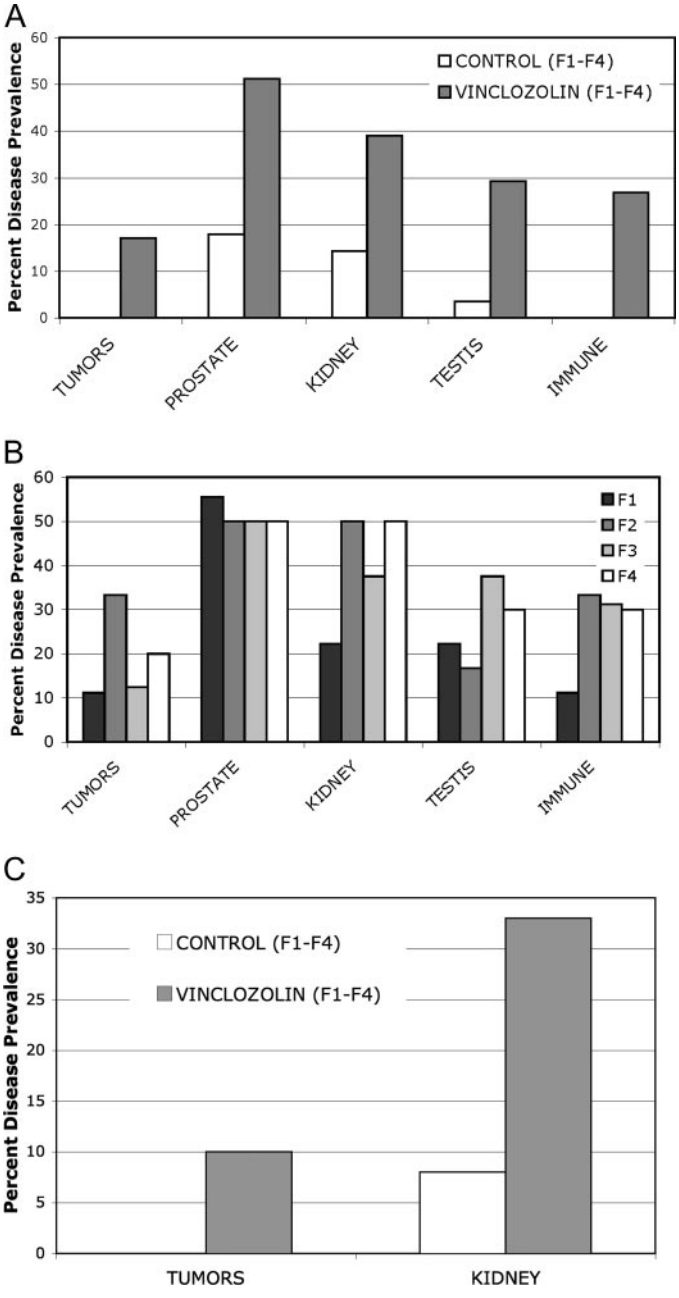

FIG. 1. Disease states or abnormalities in vinclozolin and control generation (F1–F4) animals. A, Males from combined (F1–F4) generation animals. B, Males from individual (F1–F4) generation animals. C, Females from combined (F1–F4) generation animals. The percentage disease prevalence is presented for 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

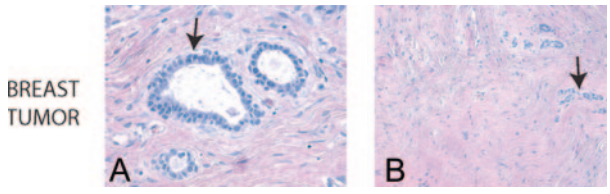

FIG. 2. Histology of representative vinclozolin generation breast fibroadenoma. A representative ×400 magnification (A) and ×200 magnification (B) micrograph is presented from a minimum of five different animals with breast adenoma analyzed. A *closed arrow* indicates a neoplastic glandular epithelial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

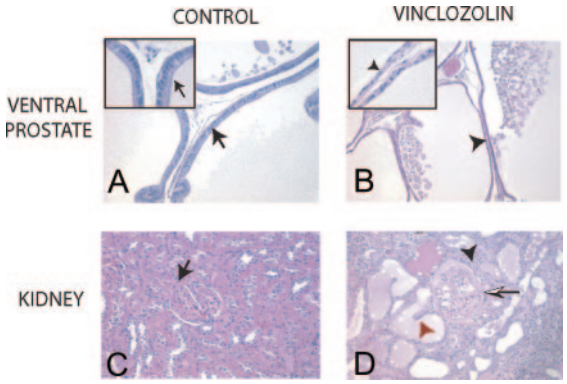

FIG. 3. Histology of representative control (A and C) and vincozolin (B and D) F2 or F3 generation tissues are presented, ventral prostate (A and B), and kidney (C and D). A representative ×200 magnification micrograph is presented from a minimum of five animals analyzed. *Insets* in A and B are ×1000 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-

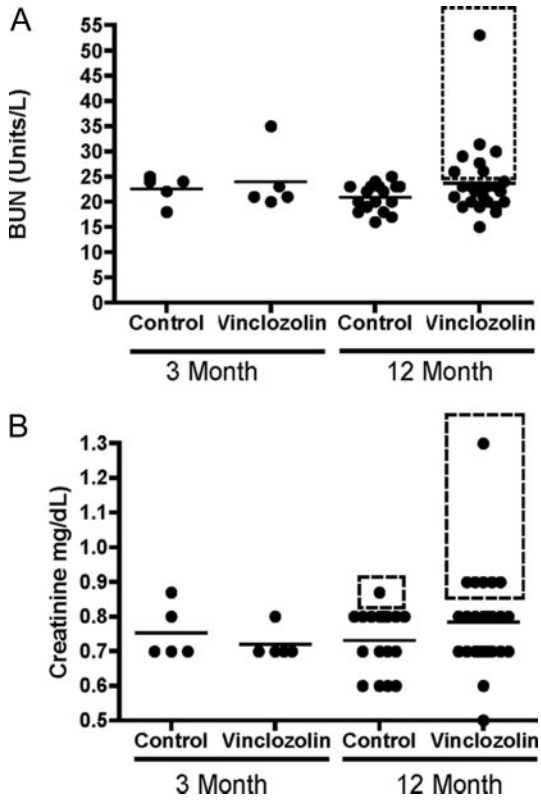

FIG. 4. Blood analysis for BUN (A) and creatinine (B) from control and vinclozolin (F1–F4) generation animals. Scatter plots are presented with a mean line indicated. Samples in the *dashed boxes* correlate with animals with diagnosed morphological kidney abnormalities and corresponding renal blood marker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

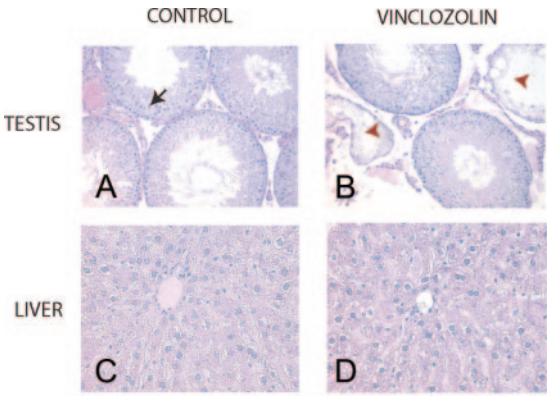

FIG. 5. Histology of representative control (A, C) and vincozolin (B and D) F2 or F3 generation tissues are presented for normal testis (A), abnormal testis (B), and liver (C and D). A representative ×200 (A and B) or ×400 (C and D) magnification micrograph is presented from a minimum of five animals 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.

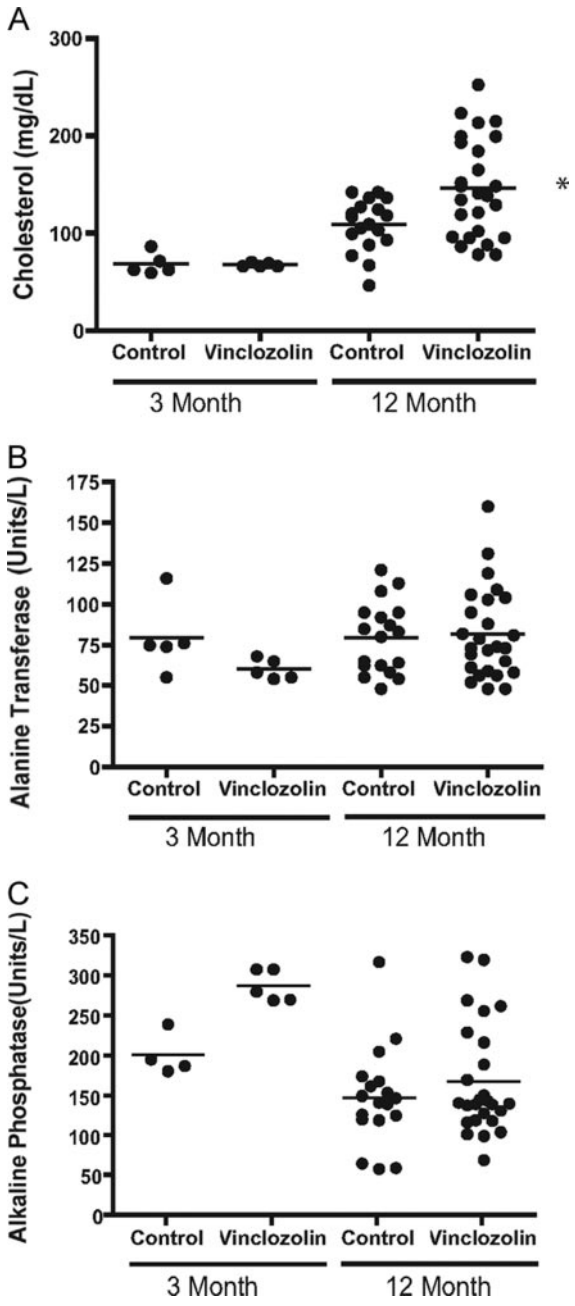

FIG. 6. Blood analysis for combined F1–F4 control and vinclozolin generation animals. A, Cholesterol concentrations and liver serum markers of (B) alanine transferase and (C) alkaline phosphatase are presented (units/liter). Scatter plots are indicated for 3-month-old and 6- to 12-month-old animals with 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

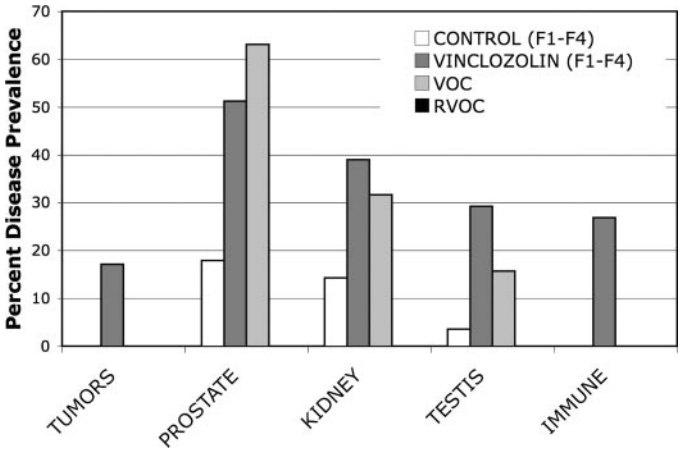

FIG. 7. Comparison of outcross disease states or abnormalities using F1–F4 control and vinclozolin generation-treated cross compared with VOC (F2 vinclozolin generation male and wild-type female) and a RVOC (F2 vinclozolin generation female and wild-type male). The percentage disease prevalence is presented 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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Environmentally Induced Epigenetic Transgenerational Inheritance of Ovarian Disease

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Abstract

The actions of environmental toxicants and relevant mixtures in promoting the epigenetic transgenerational inheritance of ovarian disease was investigated with the use of a fungicide, a pesticide mixture, a plastic mixture, dioxin and a hydrocarbon mixture. After transient exposure of an F0 gestating female rat during embryonic gonadal sex determination, the F1 and F3 generation progeny adult onset ovarian disease was assessed. Transgenerational disease phenotypes observed included an increase in cysts resembling human polycystic ovarian disease (PCO) and a decrease in the ovarian primordial follicle pool size resembling primary ovarian insufficiency (POI). The F3 generation granulosa cells were isolated and found to have a transgenerational effect on the transcriptome and epigenome (differential DNA methylation). Epigenetic biomarkers for environmental exposure and associated gene networks were identified. Epigenetic transgenerational inheritance of ovarian disease states was induced by all the different classes of environmental compounds, suggesting a role of environmental epigenetics in ovarian disease etiology.

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Introduction

Environmental exposures during fetal and early postnatal development can lead to an increased incidence of later life adult-onset diseases [1,2,3,4]. Such environmental factors include nutritional abnormalities, stress and exposure to toxicants. Examples include fetal exposures to plasticizers such as bisphenol A leading to immune abnormalities [5], maternal smoking leading to increased pulmonary disease in adulthood [6], nutrition defects leading to hypertension in offspring [7,8] and therapeutic drug exposure leading to vascular defects [9]. In addition to these direct effects of early life exposure on adult onset disease, environmental factors have been shown to affect the next F2 generation [8,10,11,12]. The subsequent generations transgenerational inheritance of epigenetic changes in the genome now provides an additional molecular mechanism, along with classic induction of genetic mutations, for the germ line transmission of environmentally induced phenotypic change [2,13,14].

Effects on the F1 and F2 generation can be due to direct multigenerational exposure to the environmental factor [13,15]. If a gestating female is defined as the F0 founder generation, then the fetal offspring are the F1 generation, and the germ cells present in those developing fetuses will eventually become the eggs or sperm that would form the F2 generation. An environmental exposure of an F0 generation gestating female directly exposes both the F1 generation fetuses and the germ cells present in those fetuses that will generate the F2 generation [2,13,15]. The subsequent F3 generation would be the first generation that would not have been directly exposed to the environmental factor. Therefore, effects on the F1 and F2 generation can be due to direct exposure and so should be considered multigenerational effects [13]. In contrast, a transgenerational effect following exposure of a F0 generation gestating female is defined as an effect seen in the F3 or later generations [15]. Transgenerational phenomena by definition do not involve direct exposure and have been shown to involve epigenetic changes induced in the germ line [16,17,18,19].

The initial report of epigenetic transgenerational inheritance of adult onset disease was from gestating female rats exposed to the fungicide vinclozolin, in which F3 generation male offspring showed defects in sperm production [16,20]. Transgenerational effects have also been reported after exposure of gestating rats to bisphenol A (BPA), where decreased fertility was seen in the F3 generation males [21]. Decreased fertility was also seen in F3 and F4 generation female mice after the gestating F0 generation was exposed to dioxin [22]. Similarly in mice, male F3 generation offspring showed changes in the methylation pattern of imprinted genes in sperm following exposure of the gestating F0 generation female to the agricultural fungicide vinclozolin [23]. Recently, a number of different exposures to environmental toxicants including BPA, phthalates, dioxin, pesticide, DEET and jet fuel hydrocarbons were found to promote epigenetic alterations in sperm and transgenerational inheritance of reproduction defects [19]. Therefore, a number of different environmental toxicants and other factors such as nutrition [4] can promote epigenetic transgenerational inheritance of adult onset disease.

In women, adult-onset diseases of the ovary that can dramatically affect fertility are primary ovarian insufficiency and polycystic ovarian disease. Primary ovarian insufficiency (POI) is

characterized by a significant reduction in the primordial follicle pool of oocytes (eggs) that appears intrinsic to the ovary, and induction of menopause prior to age 40 [24]. This is associated with decreased estrogen and elevated gonadotropin levels in the blood. POI affects about 1% of women [25,26,27]. A reduced primordial follicle pool size correlated with POI has been shown in sheep and primates to also associate with polycystic ovarian disease [28,29]. POI is often thought to have a genetic basis since chromosomal abnormalities and single gene mutations are associated with a percentage of POI cases. However, only a minority (4–20%) of human cases can be ascribed a genetic basis [26,27,30,31,32,33,34,35,36].

Polycystic ovarian (PCO) disease or polycystic ovary syndrome (PCOS) is a common endocrine disorder that affects 6–18% of women [37,38,39,40,41,42]. It is characterized by infrequent ovulation or anovulation, high androgen levels in the blood, and the presence of multiple persistent ovarian cysts [43]. PCOS patients often show insulin resistance and a heightened risk for diabetes [44]. Current thought on the etiologies that lead to development of PCOS is that there are both genetic and environmental causal factors. A genetic predisposition in an individual may combine with an early-life environmental impact such as fetal stress or increased androgens *in utero* and lead to development of PCOS in adulthood [44,45,46]. Fetal or early postnatal exposure to androgens (e.g. di-hydrotestosterone) has been shown to promote PCO and associated clinical parameters (e.g. metabolic abnormalities, adiposity and endocrine abnormalities) in rats, mice and sheep [47,48,49]. Therefore, the rodent PCO model has many of the same clinical correlations that are seen with PCOS in humans. Sequence variations in several genes have been associated with PCOS [46,50,51], although at very low frequency and none are highly predictive. Epigenetic abnormalities such as those associated with non-random X-chromosome inactivation have also been linked to PCOS [51,52,53,54]. Several diseases are now known to have an important epigenetic component, such as allergies [55], hepatic cancer [56], gastric cancer [57], asthma [58], colorectal cancer [59], prostate cancer [60], HIV latency [61] and psychiatric disorders [62]. The current study was designed to investigate the role of environmental epigenetics in ovarian disease.

A rat model is used to evaluate whether adult onset ovarian diseases are induced transgenerationally after exposure of a gestating F0 generation female to known environmental toxicants. The exposures are during days 8–15 of fetal development, which is the time of gonadal sex determination. The exposure compounds were: 1) Vinclozolin, an agricultural fungicide previously shown to cause transgenerational epigenetic disease [13,20,23]; 2) A mixture of permethrin, the most commonly used human insecticide shown to have minor toxicologic effects in mammals [63] and DEET, an insect repellent reported to have negligible toxic effects [63]; 3) A plastic mixture of bisphenol A (BPA), dibulylphthalate (DBP) and bis(2-ethylexyl)phthalate (DEHP), all plasticizer chemicals that commonly appear together from plastics with in vitro and in vivo toxic effects [64]; 4) Dioxin (TCDD), a by-product of some commercial chemical syntheses that has been shown to induce adult-onset diseases including premature acyclicity [65,66]; and 5) Jet fuel (JP8), a hydrocarbon mixture (i.e. C3->C20) often used for dust control on road surfaces, with known toxicologic effects [67,68], but is not known to induce reproductive defects [69]. The United States Department of Defense assisted in the selection of these toxicants and mixtures due to their relevance for exposures anticipated for military personnel. The plastic mixture included the three common toxicants present in heated bottled water, the pesticide mixture is the most common used in humans and the hydrocarbon mixture (i.e. jet fuel JP8) is commonly used in dust control on road surfaces. All of the above environmental toxicants have been implicated in inducing transgenerational disease phenotypes [13,19,21,22]. The current study used pharmacological doses and administration to assess potential transgenerational actions on ovarian disease and should not be considered a risk assessment analysis. Future studies are now needed to do environmental risk assessment, based on the observations of the current studies.

The adult F3 generation females from each exposure lineage group were examined for the incidence of ovarian diseases similar to primary ovarian insufficiency and polycystic ovarian disease. The human ovarian diseases POI and PCOS have numerous other clinical conditions associated with them such as endocrine abnormalities and glucose intolerance. Therefore, the rat ovarian abnormalities/disease cannot be directly correlated to the clinical aspects of human ovarian disease, but do share the majority of morphological changes. In order to gain insight into possible cellular and molecular mechanisms involved in ovarian disease development, the granulosa cells from F3 generation vinclozolin and control lineage animals were evaluated for changes to their transcriptome and epigenome (DNA methylation pattern). All the primary cell types of an ovarian follicle such as the oocyte, theca cells and granulosa cells are anticipated to develop a transgenerationally altered transcriptome and epigenome [14], and so will participate in the adult onset disease development. The granulosa cell was selected to provide the proof of concept that such an alteration in genome activity could develop. Future studies will investigate the other cell types. The capacity of vinclozolin to directly induce oocyte loss in ovaries was also examined to clarify how F1 generation effects may develop. Observations demonstrate that the environmental toxicants examined induced transgenerational ovarian adult-onset disease, and suggest that primary ovarian insufficiency and polycystic ovarian disease can have an epigenetic transgenerational etiology.

Results

Transgenerational Ovarian Disease

Gestating female rats, designated as F0 generation animals, were treated by intraperitoneal injection daily from E8 (post conception gestational day 8) through E14. The F0 generation female rats received one of five different treatments as described in Methods: Vinclozolin, Pesticide (includes permethrin and DEET), Plastics (includes BPA, DBP and DEHP), Low-dose Plastics (50% of Plastics dose), Dioxin, Hydrocarbon (Jet fuel JP8), or DMSO vehicle as Control. The F1 generation offspring were bred to others of the same treatment group to produce an F2 generation, and F2 generation animals were similarly bred to produce an F3 generation (see Methods). No sibling or cousin breedings were used to avoid any inbreeding artifacts. Only the original F0 generation gestating female rats received the treatment exposures. Female rats from the F1 and F3 generations were kept until one year of age and then sacrificed. Ovaries were removed, fixed, sectioned and stained for histologic examination.

The number of oocytes (*i.e.* eggs) present in the ovaries was determined by counting follicles. Each ovarian follicle is composed of an oocyte surrounded by a layer of granulosa cells, a basement membrane and outer layers of thecal cells. Primordial follicles are in an arrested state of development and contain a single layer of squamous flattened granulosa cells. Developing follicles have multiple layers of proliferating granulosa cells and an increase in the oocyte diameter. Later in follicle development a fluid-filled antrum forms [70,71,72,73]. In the F1 and F3 generation, ovarian

morphological evaluation and counts were performed to determine the number of primordial follicles, pre-antral developing follicles and antral developing follicles as described in Methods [74].

In F1 generation ovaries there was a marked and statistically significant ($p<0.001$) reduction in the number of primordial follicles in all exposure groups compared to ovaries from the vehicle-treated control lineage (Fig. 1). This indicates that the female fetuses exposed to these compounds during gonadal sex determination all have a decrease in their resting pool of primordial follicles. There was no change in the number of pre-antral developing follicles or antral developing follicles, except in the case of females of the vinclozolin-treated lineage. F1 vinclozolin group ovaries had significantly ($p<0.05$) fewer preantral developing follicles compared to controls (Fig. 1). This effect of the exposures on the F1 generation is attributed to direct fetal ovarian exposure to the treatments.

Since treatment with environmental toxicants resulted in fewer oocytes being present in F1 generation adult ovaries compared to controls, an experiment was performed to assess if vinclozolin could act directly on ovaries to reduce oocyte number. Ovaries from four-day old rats, containing predominately primordial follicles, were placed into a whole-ovary culture system (see Methods) and treated *in vitro* for ten days with varying concentrations of vinclozolin. Treatment with 500 μM vinclozolin did result in a decrease ($p<0.05$) in oocyte number compared to controls (Fig. 2). The 200 μM and lower doses of vinclozolin did not significantly reduce oocyte number. Therefore, direct actions of vinclozolin on the F1 generation fetal gonad have the potential to reduce follicle numbers if the dose is sufficient.

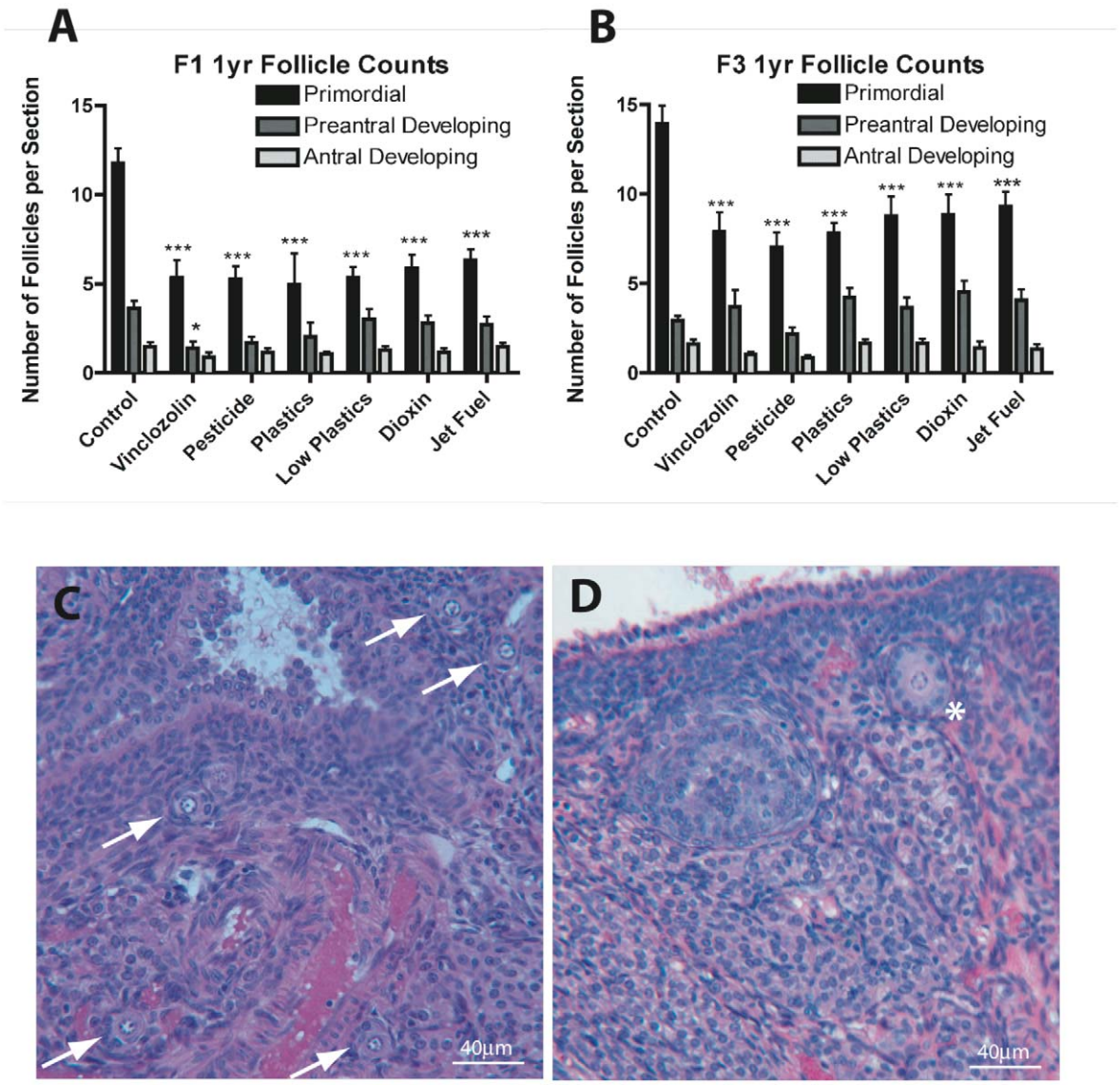

Figure 1. Follicle numbers and development. A) Number of primordial, preantral, and antral follicles per section in F1 generation ovaries. N = 9 animals per treatment group. B) Number of follicles per section in F3 generation ovaries. N = 9 animals per treatment group. Asterisks indicate groups significantly (*$p \leq 0.05$, ***$p \leq 0.005$) different than controls of their own follicle type by ANOVA followed by Dunnet's post-hoc test. C) H and E stained section of F3 generation control ovary showing several primordial follicles (arrows). D) H and E stained section of F3 generation vinclozolin lineage ovary without visible primordial follicles. Asterisk indicates a developing secondary follicle. Scale bar = 40 μm.
doi:10.1371/journal.pone.0036129.g001

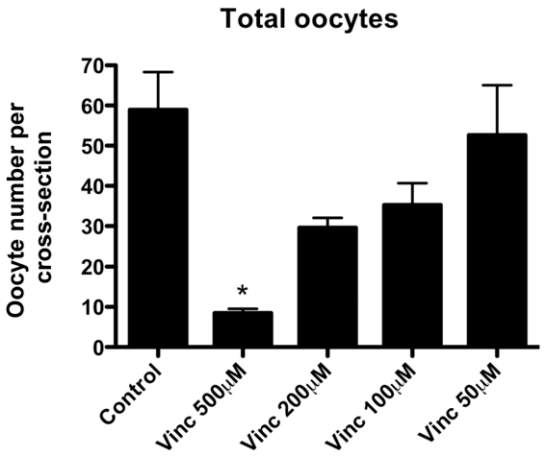

Figure 2. Number of oocytes per ovarian cross-section in ovaries taken from 4-day old rats and cultured whole for 10 days in the presence of different concentrations of vinclozolin. Data are from five different experiments performed in replicate. Asterisks indicate groups significantly (*$p \leq 0.05$) different than controls by ANOVA followed by Dunnet's post-hoc test.
doi:10.1371/journal.pone.0036129.g002

In F3 generation ovaries, similarly to F1 females, there was a significant ($p<0.001$) reduction in the number of primordial follicles in all treatment groups, compared to controls (Fig. 1). Since none of these F3 generation animals were themselves exposed to the treatment compounds, this reduction in oocyte number is a transgenerational effect. There was no change in the number of preantral developing or antral developing follicles for any exposure lineage group compared to control lineage animals. Therefore, all the exposure groups examined induced a significant transgenerational decline in the primordial follicle pool size. For the purposes of this study, the ovaries of an animal were classified as having "disease" if the ovary primordial follicle numbers were $\geq$2 standard deviations less than that seen in controls. The incidence of the follicle pool disease was 33–60% across treatment groups.

Polycystic ovarian (PCO) disease is a common disease in humans, so ovaries from F1 and F3 generation animals were evaluated for the presence of cystic structures. Ovarian cysts were defined and categorized as either small or large cysts, as described in Methods. Interestingly, an increase in the number of both small and large cysts were seen most often in F3 generation ovaries from exposure lineages, rather than in F1 generation ovaries (Fig. 3). An increase ($p<0.01$) in small cysts was seen in all F3 generation treatment groups, compared to controls. However, in the F1 generation only the low-dose plastics, jet fuel hydrocarbons and vinclozolin lineage ovaries showed an increase ($p<0.05$) in small cysts. An increase in large cysts was observed in the F3 generation ovaries of the vinclozolin, pesticide, low-dose plastics and jet fuel treatment groups (Fig. 3). However, in F1 generation ovaries only the low-dose plastics showed an increase in the incidence of large cysts compared to controls. These results indicate that development of ovarian cysts occurs more often in the F3 generation, which demonstrates a transgenerational effect of the toxicant exposures. The large cysts observed in these ovaries often were lined with a sporadic single layer of epithelial granulosa cells and were surrounded by a band of theca cells (Fig. 3F). This is consistent with these large cysts being derived from antral follicles. However, some large cysts and associated cells were morphologically identified as being from *corpora lutea* [19]. These luteal cysts were present frequently in the F3 generation jet fuel hydrocarbon exposure lineage ovaries, Figure 3.

The number of healthy-looking large antral follicles was not found to be different between exposure and control groups in either the F1 or F3 generation ovaries (Fig. 4A & B). The exception was that there were significantly fewer ($p<0.05$) large antral follicles in the F3 pesticide-lineage ovaries. Therefore, the antral follicle development process appears relatively normal in the F1 and F3 generation females independent of exposure lineage.

Previous studies have demonstrated that negligible endocrine abnormalities are detected in 120-day-old F3 generation female rats following exposure to any of the toxicants studied [19,75]. PCO has previously been associated with an increase in androgen serum levels which is due to the highly steroidogenic theca cells of the cysts. Theca cells primarily produce androstenedione so the serum androstenedione levels in the F3 generation 1-year-old females were examined. Preliminary studies show that the F3 generation control lineage had 47 ± 3 pg/mL and the vinclozolin lineage had 177 ± 82 pg/mL serum androstenedione. Therefore, the androgen levels were increased in the F3 generation vinclozolin lineage females that had the PCO disease. This increase in androgen levels requires further investigation as do the other associated clinical conditions of glucose intolerance, abnormal adiposity and hyperinsulemia.

Transgenerational Granulosa Cell Transcriptome

Previous studies demonstrated the vinclozolin induced epigenetic transgenerational inheritance of adult onset disease involving epigenetic modifications of the sperm [16] and heritable phenotypes through the paternal lineage [20]. The only cell that can transmit an altered epigenome between generations is the germline [13], however, all the cells derived from this sperm will have an altered epigenome transcriptome [14]. In order to see if transgenerational changes in gene expression are apparent in ovarian follicle cells of the exposure lineage females, the transcriptomes of granulosa cells from control and vinclozolin lineage ovaries were compared. Granulosa cells were collected from pre-ovulatory follicles of five-month old F3 generation vinclozolin and control lineage ovaries as described in Methods. Messenger RNA was isolated from the granulosa cells of each animal ($n = 24$) and RNA from four animals of the same treatment group were pooled to create three different pooled samples from each of the two treatment groups. Three F3 generation vinclozolin-lineage and three control-lineage mRNA pooled samples were used in a microarray analysis as described in Methods to evaluate alterations in gene expression. The analysis demonstrated that 523 genes were differentially expressed between control and vinclozolin lineage F3 generation granulosa cells (Table S1). The number of differentially expressed genes in each of several functional gene categories is shown in Figure 5 with the number of up-regulated and down-regulated genes indicated. Many of the differentially expressed genes were identified as contributing to metabolism or signaling processes. The complete list of differentially expressed genes is functionally categorized and presented in Table S1.

A table of cellular pathways and processes impacted by the genes differentially expressed in vinclozolin lineage F3 generation granulosa cells is presented (Table 1). In Figure S2, two of the more heavily impacted cellular pathways are shown, PPAR signaling and steroid biosynthesis, with the differentially expressed genes highlighted. These data indicate that gene expression is altered transgenerationally in granulosa cells and that specific physiological processes may be affected by these changes. Additional bioinformatic analyses examined the functional rela-

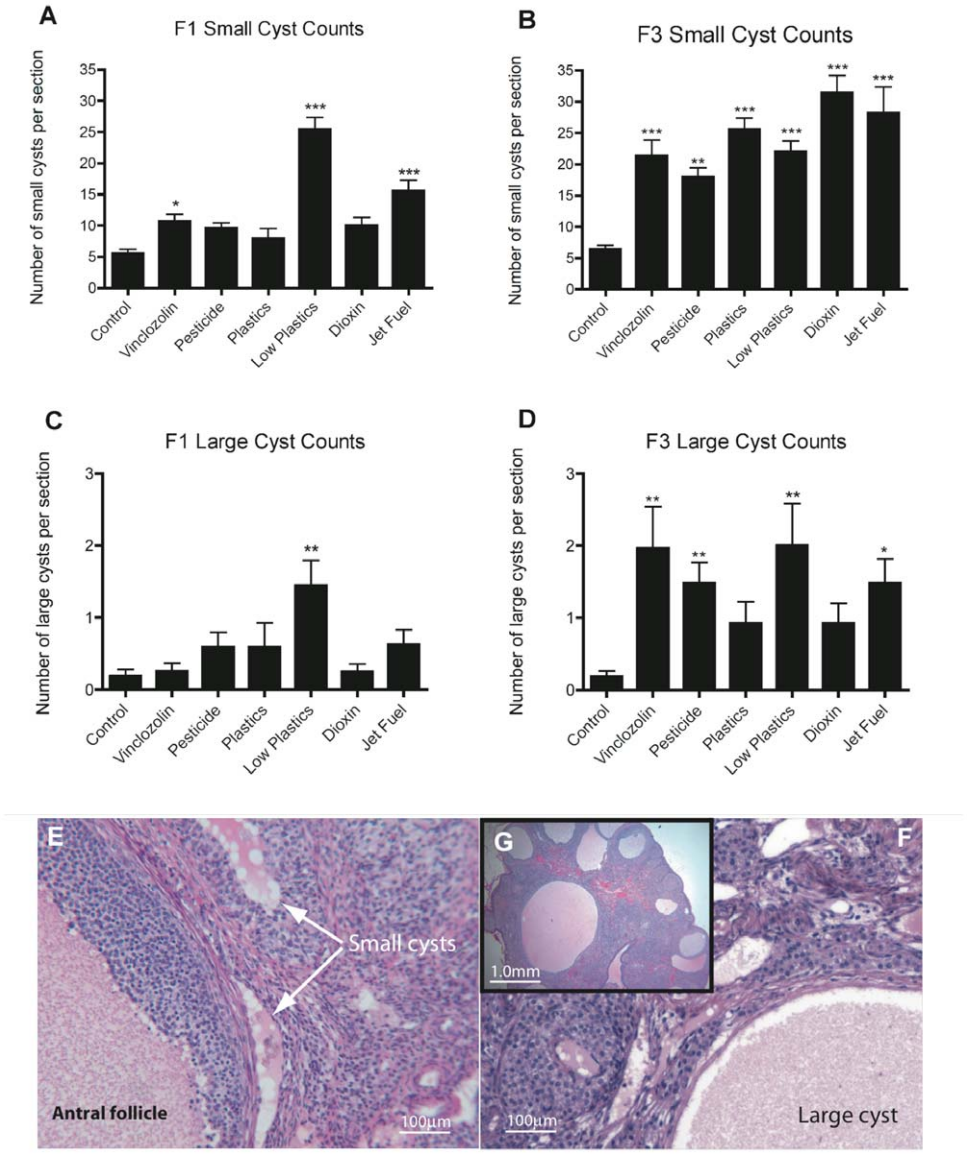

Figure 3. Ovarian cysts. Number of small (50–250 µm) cysts per section in F1 (A) and F3 (B) generation ovaries. Number of large (>250 µm) cysts per section in F1 (C) and F3 (D) generation ovaries. N = 9 animals per treatment group. Asterisks indicate groups significantly (*p≤0.05, **p≤0.01, ***p≤0.005) different than controls by ANOVA followed by Dunnet's post-hoc test. E) H and E stained section of F3 ovary showing small cysts. F) H and E stained F3 ovary showing a large cyst. G) Expanded view of small and large cysts.
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tionships among the F3 generation differentially expressed genes identified. An unbiased literature based network analysis was performed as described in Methods to determine which genes are functionally linked with respect to binding, signaling or regulation. This created a gene network of direct connections as shown in Figure 6. Some genes show significant functional connections to others, such as ESR1, MMP2 and CXCL12. Such highly connected genes may play important regulatory roles in these F3 generation granulosa cells and in the development of ovarian disease states. Therefore, a transgenerational change in the granulosa cell transcriptome was identified that may be in part a causal factor in the molecular etiology of the transgenerational ovarian disease. Further analysis of the 523 genes with transgenerational alterations in gene expression identified previously known genes involved in ovarian disease and more specifically polycystic ovarian disease. A total of 30 genes were found to be related to ovarian disease and 5 directly related to PCO disease, Figure 7. Therefore, genes known to have a relationship with PCO and ovarian disease were shown to have altered expression. The potential role of the transgenerational change in the granulosa cell epigenome to promote this transcriptome alteration is described below.

Transgenerational Granulosa Cell Epigenome

As previously described [13,16,20], an epigenetic transgenerational alteration of the sperm in vinclozolin lineage F3 generation animals can promote a transgenerational change in the epigenome unique to each cell type in all somatic cells derived from this germ line [14,76]. The F3 generation vinclozolin lineage alterations in differentially DNA methylated regions (DMR) in the granulosa cells was investigated. For this, a methylated DNA immunoprecipitation (MeDIP) procedure was used, followed by comparative

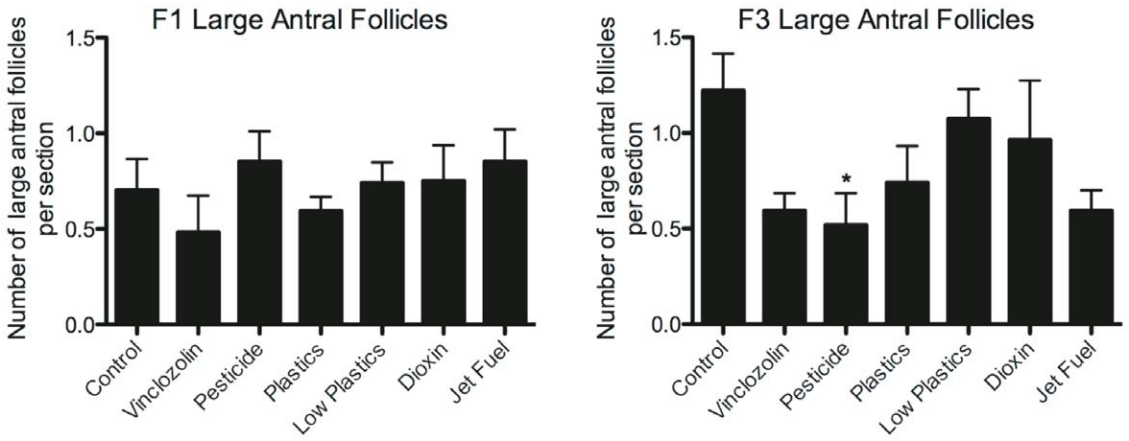

Figure 4. Large antral follicles. A) Number of large antral follicles per section in F1 generation ovaries. B) Number of large antral follicles per section in F3 generation ovaries. N = 9 animals per treatment group. Asterisks indicate groups significantly (*$p \leq 0.05$) different than controls by ANOVA followed by Dunnet's post-hoc test.
doi:10.1371/journal.pone.0036129.g004

hybridization on a genome wide promoter tiling array (Chip), termed an MeDIP-Chip assay, as previously described [16]. The MeDIP-Chip analysis of the differential DNA methylation between control and vinclozolin lineage F3 generation granulosa cells identified 43 DMR with a statistical significance $p > 10^{-7}$, Table 2. The chromosomal locations of all the DMR are presented in Figure 8 and indicates most autosomes are involved. A comparison of the 43 DMR identified with the 523 differentially expressed granulosa cell genes demonstrated only 1 gene promoter with overlap (*Plekhm1*). Analysis of the probability for a random overlap between the 43 DMR and the 523 differentially expressed genes indicated that an overlap of 1.47 genes would be expected. Therefore, the one gene overlap is likely not significant. The vast majority of differentially expressed genes did not have a DMR present in their promoters. Further analysis using statistically significant over represented clusters of differentially expressed genes identified 26 clusters from 2–5 Mbase size that had 4 to 9 genes each, Table S2. An overlap of these regulated gene clusters with the DMR identified 3 overlapped clusters, Figure 8. These 2–5 Mbase regions we refer to as potential Epigenetic Control Regions (ECR). The hypothesis is that the epigenetic regulatory site (e.g. DMR) regulates distally the expression of genes within this ECR. This is likely mediated through non-coding RNA, similar to what is seen for imprinting control regions (ICR)

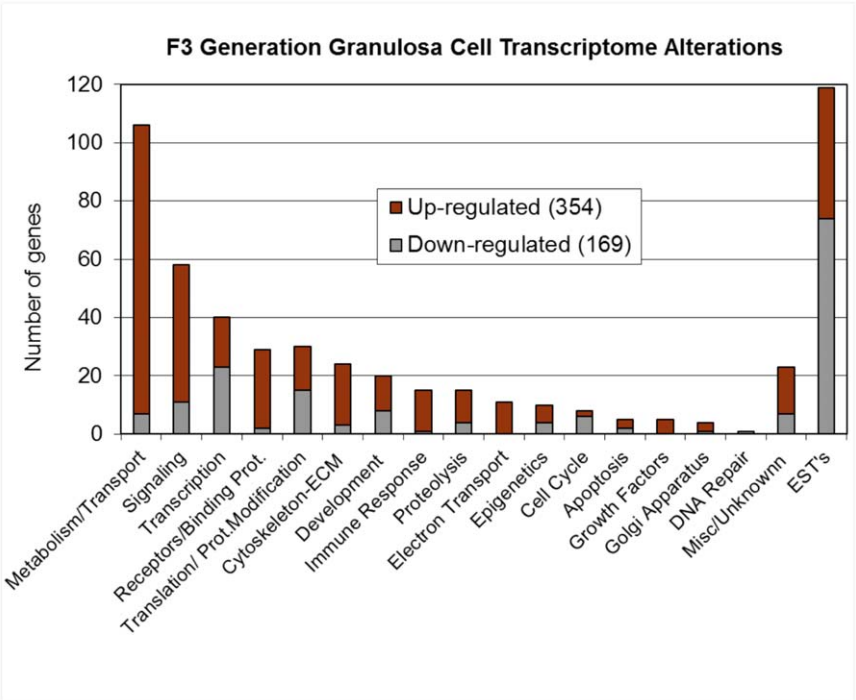

Figure 5. Number of genes with mRNA expression levels significantly different between Control and Vinclozolin-lineage F3 generation granulosa cells. Genes are placed into functional categories.
doi:10.1371/journal.pone.0036129.g005

Table 1. Physiological Pathway Enrichment.

Pathway Name	# Input Genes in Pathway	Impact Factor**
PPAR signaling pathway	11	17.0
Phagosome	11	
Cell adhesion molecules (CAMs)	10	10.3
Endocytosis	10	
Steroid biosynthesis	9	
Peroxisome	8	
Antigen processing and presentation	8	38.5
Leukocyte transendothelial migration	8	8.9
Fatty acid metabolism	6	
Valine, leucine and isoleucine degradation	6	
Spliceosome	6	
Lysosome	6	
Fc gamma R-mediated phagocytosis	6	
Regulation of actin cytoskeleton	6	4.7
Cysteine and methionine metabolism	5	
Glutathione metabolism	5	
Glycerophospholipid metabolism	5	
Biosynthesis of unsaturated fatty acids	5	10.2
MAPK signaling pathway	5	2.9
Neuroactive ligand-receptor interaction	5	1.5
Focal adhesion	5	5.0
Purine metabolism	4	
Lysine degradation	4	
Phenylalanine metabolism	4	
Sphingolipid metabolism	4	
Base excision repair	4	7.7
Calcium signaling pathway	4	4.4
Adherens junction	4	7.7
Tight junction	4	5.7
Complement and coagulation cascades	4	7.2
Jak-STAT signaling pathway	4	3.8
Pathways in cancer	4	2.3

Using 523 differentially expressed genes (F3 vinclozolin *vs.* control granulosa) in KEGG pathway database.
**Calculated by Pathway Express to estimate importance of these genes to pathway.
doi:10.1371/journal.pone.0036129.t001

previously identified [77]. A limited number of long non-coding RNA in the rat have been characterized, but of the 20 characterized 3 (NEAT1(chr1:204.8), khps1a (chr10:103.4), Zfhx2as (chr15:31.8)) had an overlap with the ECR identified. Further analysis of the rat lncRNA is needed before future correlation with the ECR can be made. The ECR provide one explanation for how a limited number of DMR can potentially control a large number of differentially regulated genes. The locations of the potential ECR are included in Figure 8 to correlate with the DMR identified.

Previously the sperm DMR identified in vinclozolin lineage F3 generation animals was reported [16]. An overlap of these sperm DMR with the current granulosa cell DMR demonstrated no overlapped sites. The lack of DMR overlap demonstrates different transgenerational epigenomes between the sperm and granulosa cell. It is anticipated that the cascade of epigenetic and transcriptome steps to achieve a differentiated somatic cell will lead to very distinct cell specific epigenomes with minimal overlap with the germ line [14]. Therefore, observations demonstrate that the F3 generation vinclozolin lineage granulosa cells have transgenerational changes in the epigenome that correlate with transgenerational changes in the transcriptome that in turn are proposed to have a role in the induction of the transgenerational ovarian disease. All the other cell types in the ovary (e.g. oocyte, theca cells, ovarian stromal cells) are also expected to have transgenerational epigenome and transcriptome changes that will also contribute to ovarian disease. The granulosa cell observations provide the proof of concept that the transgenerational disease phenotype develops from the transgenerational effects of the altered epigenome on somatic cell transcriptomes.

Discussion

The most common human diseases of the ovary are primary ovarian insufficiency and polycystic ovarian disease. These conditions can cause infertility and increase the risk for other related health issues. Primary ovarian insufficiency affects about 1% of women, while polycystic ovarian disease affects as many as 18% of women [25,26,27,37,38,39,40,41,42,44]. In the current study, F0 generation gestating female rats were exposed to various environmental compounds during fetal gonadal sex determination followed by F1 and F3 generation progeny being examined for ovarian histology. Ovarian abnormalities resembling the follicle pool depletion that precedes primary ovarian insufficiency and the cyst formation of polycystic ovarian disease were observed transgenerationally at an increased rate in the F3 generation exposure lineage animals. Molecular studies were performed comparing F3 generation control to vinclozolin lineage animals that indicated that there were transgenerational alterations in the epigenome and transcriptome of granulosa cells from ovarian follicles. These results raise the possibility that the disease etiology may in part be a result of exposure to environmental toxicants that promote epigenetic transgenerational inheritance of ovarian disease.

The analysis of ovarian follicle counts showed that there were significantly fewer oocytes in the ovaries of all of the exposure lineage females. Mean decreases in primordial follicle counts of 35% to 60% were seen in both the F1 and the F3 generation animals (Fig. 1). Since F1 generation animals were directly exposed to the environmental compounds *in utero* when the F0 gestating females were exposed, the F1 generation decrease in oocyte number compared to controls can be due to direct exposure of the follicles to the compounds. This possibility was tested using an organ culture system in which ovaries isolated from neonatal rats were treated with varying doses of vinclozolin or were left untreated as controls. A dose of 500 μM vinclozolin resulted in significantly fewer oocytes, while 200 μM and lower concentrations were not significantly different from controls (Fig. 2). F0 generation gestating female rats were treated with 100 mg/kg vinclozolin, which converts to approximately a 350 μM dose (assuming a whole-body volume of distribution). So it is conceivable that germ cells/oocytes could be lost in the F1 females when their F0 generation mothers are treated with vinclozolin. The epigenetic transgenerational inheritance of adult onset disease induced by the toxicants used in previous studies [19,78] demonstrates that the compounds or their metabolites pass the placenta to reach the fetus. Direct exposure to several of the toxicants used in this study has previously been shown to affect

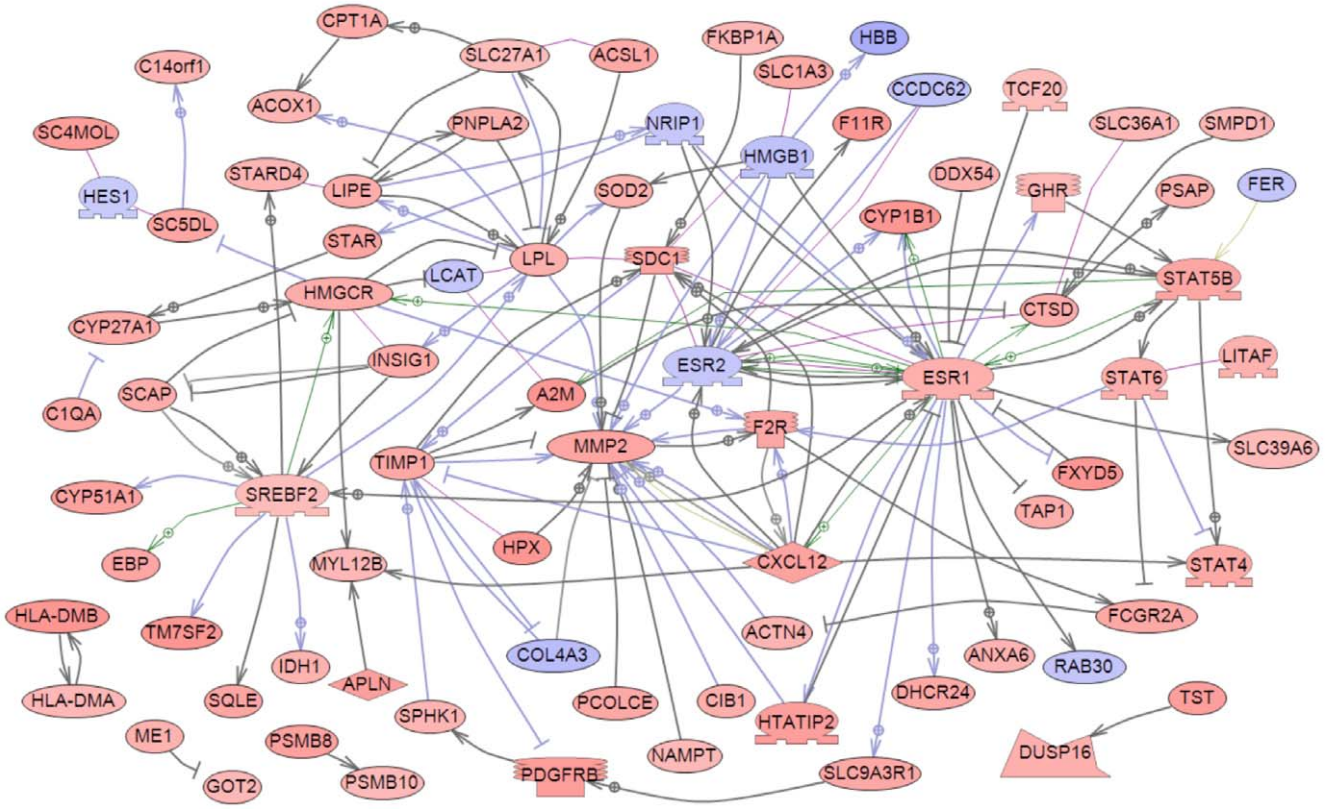

Figure 6. Gene network of known relationships among those genes found to be differentially expressed in Control compared to Vinclozolin-lineage F3 generation granulosa cells. Network is derived from an un-biased search of literature using Pathway Studio™. Node shapes code: oval and circle – protein; diamond – ligand; irregular polygon – phosphatase; circle/oval on tripod platform – transcription factor; ice cream cone – receptor. Red color represents up-regulated genes, blue color – down-regulated genes, grey rectangles represent cell processes; arrows with plus sign show positive regulation/activation, arrows with minus sign – negative regulation/inhibition. Grey arrows represent regulation, lilac – expression, green – promoter binding, olive – protein modification.
doi:10.1371/journal.pone.0036129.g006

ovarian function and development. Neonatal exposure to BPA has been shown to decrease the pool of primordial follicles and increase the incidence of ovarian cyst formation in rat ovaries, similarly to results of the current study [79,80]. Exposure to the plastic phthalate DEHP was shown to inhibit steroidogenesis in rat granulosa cells [81], and to impair oocyte growth and ovulation in zebrafish [82]. Female rats treated with dioxin both during fetal development and after birth were reported to have a premature cessation of reproductive cycles as they aged [65]. However, these direct exposure effects cannot explain the significant decrease in oocyte number seen in F3 generation exposure lineage females. F3 generation females were not directly exposed to the environmental compounds, which suggests a potential epigenetic transgenerational inheritance molecular mechanism.

Primary ovarian insufficiency in humans is characterized by an early loss of ovarian follicles and onset of menopause. This can occur when the pool of oocytes in the ovary is depleted to less than 1% of the amount present at puberty, as occurs with menopause [83,84]. The major physiological parameter of POI is the loss of the primordial follicle pool. In the current study, F1 and F3 treated-lineage groups of animals showed a 35–60% decrease in primordial follicle numbers compared to age-matched one-year-old controls. Approximately 45% of all the exposure lineage F3 generation females developed follicle pool depletion. Normal female rats usually stop cycling and enter reproductive senescence at 15–18 months of age. Other studies have shown that experimental depletion of oocyte numbers in rodents leads to an early loss of reproductive cycles [85,86,87]. Therefore, it is expected that animals from toxicant exposure lineages with follicle pool depletion would have a higher incidence of premature reproductive senescence (e.g. POI), but this remains to be investigated.

Polycystic ovarian disease is characterized by multiple persistent ovarian cysts [43]. In the current study increased numbers of ovarian cysts were seen in all the treated-lineage groups compared to the control lineage groups (Fig. 3). Interestingly, this effect was much more pronounced in the transgenerational F3 animals than in the directly exposed F1 generation. This suggests that the PCO disease identified may be due primarily to epigenetic transgenerational mechanisms and not to direct exposure. In addition, PCO disease was primarily observed in the 1 year old animals and not in young adults of 120 days of age [19], which is similar to what is observed in humans. An increase in circulating androstenedione was observed in the F3 generation vinclozolin lineage females that had PCO, similar to the clinical phenotype in women with PCO. Interestingly, research has shown that androstenedione levels are also increased in animals with follicle pool depletion [88]. The large cysts found in environmental exposure lineage females (vinclozolin, pesticide, jet-fuel and low-dose plastics groups) often had a negligible layer of epithelial/granulosa cells lining the cavity and only a stromal/thecal layer surrounding the cyst. These resemble the follicular cysts in PCOS patients [89,90,91].

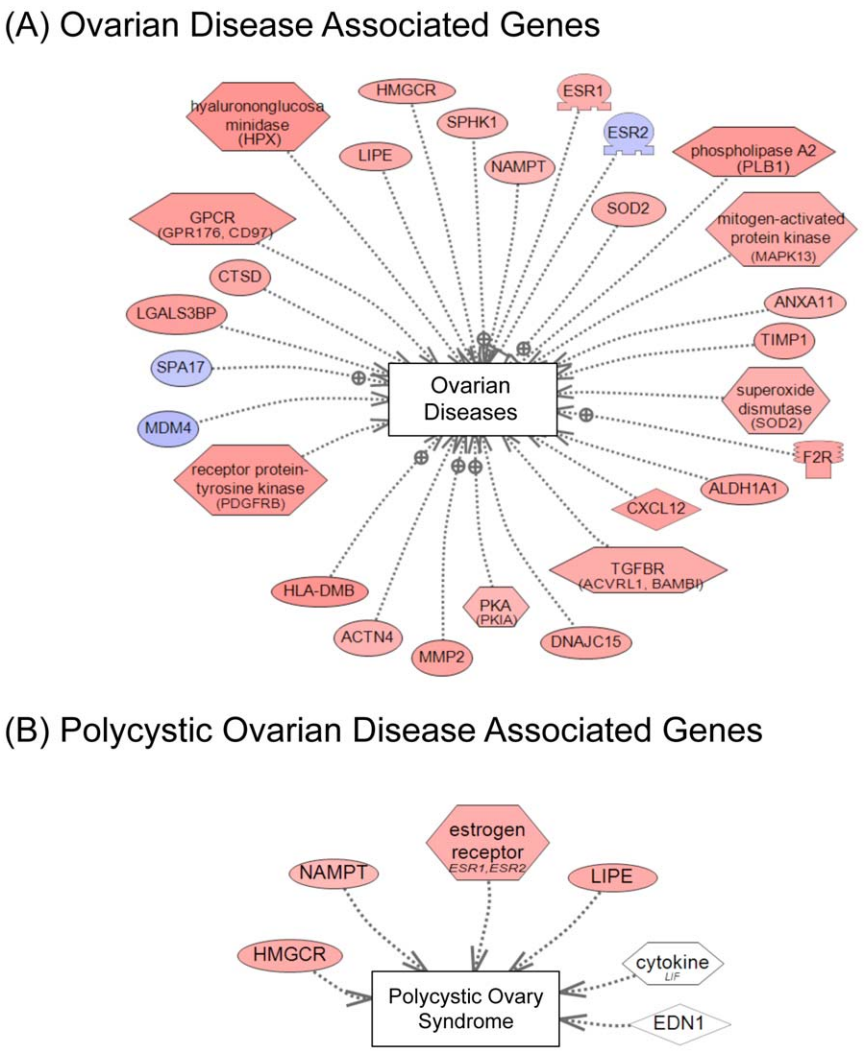

Figure 7. Ovarian diseases associated differentially expressed genes between F3 generation vinclozolin and control lineage granulosa cell. Sub-networks were identified using global literature analysis with Pathway Studio™. Node and arrow code is the same as for Figure 6. **A:** General ovarian diseases associated genes. **B:** Polycystic ovarian disease associated genes. White color nodes indicate differential methylated regions identified in this study.
doi:10.1371/journal.pone.0036129.g007

Luteal cysts were found only in the jet-fuel exposure lineage animals. These are cysts thought to form in the center of *corpora lutea* and are characterized by their surrounding band of luteal cells. This finding suggests that treatment with the different environmental toxicants can result in different transgenerational phenotypes. In contrast, all the different toxicant treatments resulted in the same increase in small ovarian cysts and in the same decrease in the primordial follicle pool. This is the case even though the different environmental exposures used are chemically dissimilar and would be expected to act through different signaling mechanisms. Observations suggest that some physiological processes in the ovary may be more prone to dys-regulation, independent of the environmental insult. For example, the complex signaling network that maintains primordial follicles and their oocytes in an arrested state [70,74,92,93] may be sensitive to dys-regulation that then leads to accelerated loss of follicles and oocytes from the ovary. Further research into this environmentally induced epigenetic transgenerational inheritance model is needed to determine the specific etiologies of adult onset ovarian diseases.

Molecular Etiology of Transgenerational Adult Onset Ovarian Disease

All the environmental exposures used in the current study induced transgenerational ovarian abnormalities. From among these exposures vinclozolin was used as a toxicant to study the molecular changes occurring transgenerationally in granulosa cells. Although all ovarian cell types (e.g. oocytes) are anticipated to develop a transgenerational alteration in the transcriptome and epigenome, granulosa cells were selected to provide the proof of concept for this phenomena. Vinclozolin is an agricultural fungicide with anti-androgenic endocrine disrupting activity [94]. Several studies have shown transgenerational effects following exposure of gestating rats during the period of fetal gonadal sex determination to vinclozolin [13]. These effects in the F3 generation animals include increased incidence of adult onset diseases such as cancer, kidney disease, immune abnormalities, prostate disease, spermatogenic defects and infertility [20,75,78,95,96]. F3 generation female rats after ancestral vinclozolin exposure have been shown to have uterine bleeding abnormalities late in pregnancy [75]. The molecular mechanism

Table 2. Differential DNA methylation regions (DMR) in F3 generation granulosa cells.

				Changed region coordinates			
Gene symbol	**Gene Description**	**Entrez gene ID**	**Significance (p≤)**	**Chr**	**Start**	**End**	**Region size (bp)**
Ceacam9	Carcinoembryonic antigen-related cell adhesion molecule 9	116711	8.53E-46	1	76960699	76961299	600
Sv2b	Synaptic vesicle glycoprotein 2b	117556	1.93E-61	1	130887128	130887728	600
Dtx4	Deltex homolog 4 (Drosophila)	293774	4.98E-14	1	215416136	215416736	600
Vdac1	Voltage-dependent anion channel 1	83529	2.12E-11	10	37793541	37794141	600
Rpl26	Ribosomal protein L26	287417	1.75E-28	10	55660989	55661674	685
Olr1468	olfactory receptor 1468	404977	1.23E-13	10	60268062	60268662	600
Cuedc1	CUE domain containing 1	303419	1.28E-08	10	76394145	76394745	600
Plekhm1	pleckstrin homology domain containing, family M (with RUN domain) member 1	303584	1.17E-34	10	92604304	92604904	600
RGD1563888	similar to DNA segment, Chr 16, ERATO Doi 472, expressed	360692	1.71E-31	11	17477532	17478132	600
Olr1567	olfactory receptor 1567	287970	2.23E-29	11	83619053	83619653	600
Selplg	selectin P ligand	363930	8.29E-10	12	43842412	43843012	600
Prom1	prominin 1	60357	3.57E-08	14	72118855	72119731	876
Lif	leukemia inhibitory factor	60584	5.55E-08	14	84886415	84887207	792
C1d	C1D nuclear receptor co-repressor	289810	1.46E-08	14	98152531	98153207	676
Zrsr1	zinc finger (CCCH type), RNA binding motif and serine/arginine rich 1	498425	1.69E-10	14	103657219	103657819	600
Gnpnat1	glucosamine-phosphate N-acetyltransferase 1	498486	8.05E-104	15	21287426	21288462	1036
Hars	histidyl-tRNA synthetase	307492	2.25E-08	15	60151437	60152140	703
LOC689713	LRRGT00175	689713	5.22E-30	16	83676467	83677258	791
Edn1	endothelin 1	24323	1.12E-11	17	28311735	28312730	995
Pcdha5	protocadherin alpha 5	393087	1.40E-39	18	29691331	29692216	885
Dtwd2	DTW domain containing 2	361326	3.36E-18	18	44718539	44719362	823
Mcm5	minichromosome maintenance complex component 5	291885	6.05E-15	19	13975188	13975788	600
Adcy7	adenylate cyclase 7	84420	4.10E-08	19	20076391	20076991	600
Dhps	deoxyhypusine synthase	288923	7.38E-10	19	24744799	24745688	889
Sv2a	synaptic vesicle glycoprotein 2a	117559	8.57E-27	2	190988570	190989259	689
Olr1686	olfactory receptor gene	294152	2.06E-09	20	417292	417892	600
Agpat1	1-acylglycerol-3-phosphate O-acyltransferase 1 (lysophosphatidic acid acyltransferase, alpha)	406165	1.91E-15	20	4243966	4244790	824
LOC686922	glutathione S-transferase, theta 4	686922	1.22E-09	20	13236150	13237231	1081
Unc5b	unc-5 homolog B (C. elegans)	60630	2.06E-21	20	28165595	28166390	795
Lcn11	lipocalin 11	100169711	3.73E-15	3	3888286	3888886	600
Lamc3	laminin gamma 3	311862	1.11E-21	3	10985872	10986472	600
Olr425	olfactory receptor 425	296687	1.05E-09	3	16610744	16611459	715
Serf2	small EDRK-rich factor 2	502663	4.86E-14	3	108254804	108255580	776

Table 2. Cont.

				Changed region coordinates			
Gene symbol	**Gene Description**	**Entrez gene ID**	**Significance (p≤)**	**Chr**	**Start**	**End**	**Region size (bp)**
Vom1r102	vomeronasal 1 receptor 102	286957	9.69E-08	4	124241988	124242588	600
Ppap2b	phosphatidic acid phosphatase type 2B	192270	4.56E-31	5	126121326	126122026	700
Ctrc	chymotrypsin C (caldecrin)	362653	2.41E-27	5	160755960	160756730	770
Clip4	CAP-GLY domain containing linker protein family, member 4	298801	3.02E-17	6	23831232	23832132	900
Olr1016	olfactory receptor 1016	288858	6.29E-25	7	6876471	6877071	600
Rabl2b	RAB, member of RAS oncogene family-like 2B	362987	1.17E-28	7	127910262	127910992	730
Nfkbie	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, epsilon	316241	5.02E-12	9	11053187	11053787	600
Aox3	aldehyde oxidase 3	493909	1.65E-14	9	56779670	56780578	908
Rpl39	ribosomal protein L39	25347	9.66E-17	X	7824369	7825465	1096
Nxf7	nuclear RNA export factor 7	501621	8.46E-20	X	122897736	122898626	890

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involved in epigenetic transgenerational inheritance requires an epigenetic alteration in the germline (egg or sperm) to transmit the phenotype [13]. An environmental exposure during fetal gonad sex determination appears to be required due to the epigenetic programming of primordial germ cells during this developmental period. Previous research with vinclozolin lineage rats has shown that permanent alterations in the male germ line epigenome are transmitted to subsequent generations and do not get erased after fertilization during early embryonic development, in a manner similar to imprinted genes [16]. A recent study demonstrates all the exposures used in the current study promote exposure specific epigenetic transgenerational alterations in the sperm epigenome [19]. Since the altered base-line epigenome of the sperm promotes an altered epigenome in cells and tissues that develop from that sperm, all tissues, including the ovary, are anticipated to have altered genome activity and develop a susceptibility to develop disease [76].

In the current study granulosa cells from large antral follicles of F3 generation females were evaluated for differences in either the gene expression profile or the epigenetic pattern of vinclozolin-lineage granulosa compared to controls. The gene expression of granulosa cells from F3 generation vinclozolin and control lineage animals was evaluated by microarray analysis. More than 500 genes were found to be differentially expressed compared to controls (Table S1). This is a transgenerational effect of the environmental compound exposure. The altered gene expression profile of vinclozolin lineage granulosa cells could contribute to the adult-onset development of abnormalities such as primary ovarian insufficiency or polycystic ovarian disease. Bioinformatic analysis of the differentially expressed gene list indicated that certain well-characterized cellular pathways and processes could be affected by changes in these genes (Table 1, Figure S2a, S2b). Interestingly, many genes involved in lipid metabolism and steroid precursor synthesis had altered expression, and this has been shown to potentially be involved in the pathology of polycystic ovarian disease [43]. Analysis of genes present within the 523 differentially expressed gene set that have previously been correlated to ovarian disease revealed 30 genes, Figure 7. In addition, 5 genes have been shown to be directly correlated to polycystic ovarian disease. Therefore, the current study involving an environmental toxicant induced epigenetic transgenerational inheritance of adult onset ovarian disease also identified a number of genes previously shown to be associated with ovarian disease. A previous study compared the transcriptomes of cumulus granulosa cells from human PCOS patients and normal control women after culturing the cumulus cells. Similarly to the current study, genes in the MAPK signaling pathway and in extracellular matrix/cell adhesion were found to be affected. However, few specific differentially expressed genes were found in common with the current study [97].

A gene network analysis of the transgenerationally altered granulosa cell transcriptome generated a highly connected set of potential regulatory genes (Fig. 6) associated with the ovarian abnormalities identified. This regulatory gene network provides potential new therapeutic targets and diagnostic markers to consider in ovarian disease etiology. Critical gene targets for future studies to be considered include *Esr1*, *Esr2*, *Srebf2*, *Mmp2*, *Cxcl12*, *Lpl*, *Stat5b* and *Hmgcr*.

The F3 generation granulosa cell epigenome analysis of differential DNA methylation demonstrated 43 different DMR in promoters. The MeDIP-Chip analysis used a comparative hybridization of F3 generation granulosa cell DNA for vinclozolin versus control lineage animals to increase sensitivity. A promoter tiling array Chip was used, so the majority of the genome was not examined. Therefore, the 43 DMR identified in promoters are a

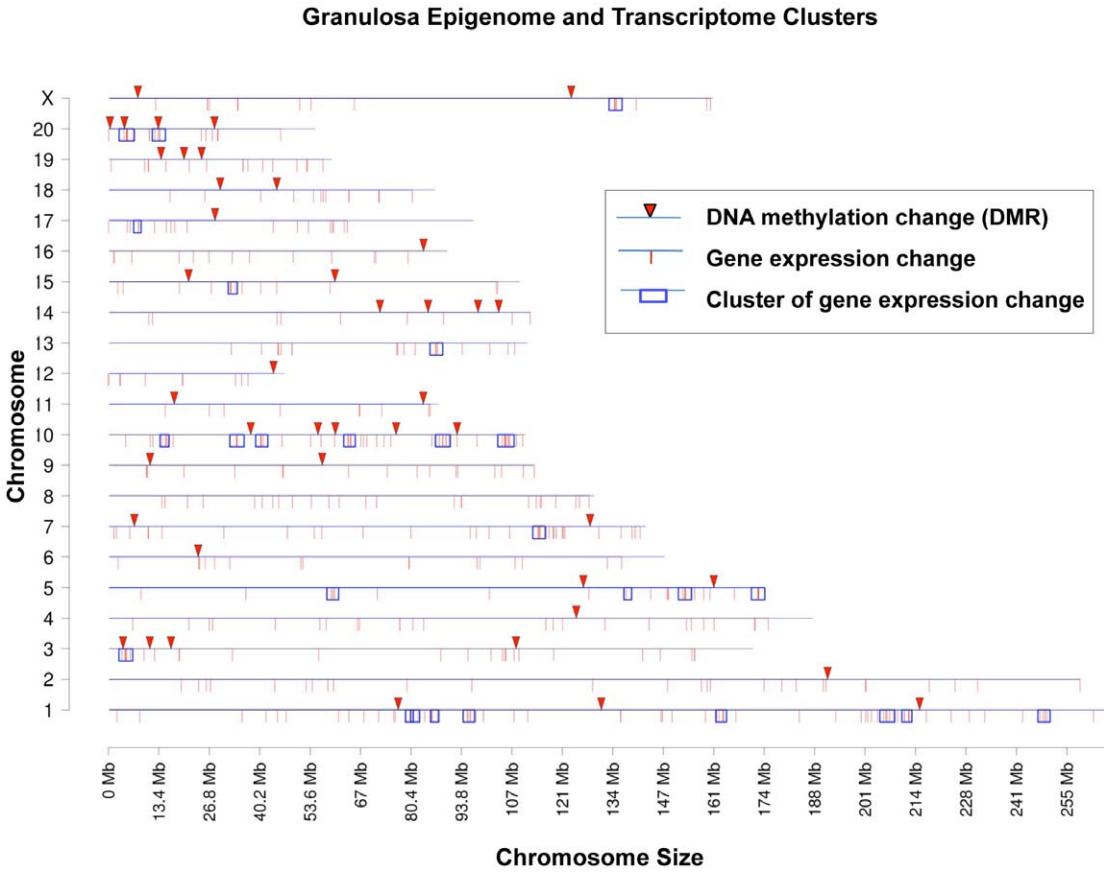

Figure 8. Chromosomal plot showing regions with vinclozolin-induced transgenerational changes in granulosa cells. Differential DNA methylation regions are displayed as inverted red triangles, changes in gene expression are displayed as red ticks, and a significant gene cluster of these genes with changed expression is delineated with blue open boxes. The chromosome number on Y-axis and size on X-axis are presented.
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sub-set of the total epigenetic modifications possible. The anticipation is a larger set of epigenetic modifications are present genome wide. The 43 DMR identified in the F3 generation granulosa cell epigenome demonstrates an environmental induced transgenerational alteration that is correlated to the onset of ovarian abnormalities. The DMR were present on most autosomes. The CpG content of these DMR was 1–10 CpG per 100 bp. Previously, low density CpG regions have been shown to be involved in epigenetic transgenerational alterations in sperm [19,98]. This genomic feature is speculated to be important in the epigenetic programming mechanism [19]. Interestingly, only one DMR (*Plekhm1*) was present in the promoter of one of the transgenerational 523 differentially expressed granulosa cell genes. This is likely due to a random overlap. Therefore, a relatively low number of epigenetic DMR sites could not explain the relatively large number of differentially expressed genes observed. Direct epigenetic regulation of individual promoters appears not to be involved. The hypothesis developing is that the epigenetic regulatory sites associated with the DMR may influence distal gene expression through non-coding RNA and are termed epigenetic control regions (ECR). This is similar on a molecular level to the imprinting control regions (ICR) previously identified (e.g. IGF2 and H19) [77]. The transgenerational differentially expressed gene set of 523 genes was examined in regards to chromosomal location and 26 gene clusters of 2–5 Mbase were identified with a statistically significant ($p<0.05$) over-represented set of genes, Figure 8 and Table S2. Several of these gene clusters correlated to the location of a DMR (approximately 15%). In addition, the small number (i.e. 20) of characterized rat long non-coding RNA (lncRNA) had 3 sites that overlapped with the ECR, but further characterization of the rat lncRNA's is required before functional associations between ECR and lncRNA can be elucidated. Future studies will be needed to determine the functional significance of these potential ECR sites, but the current study suggests the potential presence of such sites. The potential presence of DMR regulating such an ECR is speculated to clarify how a limited number of alterations in the epigenome may influence a large number of differentially expressed genes.

The molecular factors involved in epigenetic regulation of genome activity (i.e. DNA methylation, histone modifications, chromatin structure and non-coding RNA) can all regulate proximal promoter activity and gene regulation. Epigenetic factors such as DNA methylation, chromatin structure and non-coding RNA can also regulate distal gene expression, independent of classic genetic mechanisms. In the current study environmentally induced transgenerational effects on the germ line promoted epigenome and transcriptome effects in the granulosa cell that correlate with adult onset ovarian abnormalities. The etiology of ovarian diseases such as PCO and POI appear to in part involve: 1) environmental toxicant induced epigenetic alterations in the germ-line (sperm) during fetal gonadal development; 2) permanent alterations in the epigenome that are transmitted to subsequent generations through the sperm; 3) induction of alterations in the epigenome and transcriptome of all organs, such as the ovary, and

cells such as granulosa cells; and 4) an increased susceptibility to develop adult onset ovarian disease such as polycystic ovarian disease or primary ovarian insufficiency. Although the current study establishes the proof of concept such a mechanism exists, the degree the environment and epigenetic transgenerational inheritance is involved in human ovarian disease now needs to be investigated. Future studies are needed to clarify the F1, F2 and F3 generation sperm epigenome alterations in relation to each other, functional links of the DMR with the lncRNA and ECR, and to characterize transgenerational developmental changes in the transcriptome and epigenome of all ovarian cell types (e.g. oocyte, granulosa and theca). Elucidation of these molecular processes and mechanism will provide insights into the molecular etiology of ovarian disease.

Summary

An outbred rodent rat model was used to investigate the potential role of environmental epigenetics and epigenetic transgenerational inheritance in the etiology of ovarian disease. One of the ovarian abnormalities observed involved a decrease in the ovarian follicle pool size which correlates with the biology of primary ovarian insufficiency. The development of small and large ovarian cysts and the morphology of the cysts correlates with the biology of polycystic ovarian disease. However, these ovarian diseases as defined in humans are broader in concept to include correlated endocrine abnormalities and associated disease such as insulin resistance and diabetes. Therefore, the current ovarian abnormalities and disease in rats cannot be directly correlated to the human polycystic ovarian syndrome (PCOS) nor human primary ovarian insufficiency (POI) and loss of fertility. Although the rat ovarian abnormalities are consistent with these disease states, further research will be needed to clarify the role of environmental epigenetics and epigenetic transgenerational inheritance of ovarian disease in humans. Elucidation of such a disease etiology could help provide insight into clarifying the rapid increase in incidence of ovarian disease and apparent environmental impacts.

The environmental toxicants vinclozolin, dioxin and bisphenol-A have been shown in previous research to have transgenerational effects [21,22,75,78]. In recent research from our laboratory, all of the environmental toxicants used in the current study were shown to cause transgenerational disease in rats [19]. In the current study DEHP and DBP were used in combination with bisphenol-A as a single treatment, so it is uncertain if alone the compounds can promote transgenerational disease in ovaries. Similarly, permethrin was used in combination with DEET, so evidence suggests the mixture can promote epigenetic transgenerational disease. The hydrocarbon mixture jet fuel (JP8) also promoted a transgenerational increase in the incidence of ovarian disease in these studies. Results suggest that all these compounds should now be considered as potentially able to promote transgenerational ovarian disease.

The current study used pharmacologic doses of all the compounds and mixtures based on approximately 1% of the oral LD50 dose for most compounds, Table S3. The objective was to determine if these exposures have the capacity to promote epigenetic transgenerational inheritance of a disease phenotype, and not to do risk assessment of the exposures. Now that the current study has established the transgenerational actions of these compounds, risk assessment toxicology involving dose curves of relevant environmental doses are needed. In addition to considering the mode of administration and dose, the critical window of exposure to promote the epigenetic transgenerational phenotype (gonadal sex determination) needs to be considered, which for the human is 6–18 weeks of gestation. The gestating women in the first half of pregnancy would be the population most sensitive to environmentally induced epigenetic transgenerational inheritance of disease phenotypes.

In summary, gestating F0 generation rats were treated with environmental toxicants transiently during fetal gonadal sex determination. Adult-onset ovarian diseases resembling primary ovarian insufficiency and polycystic ovarian disease were seen at an increased rate in both the directly exposed offspring (F1), and transgenerationally (F3). There was a significant transgenerational alteration in both the transcriptome and the epigenome of vinclozolin-lineage granulosa cells. Therefore, ancestral toxicant exposure can contribute to the development of these disease states. These results suggest a new paradigm be considered for the etiology of ovarian disease. In addition to genetic abnormalities being causative, epigenetic abnormalities can also cause changes in gene expression during development that lead to these adult-onset diseases. These epigenetic abnormalities can be induced by exposure to a variety of environmental toxicants. If the exposure occurs during a susceptible period of an animal's development, then these epigenetic abnormalities can be fixed into the germ line (*i.e.* eggs or sperm) and be passed transgenerationally. Ovarian disease such as PCO has impacts on other diseases such as diabetes and adverse pregnancy outcomes [99]. Therefore, further elucidation of the etiology of ovarian disease and potential role of environmental epigenetics and epigenetic transgenerational inheritance will provide insights into the prevention and therapeutic strategies for female health.

Methods

Animals and treatments

All experimental protocols involving rats were pre-approved by the Washington State University Animal Care and Use Committee (IACUC approval # 02568-026). Hsd:Sprague Dawley®^{TM}SD®^{TM} female and male rats of an outbred strain (Harlan) were maintained in ventilated (up to 50 air exchanges/hour) isolator cages (cages with dimensions of 10 ¾″W×19 ¼″D×10 ¾″H, 143 square inch floor space, fitted in Micro-vent 36-cage rat racks; Allentown Inc., Allentown, NJ) containing Aspen Sani chips (pinewood shavings from Harlan) as bedding, on a 14 h light: 10 h dark regimen, at a temperature of 70 F and humidity of 25% to 35%. Rats were fed ad libitum with standard rat diet (8640 Teklad 22/5 Rodent Diet; Harlan) and ad libitum tap water for drinking.

At proestrus as determined by daily vaginal smears, the female rats (90 days of age) were pair-mated with male rats (120 days). On the next day, the pairs were separated and vaginal smears were examined microscopically. If sperm were detected (day 0) the rats were tentatively considered pregnant. Vaginal smears were continued for monitoring diestrus status in these rats until day 7. Pregnant rats were then given daily intraperitoneal injections of any one of the following single chemicals or mixtures with an equal volume of sesame oil (Sigma) on days E-8 through E-14 of gestation [75], as seen in Table S3. Treatment groups were Control (DMSO vehicle), Vinclozolin, Pesticide/repellent (includes: Permethrin (insecticide) and DEET (insect repellent)), Plastics (Bisphenol-A, DBP and DEHP), Low-dose plastics, Dioxin (TCDD), and Jet Fuel (JP8 hydrocarbon). The pregnant female rats treated with various mixtures were designated as the F0 generation. A drop in litter size was noted in the F1 generation of the Plastics group, so another treatment group was included with only half the dose of Bisphenol-A, DBP and DEHP and this group was designated the 'Low Dose Plastics' group. Doses, percent of oral LD50, and sources of the compounds are given in Table S3.

Breeding for F1, F2, and F3 generations

The offspring of the F0 generation were the F1 generation. The F1 generation offspring were bred to other F1 animals of the same treatment group to generate an F2 generation and then F2 generation animals bred similarly to generate the F3 generation animals. No sibling or cousin breedings were performed so as to avoid inbreeding. Note that only the original F0 generation pregnant females were injected with the treatment compounds.

Evaluation of adult ovaries

Ovaries taken from rats at the time of sacrifice (one year of age) were fixed in Bouin's solution, paraffin embedded and sectioned at 5 μm thickness. 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, developing pre-antral follicles, small antral follicles, large antral follicles, small cystic structures and large cysts. 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. Atretic follicles have granulosa cells or oocytes with pyknotic nuclei, an uneven or reduced layer of granulosa cells, and/or an uneven and less distinct basement membrane. 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 [100,101]. Normally a few primordial follicles at a time will undergo primordial to primary follicle transition and become developing follicles. Developing pre-antral follicles had one or more complete layers of cuboidal granulosa cells. Small antral follicles had a fluid-filled antrum and a maximum diameter of 510 μm measured across the outermost granulosa cell layer. Large antral follicles had a diameter greater than 510 μm. Large antral follicles may eventually ovulate. 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.

Neonatal rat ovary culture

Four-day old female Sprague-Dawley rats (Harlan Laboratories, Inc., USA) were euthanized according to Washington State University IACUC approved protocols and their ovaries removed and cultured whole as described previously [102]. Four-day old rat ovaries contain follicles that are almost exclusively of the primordial stage. Whole ovaries were cultured on floating filters (0.4 μm Millicell-CM, Millipore, Bedford, MD, USA) in 0.5 ml Dulbecco's modified Eagle's medium (DMEM)-Ham's F-12 medium (1:1, vol/vol) containing 0.1% BSA (Sigma), 0.1% Albumax (Gibco BRL, Gaithersburg, MD, USA), 27.5 μg/ml transferrin, and 0.05 mg/ml L-ascorbic acid (Sigma) in a four-well culture plate (Nunc plate, Applied Scientific, South San Francisco, CA, USA) for ten days. Previous studies have shown that four-day-old ovaries cultured for ten days have good cell viability [103]. The medium was supplemented with penicillin and streptomycin to prevent bacterial contamination. Ovaries were randomly assigned to treatment groups, with 1–3 ovaries per floating filter per well. Culture medium was changed and wells were treated every two days with vinclozolin (50 μM, 100 μM, 200 μM, or 500 μM), or were treated with 0.1% DMSO as a vehicle control. After culture, ovaries were fixed in Bouin's fixative (Sigma) for two hours and then equilibrated in 70% ethanol. Ovaries were then embedded in paraffin, sectioned at 3 μm and stained with hematoxylin/eosin for use in morphological analysis.

For each ovary the number of oocytes per section was counted and the counts were averaged across the two consecutive histological sections that had the largest ovarian cross section. The oocyte nucleus had to be visible for an oocyte to be counted. Normally, between 50 and 150 follicles were present in each cross-section.

Blood samples were collected, allowed to clot, centrifuged and serum samples stored for hormone assays. The androstenedione levels in serum were determined with a radio-immunoassay (RIA) performed by the Center for Reproductive Biology Assay Core at Washington State University.

Super-ovulation and collection of granulosa cells

F3 generation rats from both vinclozolin-treated and control lineages were treated with Pregnant Mare Serum Gonadotropin (Sigma cat, St. Louis, MO)(30 IU PMSG injected IP) at five to six months of age. Two days later animals were sacrificed and ovaries removed. The ovarian bursa and its adherent fat was removed from each ovary and the ovaries processed for granulosa cell collection [104]. The ovaries were suspended in the base medium used for all procedures was Ham's F-12 (Thermo Scientific). Following sequential 30 minute incubations at 37 °C in 6 mM EGTA in F-12 (to decrease Ca^{2+} - 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 each rat were collected into 1.5 mL tubes, allowed to settle for 10 minutes and supernatant removed. 1.0 mL of Trizol^{TM} (Invitrogen) was added to each sample, and then samples were stored at -70° until the time of RNA and DNA isolation.

Microarray transcriptome analysis

Messenger RNA was isolated from Trizol^{TM} for each animal as per manufacturers protocol. Messenger RNA from four animals of the same treatment group were pooled to create three different pooled samples from each of the two treatment groups. The mRNA processing and hybridization were performed at the Genomics Core Laboratory, Center for Reproductive Biology, Washington State University, Pullman, WA using standard Affymetrix reagents and protocol. Briefly, mRNA was transcribed into cDNA with random primers, then cRNA was transcribed from the cDNA, and from that, single-stranded sense DNA was synthesized which was fragmented and labeled with biotin. Biotin-labeled fragmented ssDNA was then hybridized to the Rat Gene 1.0 ST microarrays containing more than 27,000 transcripts (Affymetrix, Santa Clara, CA, USA). Hybridized chips were scanned on an Affymetrix Scanner 3000. CEL files containing raw data were then pre-processed and analyzed with Partek Genomic Suite 6.5 beta software (Partek Incorporated, St. Louis, MO) using an RMA and GC-content adjusted algorithm (Figure S1). The signals from an average of 28 different probes for each transcript were compared to give a single value. Lists of differentially expressed genes for each treatment were generated using the following cut off criteria: signal ratio Treatment/Control >1.20 change, mean difference for un-logged signals between control and treatment >10, t-test p-values<0.05 using an analysis correcting for organ culture date batch effects.

CEL files from this study have been deposited with the NCBI gene expression and hybridization array data repository (GEO, http://www.ncbi.nlm.nih.gov/geo, #GSE 33423) and can be also

accessed through www.skinner.wsu.edu. For gene annotation, Affymetrix annotation file RaGene1_0stv1.na31.rn4.transcript.csv was used unless otherwise specified.

To look for known functional relationships among the F3 generation differentially expressed genes identified above, KEGG pathways were interrogated using the http://www.genome.jp/kegg/ website (Kyoto Encyclopedia for Genes and Genome, Kyoto University, Japan), and also using Pathway Express, a web-based tool freely available as part of the Onto-Tools website (http://vortex.cs.wayne.edu) [105].

To further look for known functional relationships among the F3 differentially expressed genes, an unbiased, automated survey of published literature was performed to determine which genes are functionally linked with respect to binding, up-regulation, down-regulation, *etc*. Global literature analysis of differentially expressed genes was performed using Pathway Studio software (Ariadne, Genomics Inc. Rockville MD), which performs an interaction analysis and builds sub-networks of genes and the cellular processes that connect them to each other.

Previous studies have demonstrated that microarray data are validated with quantitative PCR data [106,107]. Due to the presence of an average of 28 different oligonucleotide probes for each specific gene being used on the microarray versus only a single primer set for a gene in a quantitative PCR, the microarray is more effective at eliminating false positive or negative data and provides a more robust quantification of changes in gene expression.

Methylated DNA immunoprecipitation (MeDIP)

DNA was collected from the same granulosa cell Trizol^{TM} preparations that were used for RNA isolation. The DNA Trizol^{TM} fractions from four animals of the same treatment group were pooled to create three different pooled DNA samples from each of the two treatment groups. These DNA samples were then used for methylated DNA immunoprecipitation (MeDIP). MeDIP was performed as follows: 6 µg of genomic DNA was subjected to series of three 20 pulse sonications at 20% amplitude and the appropriate fragment size (200–1000 bp) was verified through 2% agarose gels; the sonicated genomic DNA was resuspended in 350 µl TE and denatured for 10 min at 95°C and then immediately placed on ice for 5 min; 100 µl of 5× IP buffer (50 mM Na-phosphate pH7, 700 mM NaCl, 0.25% Triton X-100) was added to the sonicated and denatured DNA. An overnight incubation of the DNA was performed with 5 µg of antibody anti-5-methylCytidine monoclonal from Diagenode S.A (Denville, NJ) at 4°C on a rotating platform. Protein A/G beads from Santa Cruz (Santa Cruz, CA) were prewashed on PBS-BSA 0.1% and resuspended in 40 µl 1× IP buffer. Beads were then added to the DNA-antibody complex and incubated 2 h at 4°C on a rotating platform. Beads bound to DNA-antibody complex were washed 3 times with 1 ml 1× IP buffer; washes included incubation for 5 min at 4°C on a rotating platform and then centrifugation at 6000 rpm for 2 min. Beads-DNA-antibody complex were then resuspended in 250 µl digestion buffer (50 mM Tris HCl pH 8, 10 mM EDTA, 0.5% SDS) and 3.5 µl of proteinase K (20 mg/ml) was added to each sample and then incubated overnight at 55°C on a rotating platform. DNA purification was performed first with phenol and then with chloroform:isoamyl alcohol. Two washes were then performed with 70% ethanol, 1 M NaCl and glycogen. MeDIP selected DNA was then resuspended in 30 µl TE buffer. Whole-genome amplification was then performed with the WGA2 kit (Sigma-Aldrich #WGA2) on each MeDIP sample to be used in the microarray comparative hybridization analysis.

Tiling Array MeDIP-Chip Analysis

Roche Nimblegen's Rat DNA Methylation 3x720K CpG Island Plus RefSeq Promoter Array was used, which contains three identical sub-arrays, with 713,670 probes per sub-array, scanning a total of 15,287 promoters (3,880 bp upstream and 970 bp downstream from transcription start site). Probe sizes range from 50–75 mer in length with a median probe spacing of 100 bp. Three different comparative (amplified MeDIP vs. amplified MeDIP) hybridizations experiments (3 sub-arrays) were performed, each encompassing DNA samples from 24 animals (3 treatment and 3 control groups). MeDIP DNA samples from experimental groups were labeled with Cy3 and MeDIP DNA samples from the control groups were labeled with Cy5.

Bioinformatic and Statistical Analyses of Chip Data

For each comparative hybridization experiment, raw data from both the Cy3 and Cy5 channels were imported into R (R Development Core Team (2010), R: A language for statistical computing, R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org), checked for quality and converted to MA values ($M = Cy5 - Cy3$; $A = (Cy5+Cy3)/2$). The following normalization procedure was conducted. Within each array, probes were separated into groups by GC content and each group was separately normalized, between Cy3 and Cy5 using the loess normalization procedure. This allowed for GC groups to receive a normalization curve specific to that group. After each array had its CG groups normalized within the array, the arrays were then normalized across arrays using the A quantile normalization procedure.

Following normalization each probe within each array was subjected to a smoothing procedure, whereby the probe's normalized M values were replaced with the median value of all probe normalized M values across all arrays within a 600 bp window. If the number of probes present in the window was less than 3, no value was assigned to that probe. Each probe's A values were likewise smoothened using the same procedure. Following normalization and smoothing each probe's M value represents the median intensity difference between vinclozolin generation and control generation of a 600 bp window. Significance was assigned to probe differences between treatment-generation samples and control generation samples by calculating the median value of the intensity differences as compared to a normal distribution scaled to the experimental mean and standard deviation of the normalized M. A Z-score and P-value were computed for each probe from that distribution. The statistical analysis was performed in pairs of comparative IP hybridizations between treatment lineage (T) and control lineage (C). T1-C1 and T2-C2 gave 333 sites; T1-C1 and T3-C3 gave 327 sites; T2-C2 and T3-C3 gave 340 sites. In order to assure the reproducibility of the candidates obtained, only the candidates showing significant changes in all three of the paired comparisons were chosen as having a significant change in DNA methylation between the experimental group and controls. This is a very stringent approach to select for changes, since it only considers those changes repeated in all paired analyses.

Clustered Regions of interest were then determined by combining consecutive probes within 600 bases of each other, and based on whether their mean M values were positive or negative, with significance p-values less than 10^{-5}. 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 $\geq$1 CpG/100 bp.

Statistical Analysis for ovarian morphological data

Treatment groups are compared using analysis of variance (ANOVA) followed by Dunnet's post-hoc tests where appropriate. Groups were considered statistically significant with $P \leq 0.05$. Statistics for ovary counts were calculated using Graph Pad Prism version 5.0 b for Macintosh, Graph Pad Software, San Diego, CA, USA.

Supporting Information

Figure S1 Sample histograms and box plots for granulosa cell microarray signal values after pre-processing with RMA, GC-content adjusted algorithm. Plots for F3 generation control (red) and F3 generation vinclozolin (blue) microarrays.
(PDF)

Figure S2 (A): Steroid Biosynthesis Pathway; and (B): PPAR Signaling Pathway showing granulosa cell differentially expressed genes between F3 generation vinclozolin and control lineage rats: red or red-countered boxes represent up-regulated genes, green down-regulated and white boxes – not affected genes.
(PDF)

Table S1 Rat granulosa cell genes differentially expressed between F3 generation vinclozolin and control lineage animals (523 genes).
(PDF)

Table S2 Differential expressed gene clusters.
(PDF)

Table S3 Doses and sources of chemicals used.
(PDF)

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Author Contributions

Conceived and designed the experiments: MKS. Performed the experiments: EN GL MM CGB MIS. Analyzed the data: MKS EN GL MM CGB MIS. Wrote the paper: MKS EN. Edited the manuscript: MKS EN GL MM CGB MIS.

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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.

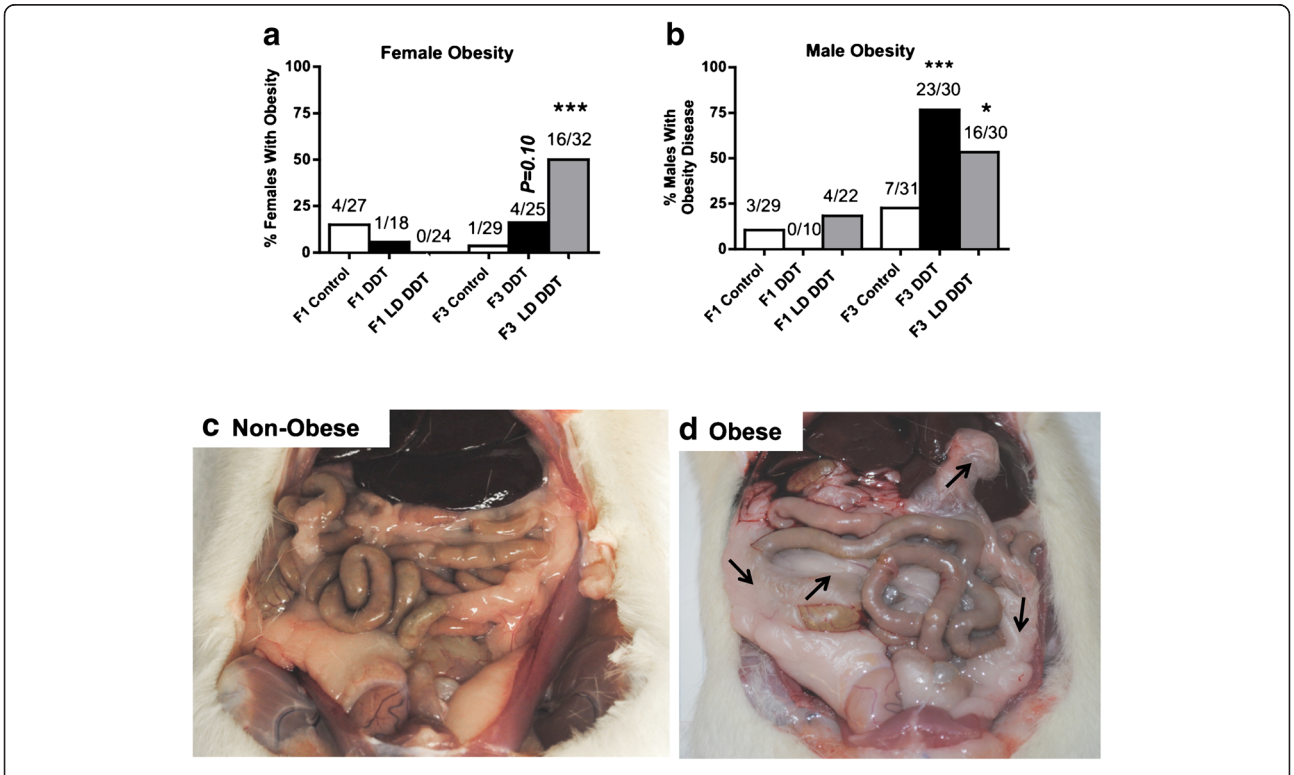

Figure 1 Transgenerational obesity. Percentages of females **(a)** or males **(b)** with obesity in the F1 and F3 generations. The dichlorodiphenyltrichloroethane (DDT) or lower dose (LD) DDT is indicated. The number of diseased rats/total number of rats (n value) is shown above the respective bar graphs. 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.

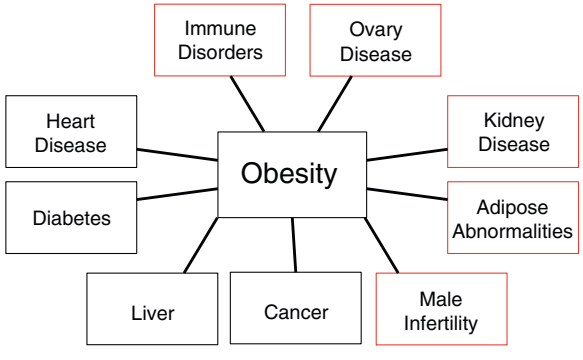

Figure 2 Obesity and associated disease. The major diseases previously shown to be associated with obesity are indicated with those identified in the present study shown with a red box.

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

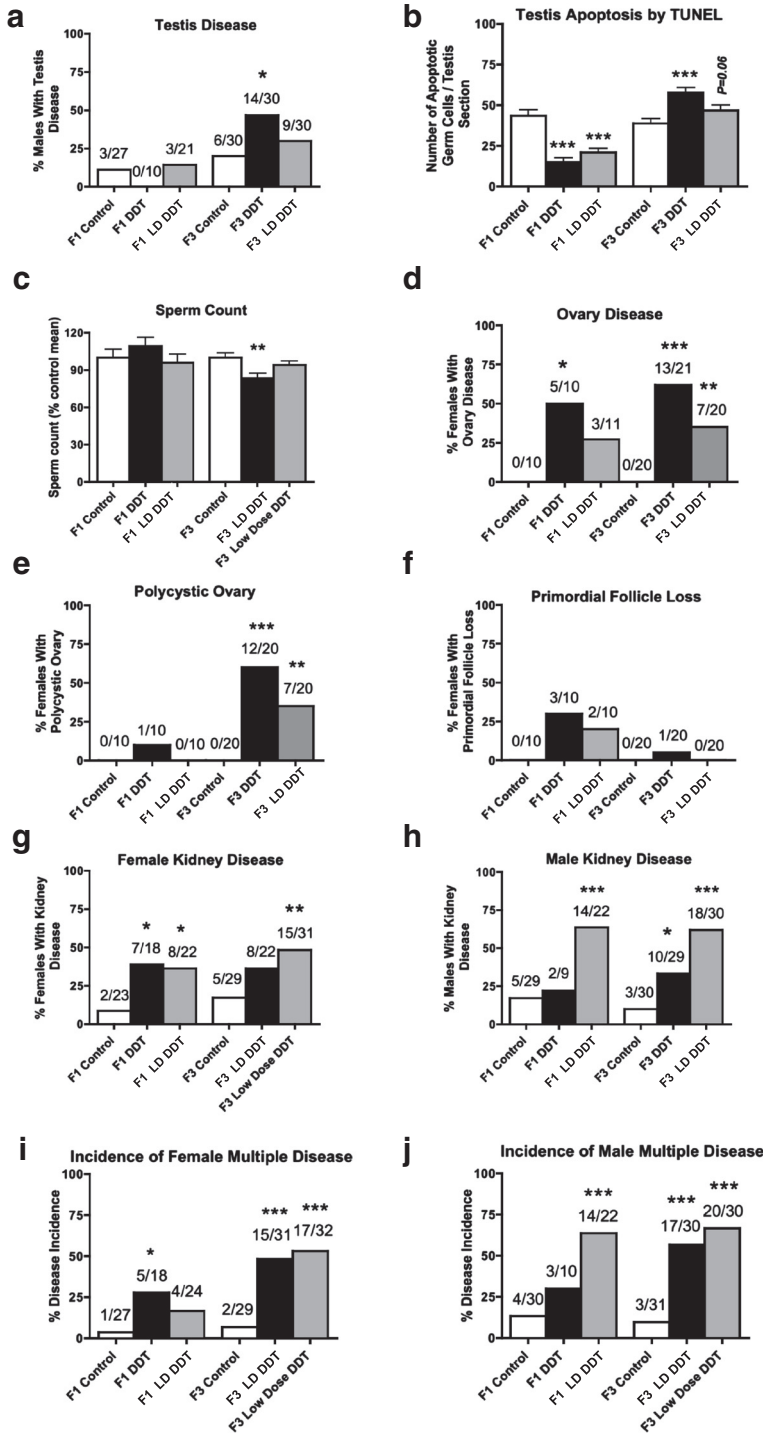

Figure 3 Transgenerational obesity and associated disease. Percentages of the F1 and F3 generation disease/abnormalities from control (open bars), dichlorodiphenyltrichloroethane (DDT) (black bars) and lower dose (LD) DDT lineages. Testis disease **(a)**, spermatogenic cell apoptosis **(b)** and sperm 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

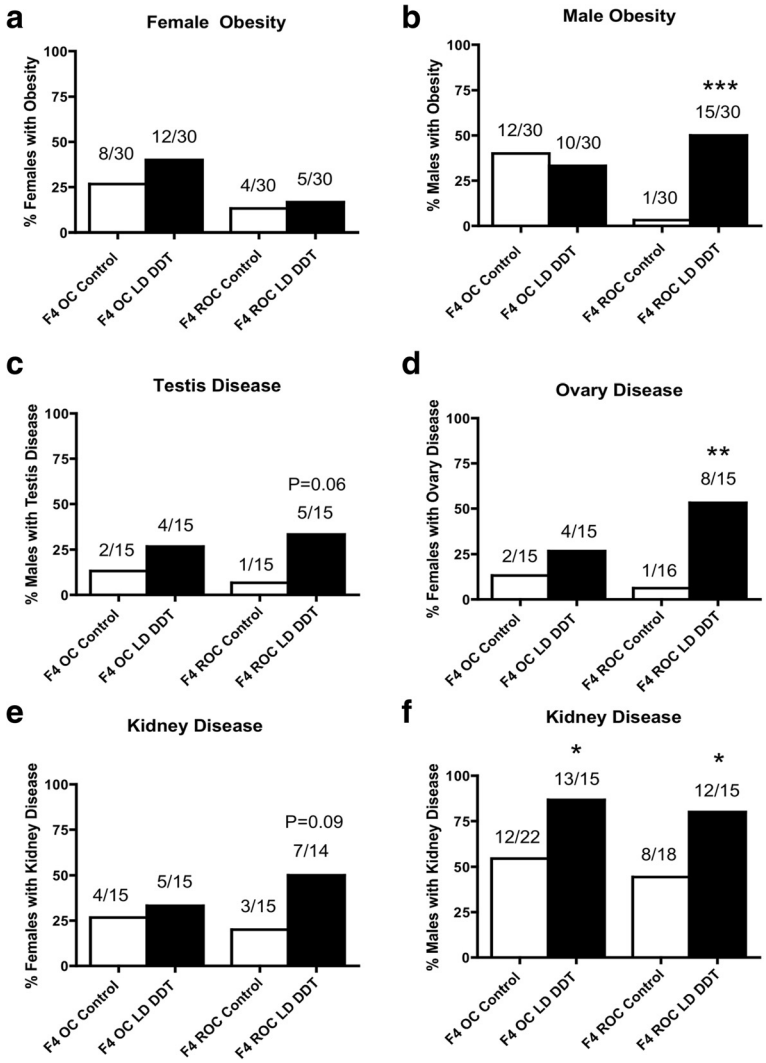

Figure 4 Transgenerational disease in F4 generation outcross or reverse outcross offspring for both male and female germline transmission. Incidences of obesity in females **(a)** and in males **(b)**, ovary disease **(c)**, testis disease **(d)**, kidney disease in females **(e)**, in males **(f)**, 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

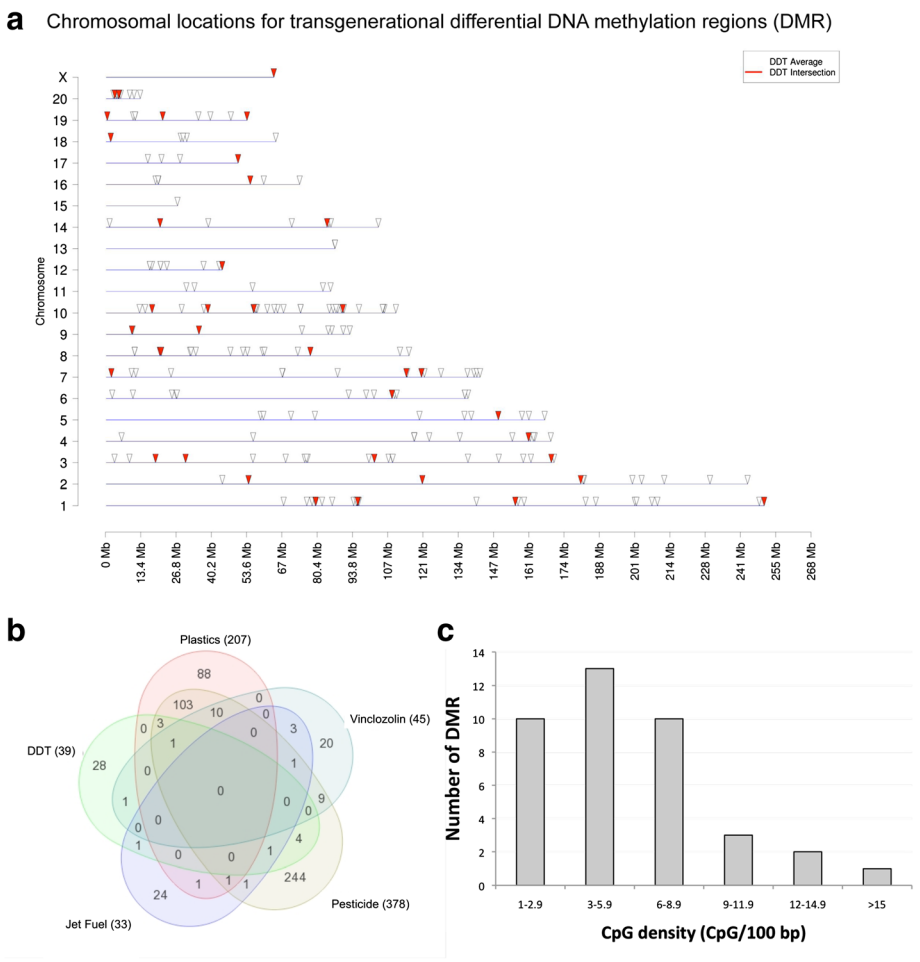

Figure 5 Transgenerational F3 generation sperm epimutations. (a) Chromosomal locations for differential DNA methylated regions (DMR) in sperm DNA from F3 generation dichlorodiphenyltrichloroethane (DDT) lineage rats compared to control lineage (arrowheads). The high stringency intersection epimutations 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

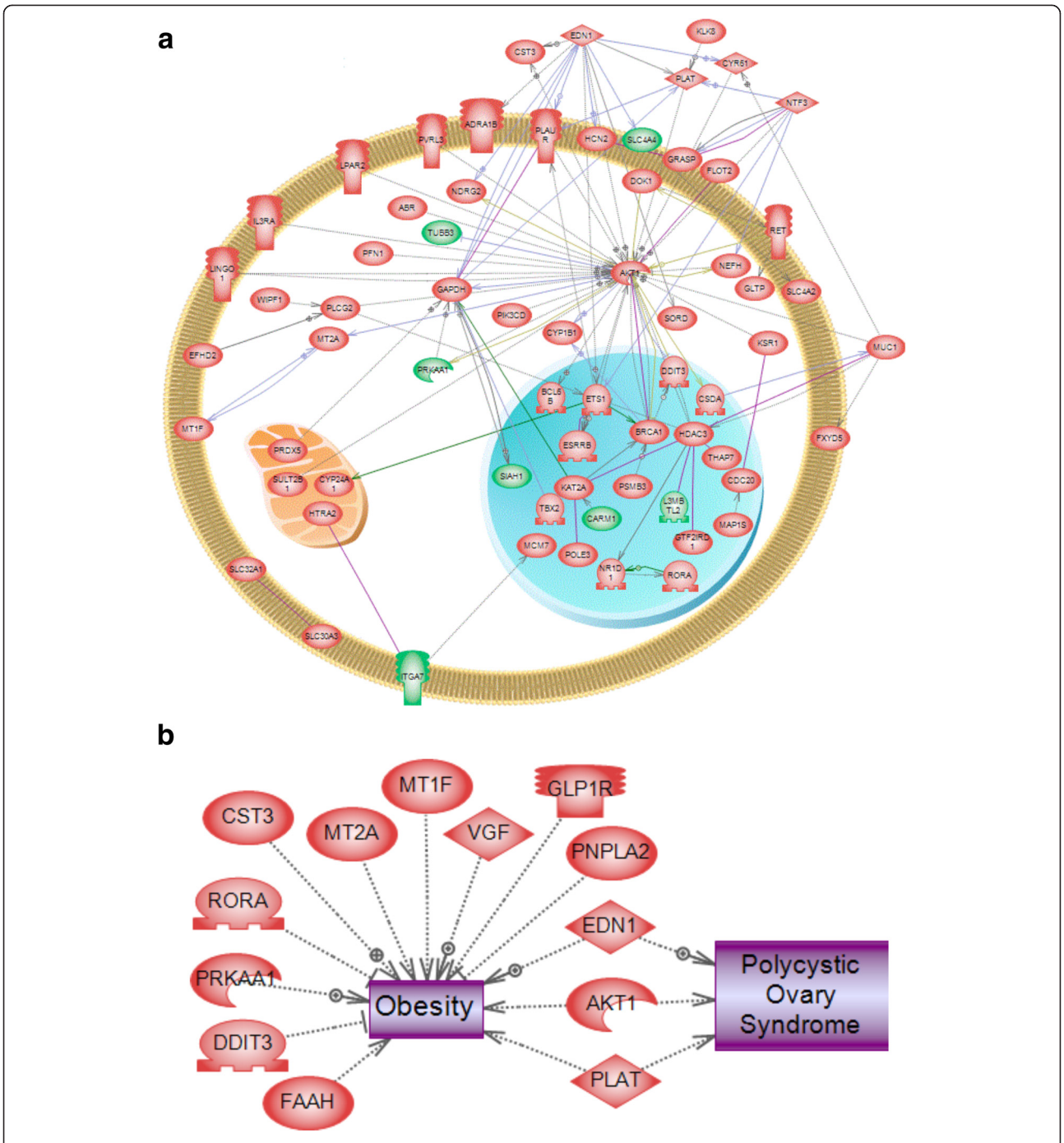

Figure 6 Transgenerational F3 generation sperm epimutation-associated gene network. (a) The gene network associated with the differential DNA methylation regions (epimutations) with cellular location indicated. The green labeled genes are those in the high stringency intersection set of 38 differential 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

William P Kabasenche[1] and Michael K Skinner[2*]

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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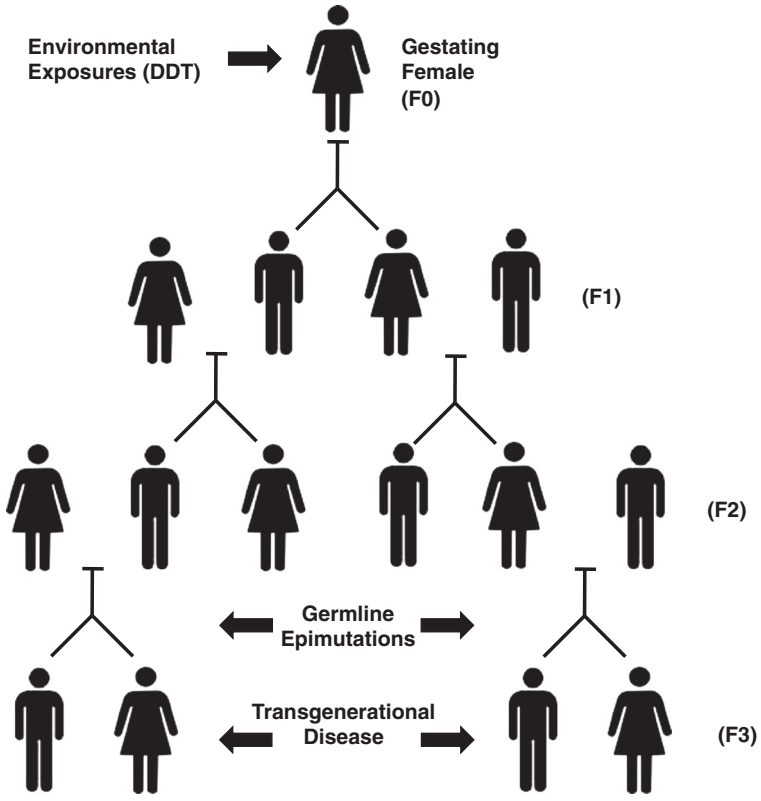

Figure 1 Scheme for DDT induced epigenetic transgenerational inheritance of disease.

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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