Spring 2021 – Epigenetics and Systems Biology
Discussion Session (Epigenetics and Disease Etiology)
Michael K. Skinner – Biol 476/576
Week 13 (April 15)

Epigenetics and Disease Etiology

Primary Papers

1. Anway et al. (2006) Endocrinology. 147(12):5515-5523. (PMID: 16973726)
2. Nilsson et al. (2018) Epigenetics. 13(8): 875-895. (PMID: 30207508)
3. King and Skinner (2020) Trends Endocrinol Metab. 31(7):478-494. (PMID: 32521235)

Discussion

Student 35 – Ref #1 above

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

Student 36 – 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 37 – Ref #3 above

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

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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.

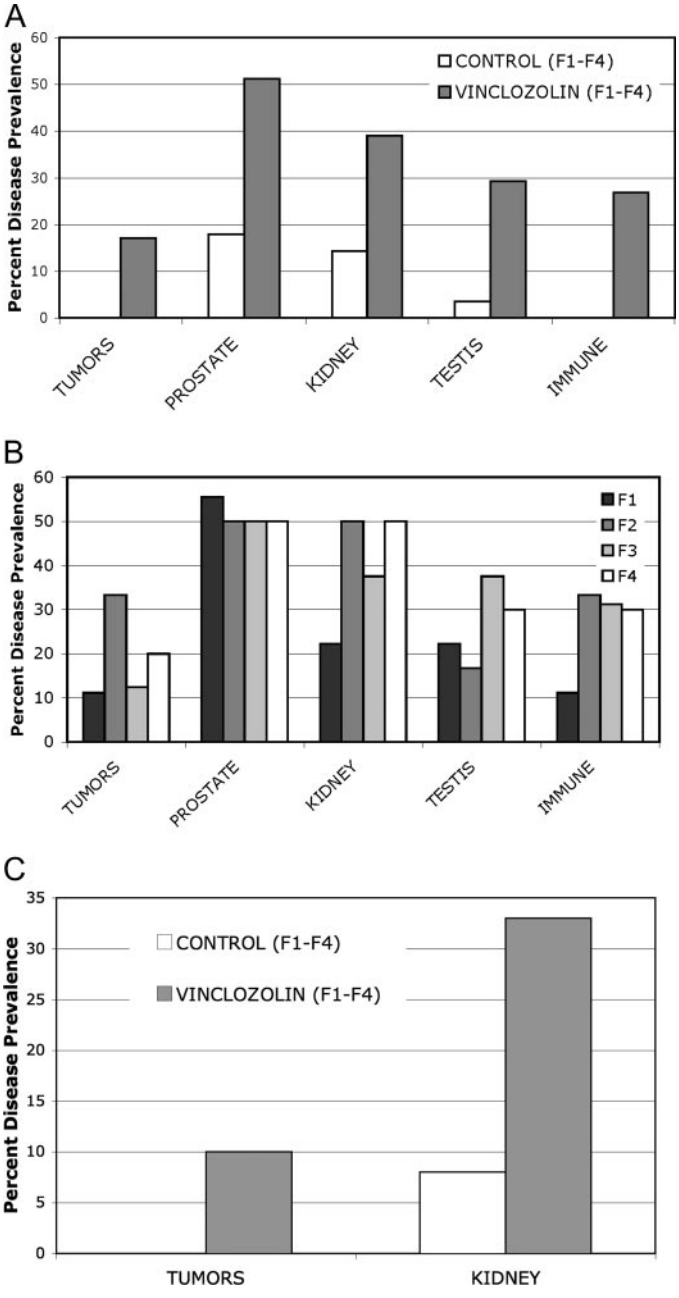

FIG. 1. Disease states or abnormalities in vinclozolin and control generation (F1–F4) animals. A, Males from combined (F1–F4) generation animals. B, Males from individual (F1–F4) generation animals. C, Females from combined (F1–F4) generation animals. The percentage disease prevalence is presented for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

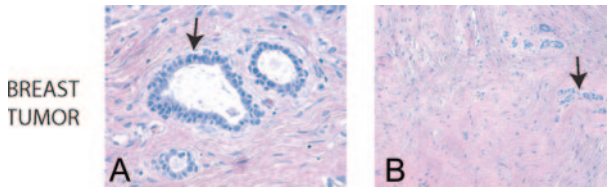

FIG. 2. Histology of representative vinclozolin generation breast fibroadenoma. A representative ×400 magnification (A) and ×200 magnification (B) micrograph is presented from a minimum of five different animals with breast adenoma analyzed. A *closed arrow* indicates a neoplastic glandular epithelial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

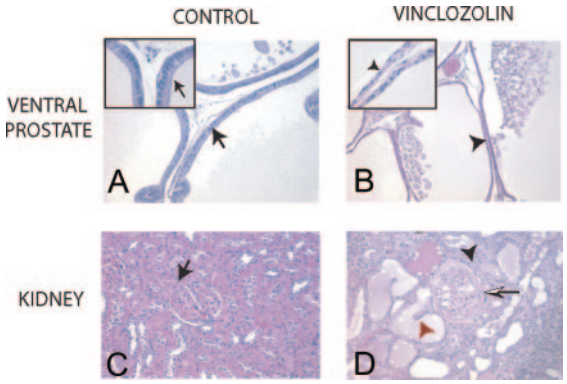

FIG. 3. Histology of representative control (A and C) and vincozolin (B and D) F2 or F3 generation tissues are presented, ventral prostate (A and B), and kidney (C and D). A representative ×200 magnification micrograph is presented from a minimum of five animals analyzed. *Insets* in A and B are ×1000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

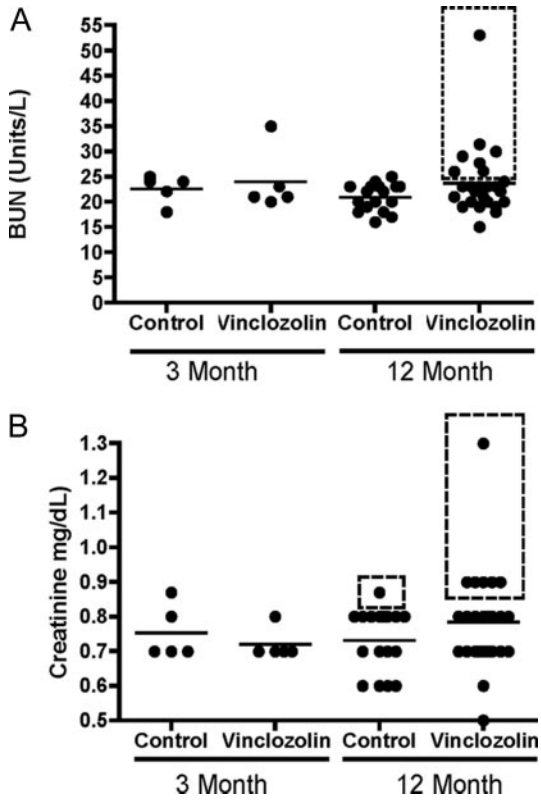

FIG. 4. Blood analysis for BUN (A) and creatinine (B) from control and vinclozolin (F1–F4) generation animals. Scatter plots are presented with a mean line indicated. Samples in the *dashed boxes* correlate with animals with diagnosed morphological kidney abnormalities and corresponding renal blood marker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

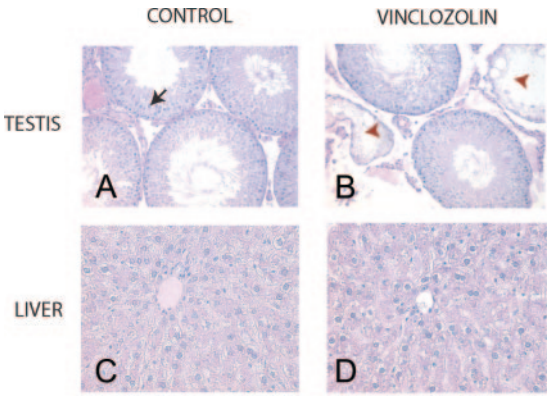

FIG. 5. Histology of representative control (A, C) and vincozolin (B and D) F2 or F3 generation tissues are presented for normal testis (A), abnormal testis (B), and liver (C and D). A representative ×200 (A and B) or ×400 (C and D) magnification micrograph is presented from a minimum of five animals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

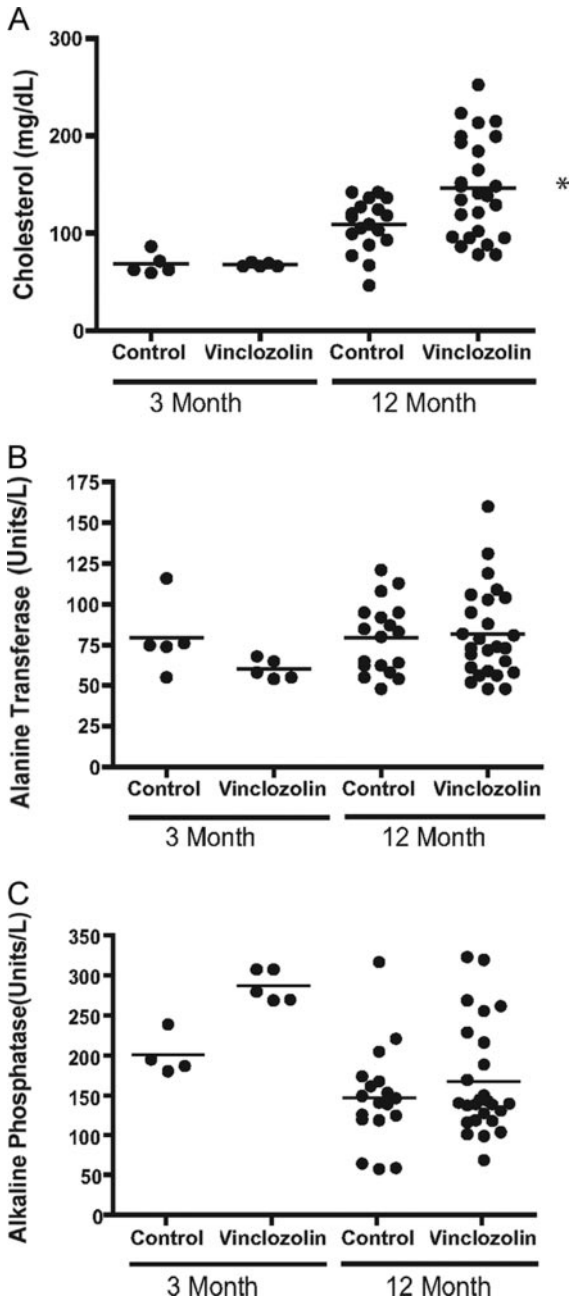

FIG. 6. Blood analysis for combined F1–F4 control and vinclozolin generation animals. A, Cholesterol concentrations and liver serum markers of (B) alanine transferase and (C) alkaline phosphatase are presented (units/liter). Scatter plots are indicated for 3-month-old and 6- to 12-month-old animals with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

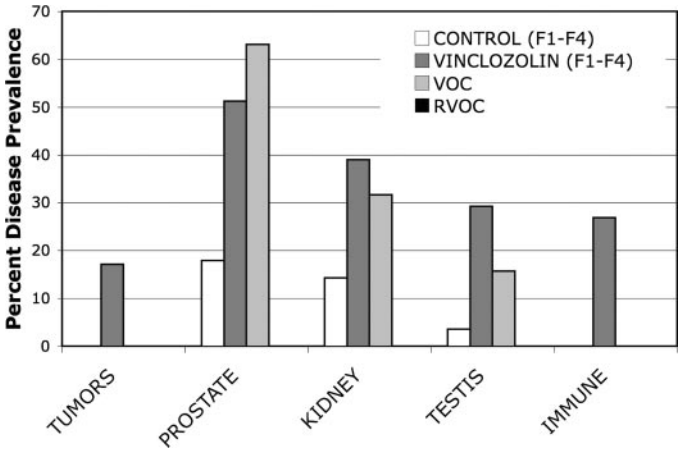

FIG. 7. Comparison of outcross disease states or abnormalities using F1–F4 control and vinclozolin generation-treated cross compared with VOC (F2 vinclozolin generation male and wild-type female) and a RVOC (F2 vinclozolin generation female and wild-type male). The percentage disease prevalence is presented 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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Environmental toxicant induced epigenetic transgenerational inheritance of ovarian pathology and granulosa cell epigenome and transcriptome alterations: ancestral origins of polycystic ovarian syndrome and primary ovarian insufiency

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ABSTRACT

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

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Introduction

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

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

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

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Supplemental data for this article can be accessed here.

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

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

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

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

Results

Ovarian pathology analysis

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

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

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

DNA methylation analysis

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

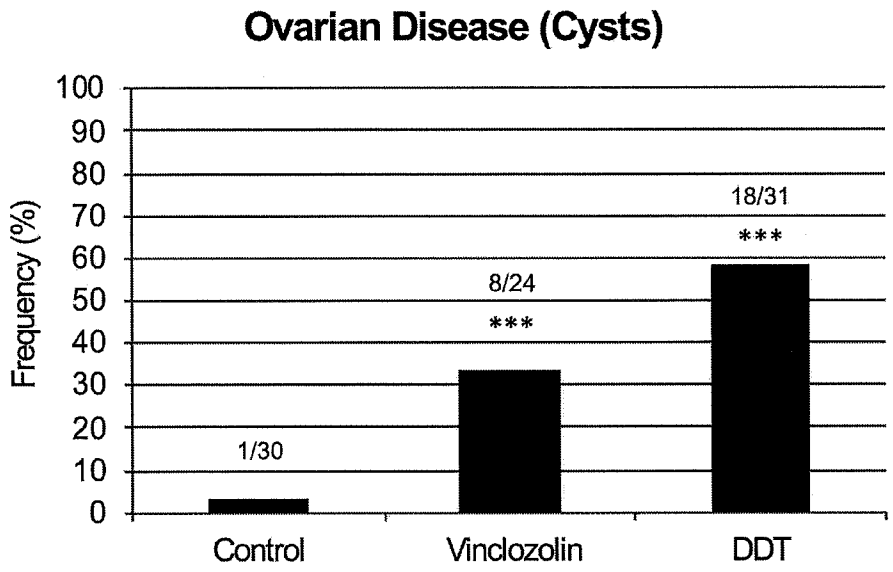

Figure 1. Ovarian pathology frequency. Transgenerational ovarian disease in F3 generation control, vinclozolin and DDT lineage rats at 1 y of age. Numbers for diseased individuals versus the total number of individuals analyzed is shown and (***) indicates statistical significance of $P < 7 \times 10^{-3}$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

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

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

Granulosa mRNA and noncoding RNA analysis

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

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

Genes and pathway associations

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

A Vinclozolin Transgenerational Granulosa DMRs

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

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

B DDT Transgenerational Granulosa DMRs

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

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

C

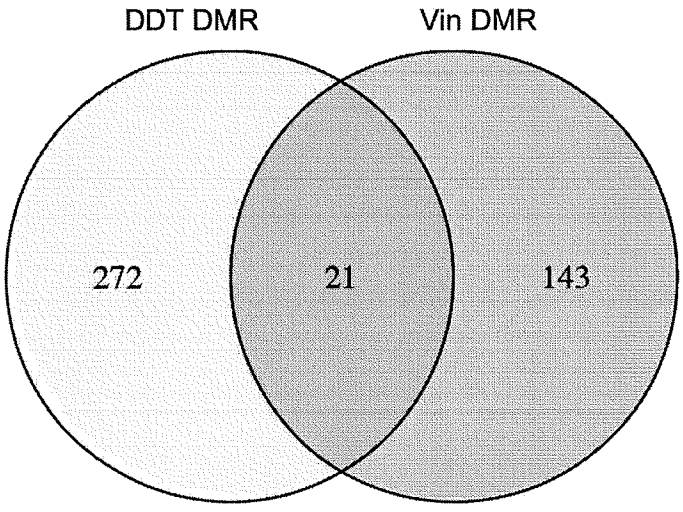

Figure 2. DMR identification. The number of DMRs found using different *P* value cutoff thresholds. The all window column shows all DMRs. The multiple window column shows the number of DMRs containing at least two significant windows. Lower table of each set shows the number of DMR having each specific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

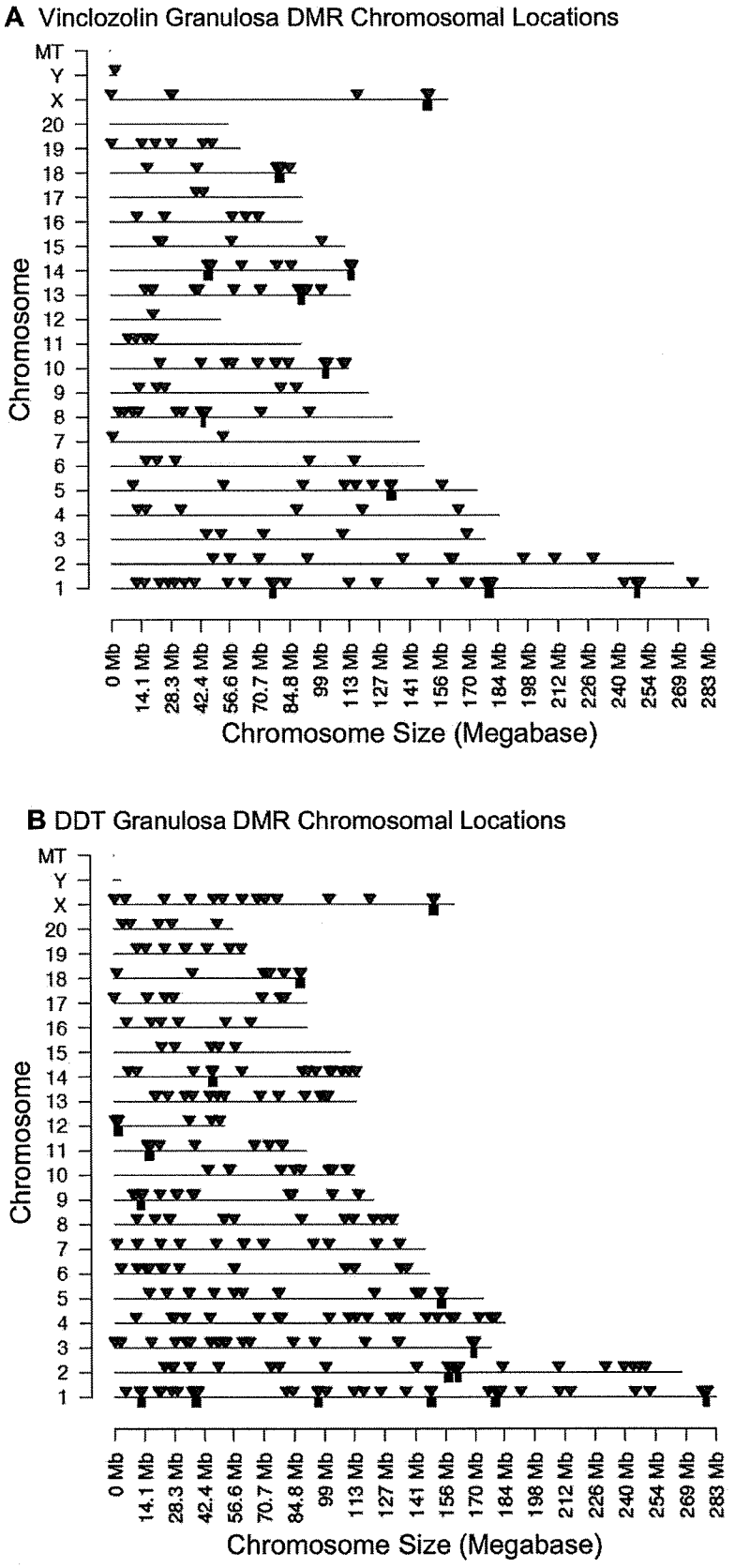

Figure 3. DMR chromosomal locations. The DMR locations on the individual chromosomes for all DMRs at a *P* value threshold of $< 1x10^{-6}$. (a) Vinclozolin F3 generation. (b) DDT F3 generation. Red arrowheads indicate positions of DMRs and black boxes indicate clusters of DMRs.

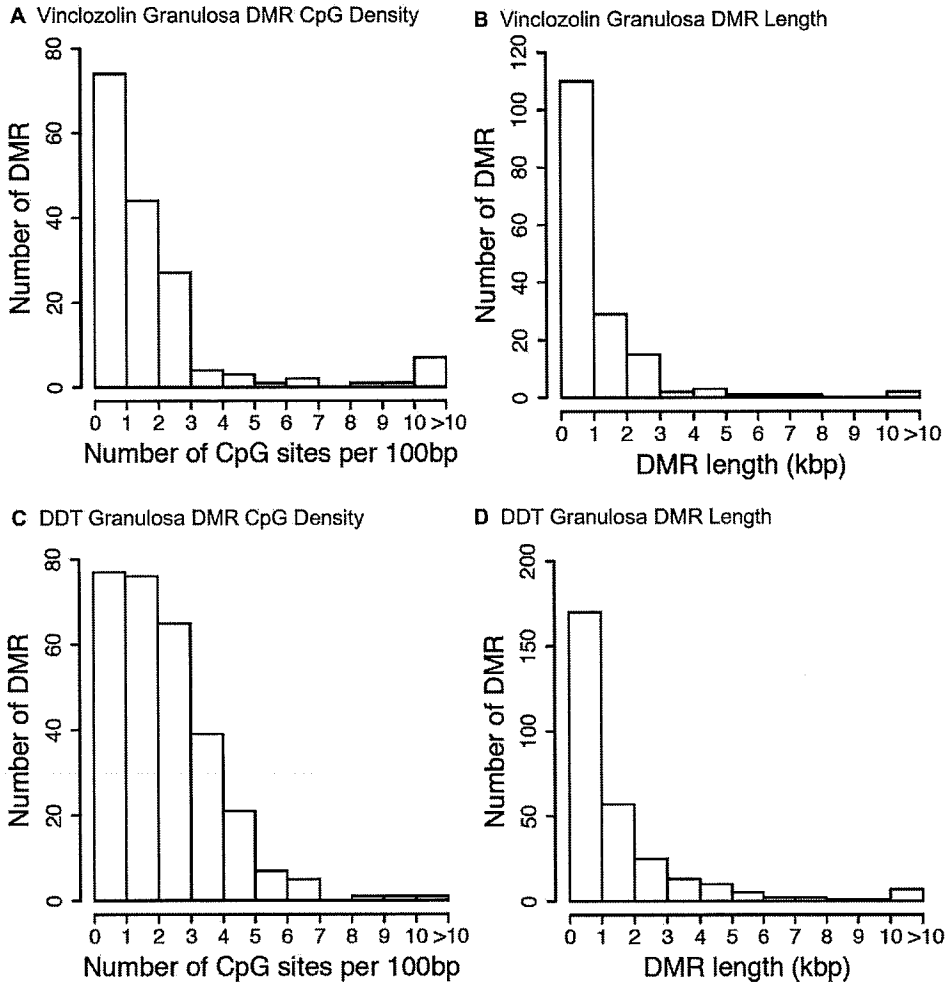

Figure 4. DMR genomic features. (a & c) The number of DMRs at different CpG densities for all DMRs at a p-value threshold of $P < 1x10^{-6}$. (b & d) The DMR lengths for all DMRs are at a P value threshold of $< 1x10^{-6}$. (a & b) Vinclozolin F3 generation. (c & d) DDT F3 generation.

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

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

A Vinclozolin Transgenerational Granulosa Differential Expressed RNA

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

B DDT Transgenerational Granulosa Differential Expressed RNA

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

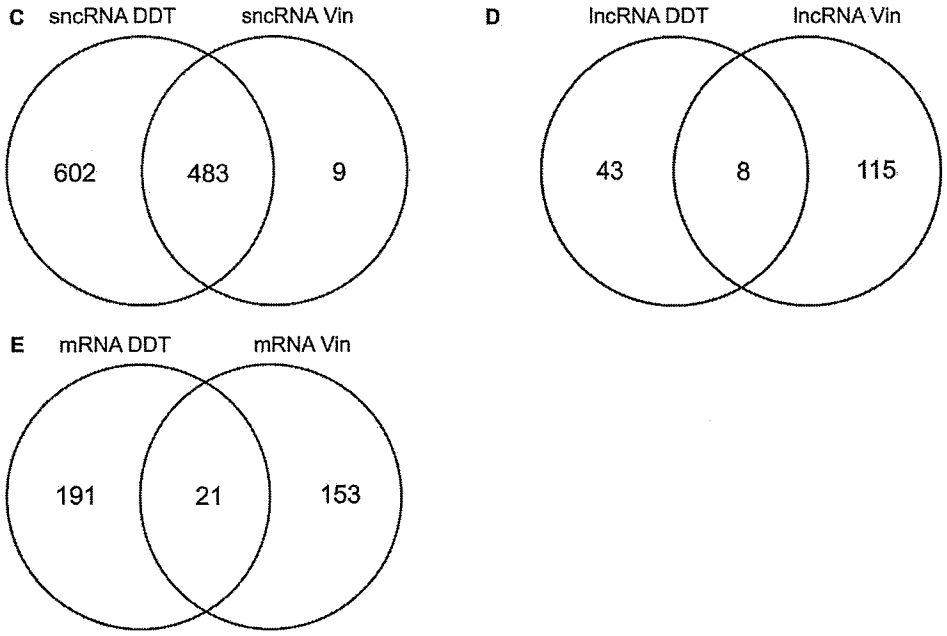

Figure 5. Differential RNA expression at different p-value thresholds for vinclozolin (a) and DDT (b). *P* value < $1x10^{-4}$ was used for subsequent analysis. Venn diagrams show overlap of RNA categories between the two lineages for (c) sncRNA, (d) lncRNA, and (e) mRNA.

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

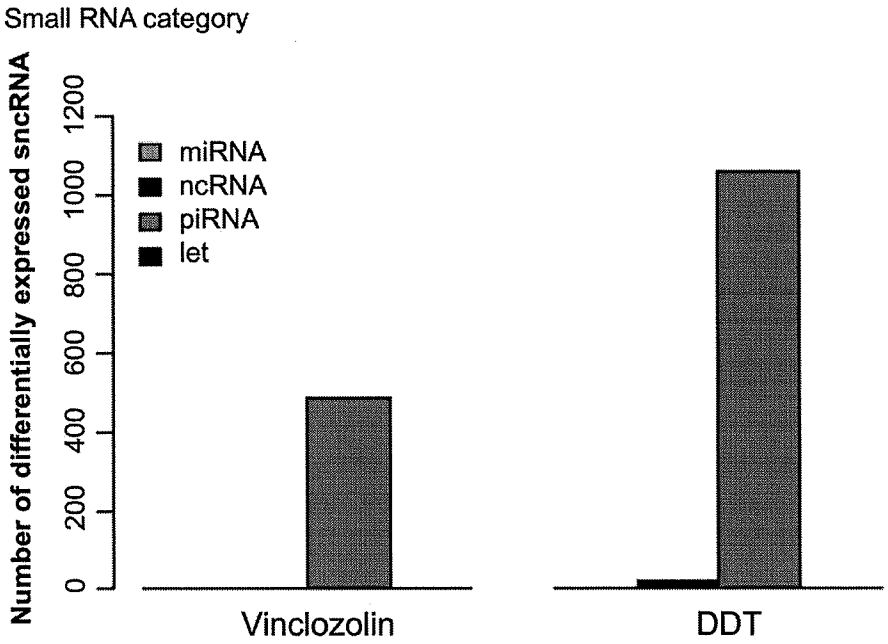

Figure 6. Differentially expressed small, noncoding RNA broken down by class and lineage. *P* value < 1 x 10^{-4} was used for analysis.

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

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

Discussion

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

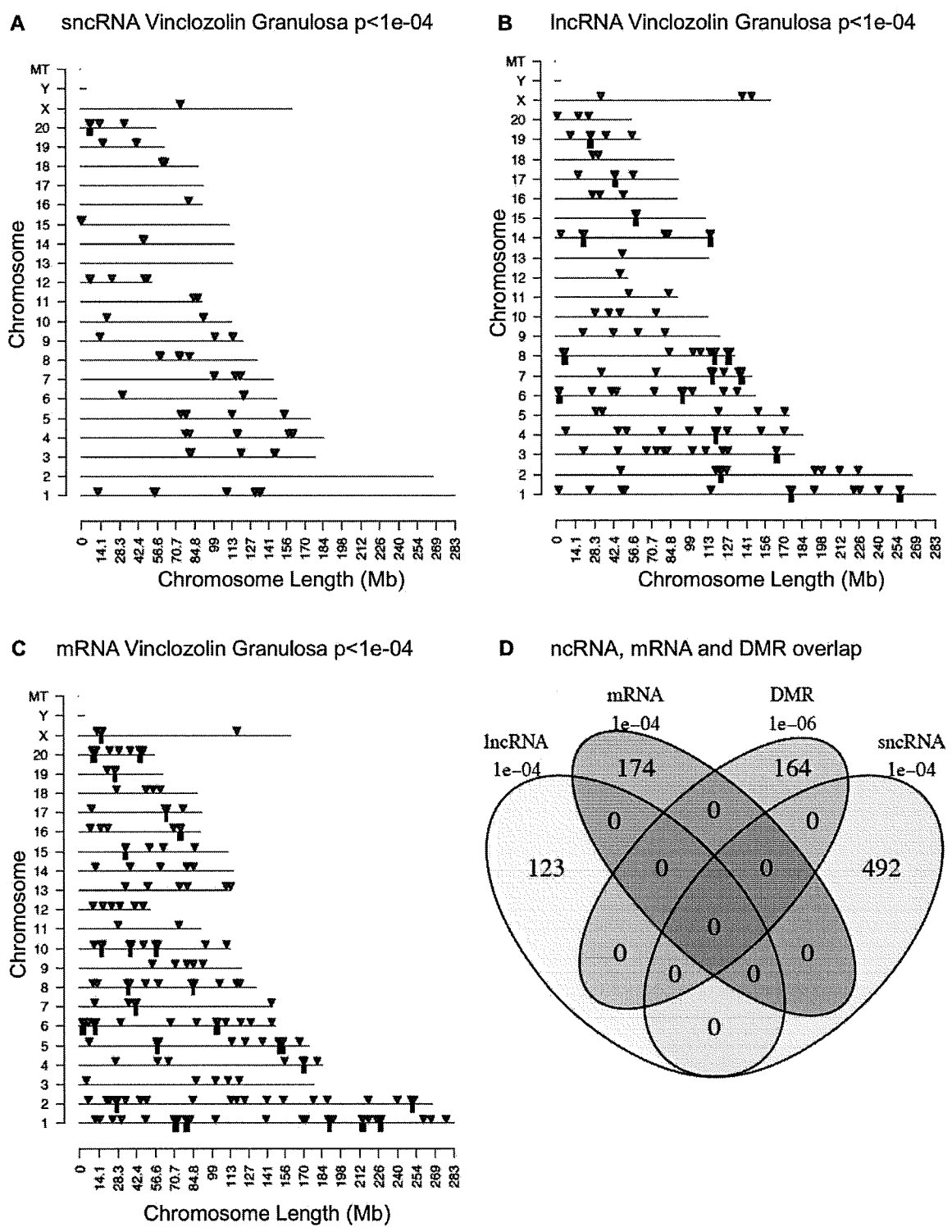

Figure 7. Differentially expressed RNAs from the vinclozolin lineage. Chromosomal locations of differentially expressed sncRNA (a), lncRNA (b), or mRNA (c). Individual RNAs are shown as red arrows and clusters are shown as black boxes. RNAs with unknown locations are not shown. (d) Venn diagram showing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

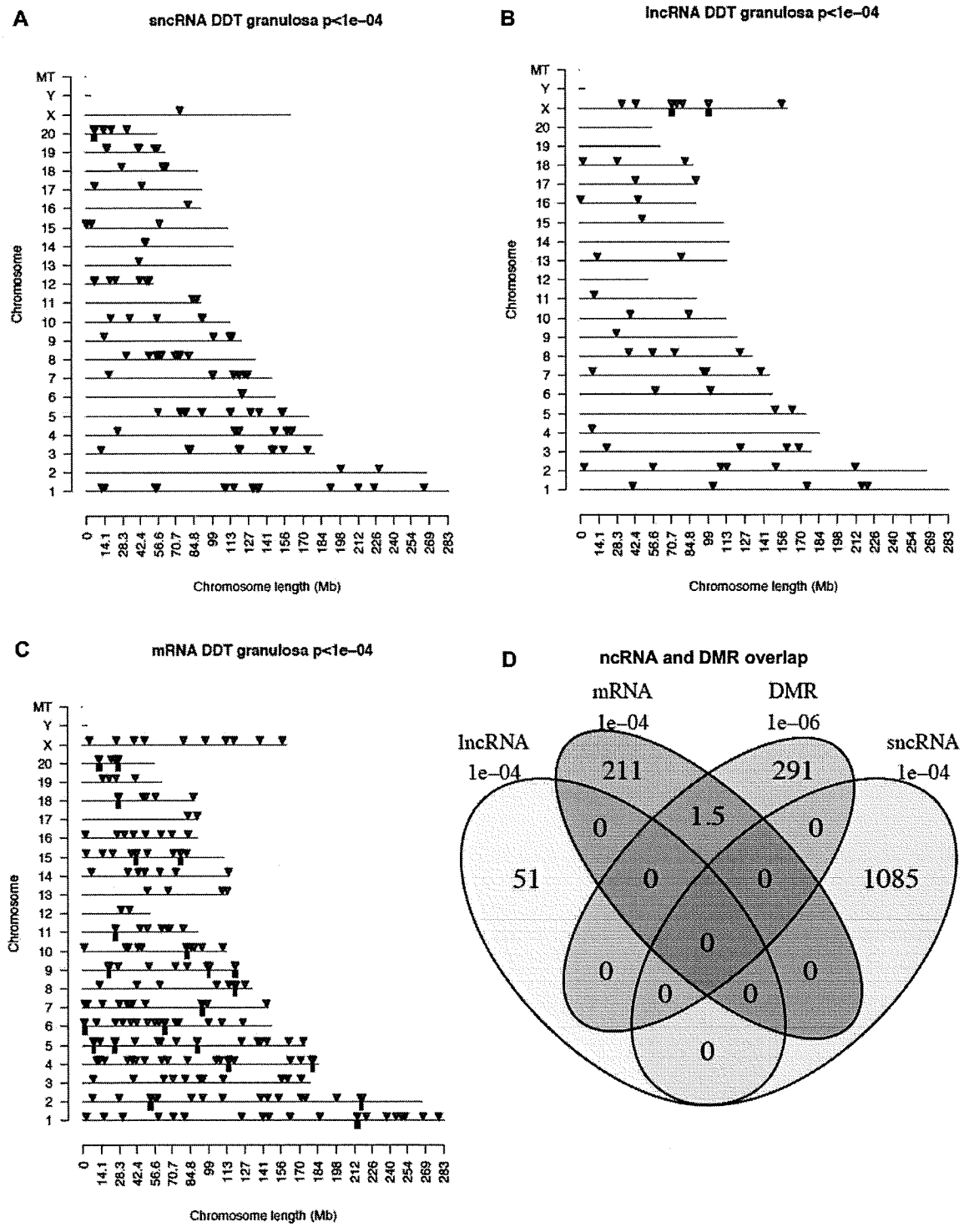

Figure 8. Differentially expressed RNAs from the DDT lineage. Chromosomal locations of differentially expressed sncRNA (a), lncRNA (b), or mRNA (c). Individual RNAs are shown as red arrows and clusters are shown as black boxes. RNAs with unknown locations are not shown. (d) Venn diagram showing overlap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

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

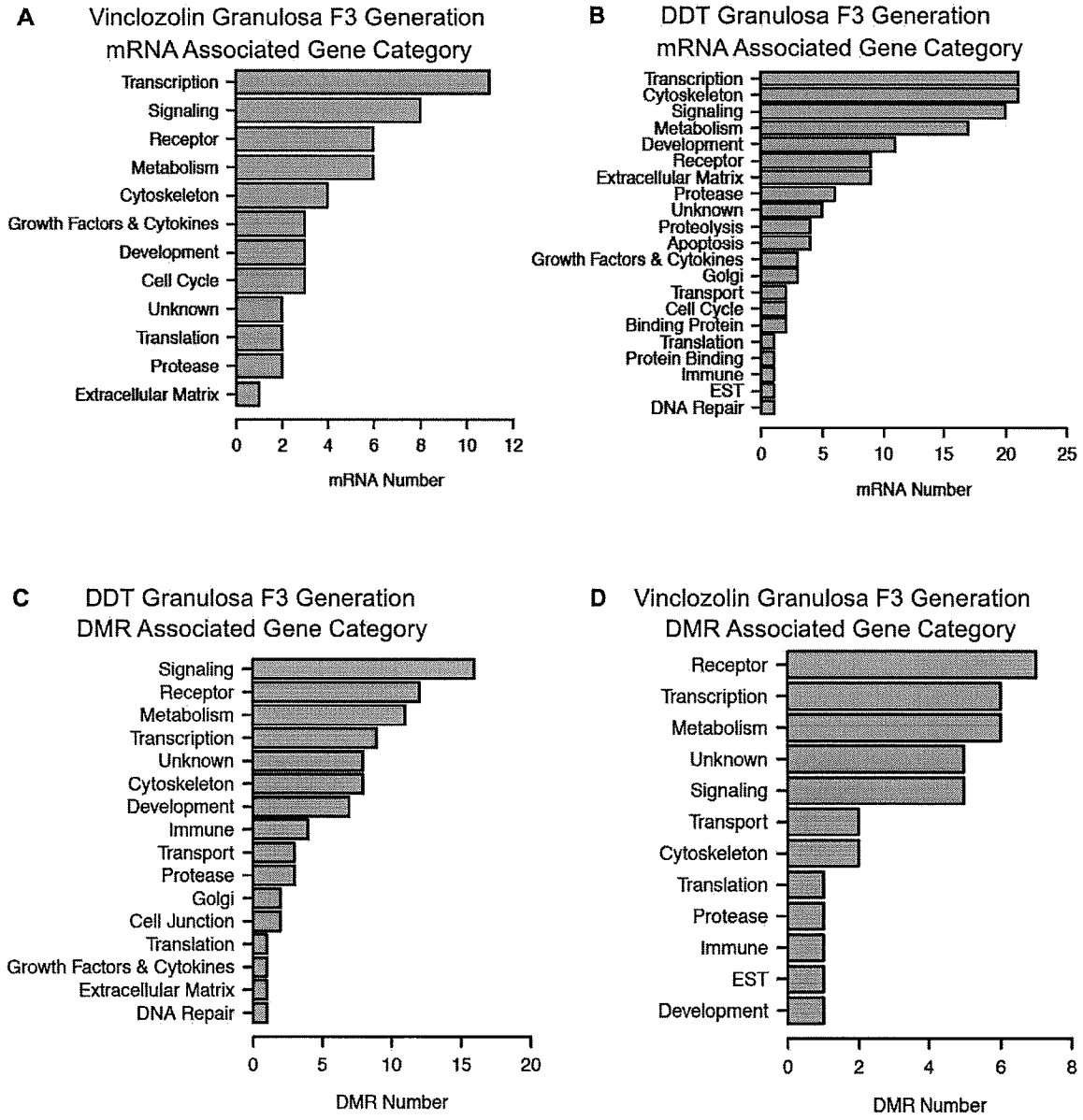

Figure 9. Differentially expressed epigenetic modifications were broken down by predicted associated gene category. Top row: mRNAs from vinclozolin (a) and DDT (b) lineages. Bottom row: DMRs from DDT (c) and vinclozolin (d). Genes or DMRs that could not be assigned a category are not shown.

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

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

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

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

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

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

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

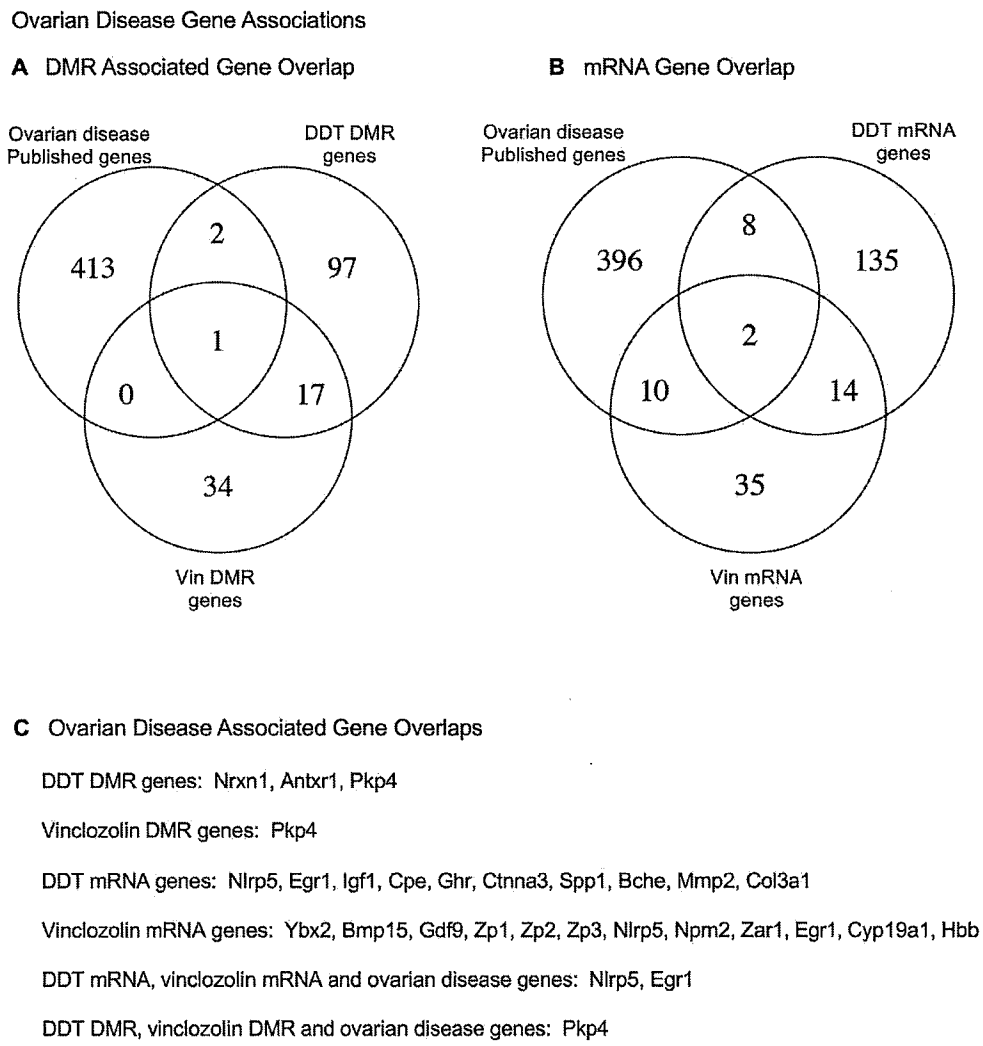

Figure 11. Ovarian disease gene associations. (a) DMR associated gene overlap with published ovarian disease genes. (b) mRNA gene overlaps with published ovarian disease genes. (c) Specific ovarian disease associated gene overlap with DMR associated genes and mRNA genes.

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

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

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

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

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

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

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

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

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

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

Methods

Animal studies and breeding

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

Histopathology and ovarian disease classification

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

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

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

Granulosa cell isolation

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

DNA isolation

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

Methylated DNA Immunoprecipitation (MeDIP)

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

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

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

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

MeDIP-seq analysis

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

RNA isolation and sequencing

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

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

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

DMR statistics and bioinformatics

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

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

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

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

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Disclosure statement

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Feature Review

Epigenetic Transgenerational Inheritance of Obesity Susceptibility

Stephanie E. King[1] and Michael K. Skinner[1,*]

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

Highlights

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

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

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

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

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

Origins of the Obesity Epidemic

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

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

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

Epigenetics and the Developmental Origins of Health and Disease

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

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

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

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

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

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

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

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

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

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

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

Glossary

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

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

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

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

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

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

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

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

Epigenetic Developmental Reprogramming

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

Epigenetic Transgenerational Inheritance Mechanisms

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

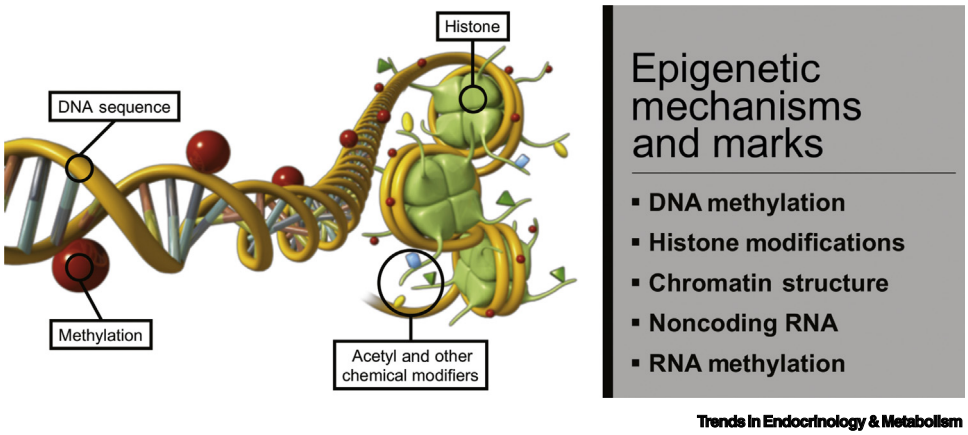

Figure 1. Epigenetic Processes and Marks. Modified from [149].

Box 3. History and Mechanisms of Epigenetics and Epigenetic Inheritance

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

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

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

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

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

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

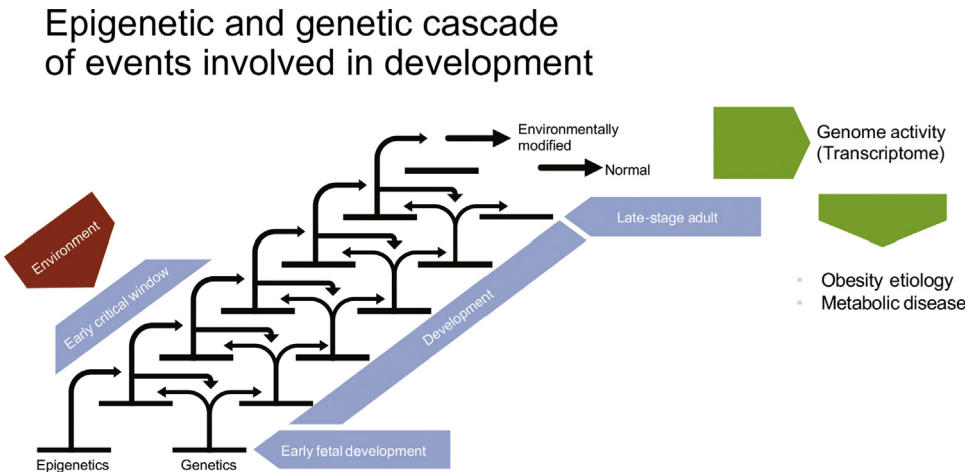

Figure 2. Epigenetic and Genetic Cascade of Events Involved in Development. Development and cellular differentiation are dependent on the impacts of a cascade of genetic and epigenetic changes. Early life environmental exposures have an increased impact on the transcriptome and physiology of an organism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

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

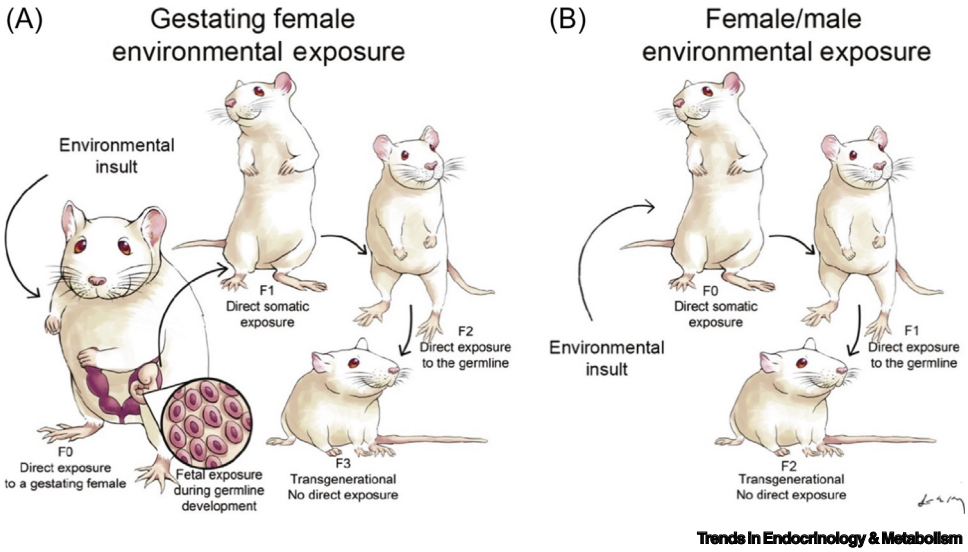

Figure 3. Exposure Mechanisms of Environmentally Induced Epigenetic Transgenerational Inheritance. (A) The exposure of an F0 generation gestating female to an environmental insult also exposes the developing F1 generation embryo. In addition, alterations of the epigenome in the developing germ cells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

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

Evidence for Epigenetic Transgenerational Inheritance

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

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

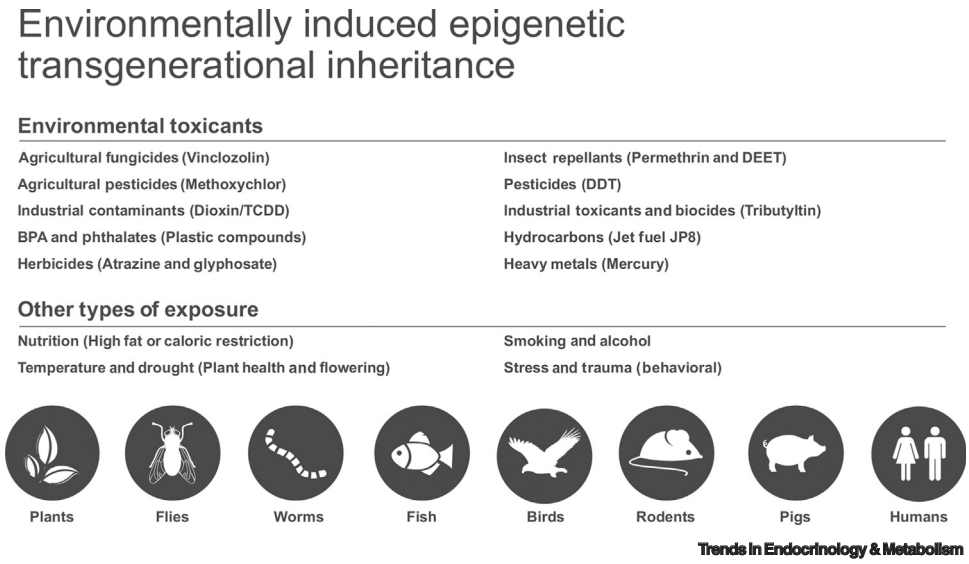

Figure 4. Environmentally Induced Epigenetic Transgenerational Inheritance. The potential for epigenetic transgenerational inheritance is induced by various environmental insults, including environmental toxicants. This phenomenon has been observed in a variety of organisms, including plants and animals.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

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

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

Table 1. Environmental Insults That Can Induce Epigenetic Transgenerational Inheritance

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

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

Epigenetic Transgenerational Inheritance of Susceptibility for Obesity and Metabolic Dysfunction

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

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

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

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

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

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

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

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

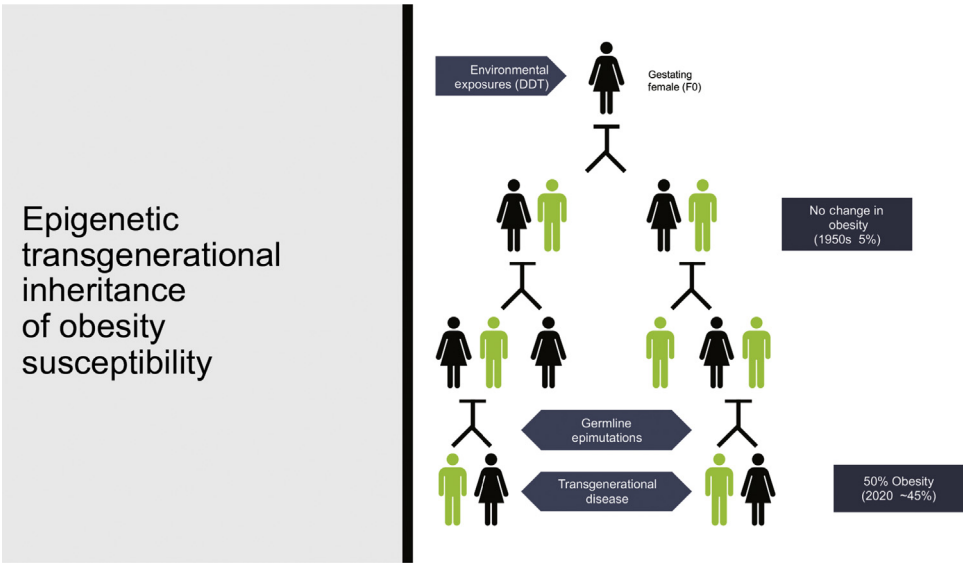

Figure 5. Environmentally [e.g., Dichlorodiphenyltrichloroethane (DDT)]-Induced Epigenetic Transgenerational Inheritance of Obesity. Obesity susceptibility epigenetically inherited from ancestral exposures. During the 1950s, the entire North American population was exposed to high levels of the pesticide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

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

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

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

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

Concluding Remarks and Future Directions

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

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

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

Outstanding Questions

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

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

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

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

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

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Resources

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

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

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

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

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