Spring 2018 – Systems Biology of Reproduction
Discussion Outline – Epigenetics and Transgenerational Reproductive Disease
Michael K. Skinner – Biol 475/575
CUE 418, 10:35-11:50 am, Tuesday & Thursday
March 8, 2018
Week 9

Epigenetics and Transgenerational Reproductive Disease

Primary Papers:

1. Manikkam, et al. (2012) PLoS ONE 7:e31901
2. Guerrero-Bosagna, et al. (2013) PLoS ONE 8:e59922
3. Nilsson, et al. (2012) PLoS ONE 7:e36129

Discussion

Student 26: Reference 1 above

- What transgenerational phenotypes were observed?
- What is the first transgeneration generation?
- What is the impact of the observations?

Student 27: Reference 2 above

- What are the technologies used and objectives?
- What was the Sertoli cell effects observed?
- What basic information on testis disease was obtained?

Student 28: Reference 3 above

- What is the experimental and systems approach?
- What was the granulosa cell effects observed?
- What gene networks and insights were identified for ovarian disease?

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Transgenerational Actions of Environmental Compounds on Reproductive Disease and Identification of Epigenetic Biomarkers of Ancestral Exposures

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Abstract

Environmental factors during fetal development can induce a permanent epigenetic change in the germ line (sperm) that then transmits epigenetic transgenerational inheritance of adult-onset disease in the absence of any subsequent exposure. The epigenetic transgenerational actions of various environmental compounds and relevant mixtures were investigated with the use of a pesticide mixture (permethrin and insect repellant DEET), a plastic mixture (bisphenol A and phthalates), dioxin (TCDD) and a hydrocarbon mixture (jet fuel, JP8). After transient exposure of F0 gestating female rats during the period of embryonic gonadal sex determination, the subsequent F1–F3 generations were obtained in the absence of any environmental exposure. The effects on the F1, F2 and F3 generations pubertal onset and gonadal function were assessed. The plastics, dioxin and jet fuel were found to promote early-onset female puberty transgenerationally (F3 generation). Spermatogenic cell apoptosis was affected transgenerationally. Ovarian primordial follicle pool size was significantly decreased with all treatments transgenerationally. Differential DNA methylation of the F3 generation sperm promoter epigenome was examined. Differential DNA methylation regions (DMR) were identified in the sperm of all exposure lineage males and found to be consistent within a specific exposure lineage, but different between the exposures. Several genomic features of the DMR, such as low density CpG content, were identified. Exposure-specific epigenetic biomarkers were identified that may allow for the assessment of ancestral environmental exposures associated with adult onset disease.

Citation: Manikkam M, Guerrero-Bosagna C, Tracey R, Haque MM, Skinner MK (2012) Transgenerational Actions of Environmental Compounds on Reproductive Disease and Identification of Epigenetic Biomarkers of Ancestral Exposures. PLoS ONE 7(2): e31901. doi:10.1371/journal.pone.0031901

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Introduction

Epigenetic transgenerational inheritance provides an alternative molecular mechanism for germ line transmission of environmentally induced phenotypic change compared to that of classic genetics [1,2]. Most factors do not have the ability to modify DNA sequence, but environmental factors such as nutrition or various toxicants can influence epigenetic processes to mediate alterations in genome activity [1,3]. Environmental epigenetics focuses on how a cell or organism responds to environmental factors or insults to create altered phenotypes or disease. Previous observations have demonstrated that the exposure of a gestating female to the environmental fungicide compound vinclozolin [4] during fetal gonadal sex determination promotes a reprogramming of the male germ line epigenome [2]. The altered DNA methylation profile in the sperm becomes permanently reprogrammed to create an abnormal epigenome in the embryo and all cells and tissues derived from that embryo [5]. Later in life the animals develop adult onset disease states such as mammary tumors, prostate disease, kidney disease, testis abnormalities, and immune abnormalities at high (20–50%) frequencies [6]. Due to the imprinted-like nature of the altered epigenetic DNA methylation sites, the germ line (sperm) transmit this epigenome and adult onset disease phenotype to subsequent generations, which is termed epigenetic transgenerational inheritance [1]. The basic mechanism involves the ability of an environmental factor (compound) to alter the germ line DNA methylation program to promote imprinted-like sites that then transmit an altered epigenome that subsequently promotes adult onset disease phenotypes transgenerationally [1,2]. The vast majority of environmental exposures act on somatic cells at critical windows of development to influence phenotype and/or disease in the individual exposed, but this will not become transgenerational [1,3]. In the event the critical window for the primordial germ cell is affected by environmental exposure, the altered germ line has the ability to promote a transgenerational phenotype for subsequent generations [1]. More recently a number of reports have documented the ability of nutritional factors [7] and environmental toxicants such as bisphenol A (BPA), dioxin, vinclozolin and methoxychlor to promote epigenetic transgenerational inheritance [2,8,9,10].

The current study was designed to investigate the potential epigenetic transgenerational actions of a variety of different toxicants or mixtures of relevant compounds. This was initiated to determine the compound specificity to promote epigenetic transgenerational inheritance and to determine if the epigenetic alterations may provide biomarkers for exposure. The environ-

mental compounds (toxicants) selected have been shown to have biological and health effects [11], and were identified as common suspected exposures of military personnel. In addition, the cellular signal transduction process affected for each exposure is unique. The first compound mixture is termed "plastics" and includes bisphenol A (BPA) and the phthalates DEHP (bis(2-ethylhexyl)phthalate) and DBP (dibutyl phthalate) which are the combined exposures from most plastics that have been shown to promote *in vitro* and *in vivo* toxicologic effects [12]. Epigenetic effects of these compounds after neonatal exposures promotes adult onset disease [13,14]. The second mixture involves the most commonly used human pesticide (permethrin) and insect repellent N,N-Diethyl-meta-toluamide (DEET), and is termed "pesticide" for this study, and has been shown to have some toxicologic effects in either *in vitro* or *in vivo* studies [15,16,17,18,19,20]. The third compound used is dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin, TCDD), which has been shown to have significant *in vitro* and *in vivo* effects in the promotion of cellular abnormalities and adult onset disease states [21]. Epigenetic parameters have been shown to be influenced by dioxin actions [22]. The fourth exposure is jet fuel (jet propellant 8, JP8) which is a "hydrocarbon" mixture that is a significant environmental exposure due to its use for dust control on road surfaces [23]. Toxicological effects have been shown in *in vitro* and *in vivo* studies with JP8 exposures [24]. The four exposures used are common environmental toxicants which have been generally shown to promote abnormal or disease phenotypes. The objective of this study was to determine the potential ability for these different compounds and mixtures to promote epigenetic transgenerational inheritance of disease and map the potential alterations in the sperm epigenome.

The potential transgenerational diseases investigated focused on pubertal onset parameters and gonadal functions associated with infertility. The incidence of altered pubertal onset has increased over the past several decades in human populations [25,26,27]. Pubertal onset can occur several years early in some women [28]. This early onset of female puberty has been shown to affect brain development, endocrine organ systems and growth, that all potentially increase disease susceptibility later in life [29]. Although environmental exposures to endocrine disrupting compounds have been suggested as a causal factor [28], the basic mechanisms involved are unknown. The other disease parameters examined were associated with testis and ovary functions that influence fertility. In regards to testis function, sperm numbers and motility were examined, as well as spermatogenic cell apoptosis. In the human male population there has been a gradual decline in sperm number in most populations [30]. Estimates of male infertility appears to be over 10% in many human male populations [31]. In regards to ovarian function, the ovarian reserve or primordial follicle pool was assessed. An increasing percentage of the female population is developing premature ovarian failure associated with a loss of the follicle pool which promotes female infertility and affects approximately 15% of many female populations [32]. The causal factors for these gonadal disease phenotypes and increase in infertility are suggested to be due in part to environmental exposures to endocrine disruptor toxicants [33], but the basic molecular mechanisms involved are not known. The potential that the exposures used in the current study may promote the epigenetic transgenerational inheritance of these disease states is investigated.

Results

The current study was designed to investigate the potential ability of various environmental compounds and mixtures to promote epigenetic transgenerational disease with a focus on pubertal and gonadal abnormalities. The alterations in the sperm epigenome were investigated to determine the similarities and differences between the different exposures on differential DNA methylation. The experimental design used pharmacologic doses, Table S1A, based on approximately 1% of the lethal oral dose 50% (LD50) for most of the compounds that previously have been shown *in vivo* to not cause direct toxicological effects. Gestating female outbred Harlan Sprague Dawley rats were given intraperitoneal (IP) injections daily between embryonic days 8–14 of fetal development correlating with gonadal sex determination. No consistent effects were observed on litter size, sex ratios or weaning weights, Figures S1 and S2. The number of litters and male and female animals obtained for each generation is presented in Table S1B and S1C. The F0 generation gestating female was the only animal injected IP. The F1 generation animals at 90 days of age were mated to the same lineage to generate the F2 generation and the F2 generation were mated to generate the F3 generation progeny as previously described [2]. No sibling or cousin breedings were used to avoid any inbreeding artifacts. No major overt toxicity was observed in F1, F2 or F3 generations, Figure S1 and S2. The only treatment that promotes some toxicity in the F1 generation was the high dose plastics, Table S1A, so a lower dose at 50% that shown in Table S1A was also used and termed "Low Dose Plastic" that had no toxicological effects, Figure S1. Anogenital distance was measured as an indicator of exposure to androgenic compounds that promote masculinization during the perinatal period [34,35]. Analysis of anogenital index (AGI) demonstrated some effects of the treatments on the F2 and F3 generation animals, but no effects at the F1 generation animals, Figure S3. These actions on the AGI in the F2 generation are possibly due to the direct exposure of fetal germ cells to the endocrine disruptor activities of several of the exposure compounds (e.g. BPA, DEHP, DBP) [12,13,14], while the increased AGI in the F3 generation appears to be transgenerational. Therefore, classic endocrine disruptor actions [36] are likely not involved in the F2 and F3 generation, but only in the F1 generation. In considering the actions of environmental exposure the direct versus indirect (e.g. epigenetic) actions are critical. The exposure of the F0 generation gestating female directly affects the F0 generation female, the F1 generation embryo and the germ line inside the F1 embryo that will be generating the F2 generation animal [1]. Therefore, phenotypes in the F0, F1 and F2 generations may be due to direct exposures and are not transgenerational effects or phenotypes observed by definition. A transgenerational phenotype or phenomenon requires the lack of direct exposure to promote a generational effect [1,3]. The actions on F0, F1 and F2 are due to a direct multi-generational exposure and only the F3 generation phenotype can be considered a transgenerational effect. Since the mechanisms promoting the F1 or F3 generation effects differ, the phenotypes can be distinct between the generations.

Puberty is a developmental process involving the hypothalamic – pituitary – gonadal axis which initiates during fetal development and matures in adolescence [25]. The onset of puberty was investigated with the different exposure lineages of control (DMSO vehicle), pesticide, low and high dose plastics, dioxin, or hydrocarbons in the F1–F3 generation rats. The analysis was initiated for females at postnatal day 30 and males at postnatal day 35 until puberty (vaginal opening or balano-preputial separation) [37]. In the F1 generation plastics promoted delayed female pubertal onset, while in the F2 generation plastics, dioxin and jet fuel promoted early onset of puberty for females, with plastics and dioxin promoting early onset of puberty in males, Figure S4. In the

transgenerational F3 generation it was demonstrated that plastics, low dose plastics, dioxin and jet fuel promote early onset of puberty in females, while having no effect on males, Figure 1A, 1B. Therefore, several of the exposures were found to promote early onset of puberty in females transgenerationally.

Gonadal function for both testis and ovary were investigated in the F3 generation at postnatal 120 days of age. Previously vinclozolin was shown to promote a transgenerational phenotype of spermatogenic cell apoptosis [2], so potential germ cell apoptosis in the testis was investigated. The jet fuel exposure was found to transgenerationally increase spermatogenetic cell apoptosis in the F3 generation male testis, Figure 1C. Epididymal sperm concentration and motility for the F3 generation were also investigated and did not provide consistent alterations transgenerationally, as previously seen with vinclozolin exposure. The F3 generation ovaries were examined for total follicle number and the individual types of primordial follicles, primary follicles and developing follicles were categorized, Figure 1D and 1E. All the exposures were found to promote a transgenerational effect on the F3 generation ovary with a significant reduction in total follicle number, Figure 1D, and the follicle class primarily affected was the primordial follicle, Figure 1E. Therefore, all the exposures promoted the transgenerational phenotype of a reduction in the primordial follicle pool size. The large developing antral follicles were counted to determine potential effects on later stage follicle development and no differences were observed between the exposures when compared to control, Figure 1F. The transgenerational action of the various exposures on the ovary was a reduction in the primordial follicle pool size. This may promote premature ovarian failure as the animals age. The testis and ovary are hormone regulated and both produce endocrine steroids. Hormone levels were analyzed to determine how the endocrine system was responding transgenerationally. The F3 generation males had a reduction in testosterone levels in the plastics, dioxin and jet fuel exposure lineages, Figure S5A, while the females had no change in progesterone levels, Figure S5B. No change in luteinizing (LH) hormone levels was detected in either male or female F3 generation animals, Figure S5C & D. Therefore, the endocrine system was altered transgenerationally in the males.

The mechanism involved in these transgenerational phenotypes is the reprogramming of the germ line (sperm) during male sex determination [1,3]. This altered sperm epigenome appears to be permanently reprogrammed and escapes the DNA methylation programming at fertilization to allow transgenerational transmission of the altered sperm epigenome, that then promotes all tissues developed from that sperm to have altered cell and tissue transcriptomes that can promote transgenerational disease [1]. Previously, vinclozolin was shown to promote a transgenerational (F3 generation) alteration in DNA methylation [2,5] and a transgenerational transcriptome alteration in tissues like the testis [38]. The F3 generation rat sperm from the control and all exposure groups were collected for genome wide promoter DNA methylation analysis [5]. The procedure involved the use of an antibody to methylcytosine to immunoprecipitate methylated DNA (MeDIP) and then a competitive hybridization tiling array (Chip) for a MeDIP-Chip analysis [5]. Differentially methylated sites were identified for all the different exposure lineages in the F3 generation sperm when compared with vehicle control F3 generation sperm. The complete lists of differential methylation regions (DMR) for each exposure in the F3 generation sperm are provided in Table S2(A–D). The overlap of the DMR sets for each exposure is shown in a Venn diagram in Figure 2A. The number of DMR for hydrocarbons (jet fuel) was 33, dioxin 50, plastics (BPA, DEHP, DBP) 197 and pesticide (permethrin and DEET) 363 with a statistically significant difference ($p<10^{-5}$). Interestingly, the majority of each DMR set was specific to an exposure group and not common with the other exposure DMRs. The only exception was an overlap between plastics and pesticide of 113 DMRs, Figure 2A. Therefore, each exposure had a unique signature of epigenetic alterations in the F3 generation sperm. The chromosomal localizations of these sites are shown in Figure 2B. The DMRs are seen on all autosomes and the X chromosome. Clustering analysis of the DMRs when over represented in specific chromosomal locations identified 35 different clusters (2–5 megabase each) of DMR between the exposures that with z-score analysis have a statistically significant difference ($p<0.05$), Figure 2B. These DMR clusters may represent ''epigenetic control regions'' where different exposure DMRs may commonly regulate genome activity. The functional significance of these DMR clusters remains to be elucidated and are identified for individual DMR in Table S2. In considering the combined DMR and associated gene promoters for all exposures, the potential cellular signaling processes affected demonstrated similar pathways are predominant, as shown in Table S3. A gene network analysis for direct connections within the total gene set associated with the DMR is shown in Figure 3 and demonstrates extracellular, membrane, cytoplasmic and nuclear associated genes are all associated with the DMR identified. Common cellular signaling pathways and processes appear to be involved from the gene network identified. Therefore, common cellular pathways and gene networks may be influenced by the different exposures and transgenerational sperm epigenomes. Although exposure specific transgenerational differential DNA methylation regions (DMR) are predominant, the common cellular processes and gene networks effected may explain the similar disease phenotypes observed.

The identification of epigenetic alterations in specific regions of the F3 generation sperm support a role for epigenetic transgenerational inheritance of the disease phenotypes observed. Several of the top exposure specific DMRs for each exposure with the highest statistical significance were selected for confirmation with quantitative PCR of the MeDIP samples. A list of the confirmed exposure specific signatures are presented in Figure 4. In addition, several of the top overlapped (common) DMR were also selected and shown. The MeDIP qPCR analysis demonstrated both increases and decreases for the exposure specific and common DMR, Figure 4B. These exposure specific DMR are considered potential epigenetic biomarkers for exposure and the transmission of transgenerational phenotypes. Further analysis of the epigenetic sites identified considered two genomic features associated with the DMRs. The first one was a DNA sequence motif termed ''Environmentally Induced DNA Methylation Region 1'' (EDM1) that was previously identified and shown to be associated with a high percentage of the vinclozolin induced sperm DMRs [5]. This motif may not be at the specific altered DNA methylation site, but is within the 400–500 bp region. A DNA sequence motif such as EDM1 may promote a region of sensitivity for these DMR's to be programmed transgenerationally. The potential presence of this EDM1 motif in the epigenetic sites (DMR) identified in the current study for all the exposures was determined. An evaluation of the presence of EDM1 using the MCAST online software revealed a statistically significant higher EDM1 presence in promoter regions of the jet fuel and dioxin exposure groups (74.19% and 57.63%, respectively) compared to a computer generated random set of 144 promoters (20.83%). The presence of EDM1 in the promoter regions of the plastics (20.47%) and pesticides (7.36%) was similar or below its presence in the random set of promoters. This suggests that the molecular

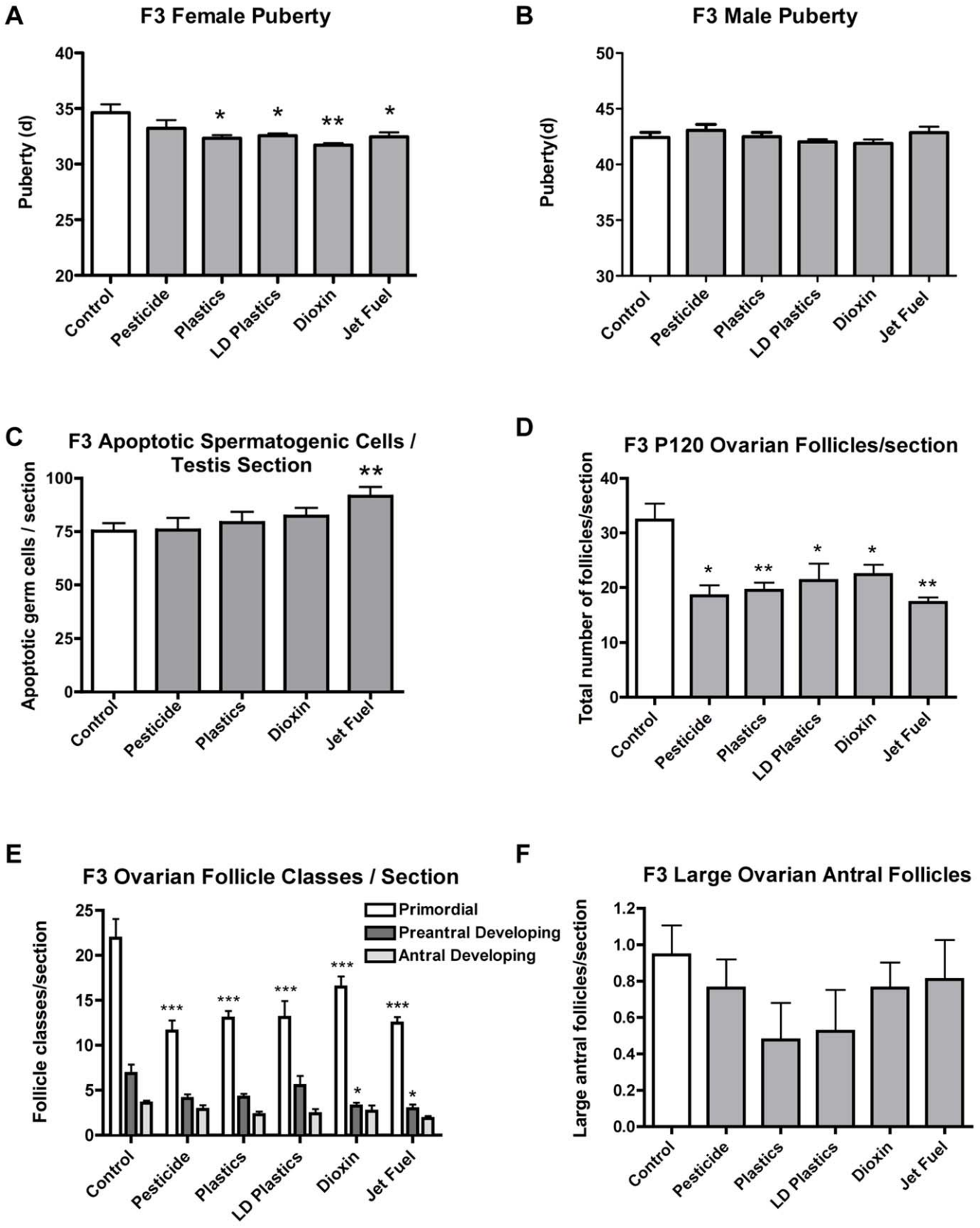

Figure 1. Ancestral (F0 generation female) exposures to environmental compounds promote transgenerational diseases, altering onset of puberty, testicular spermatogenic function and ovarian follicular development in F3 generation rat progeny. (A) Onset of female puberty was advanced from exposures to plastics, dioxin and jet fuel. (B) Onset of male puberty was unaffected from these exposures. (C) Increased apoptotic spermatogenic cells per testis section were observed from jet fuel exposure. (D) Total numbers of ovarian follicles per section

were reduced in individuals from all exposures, (E) Total numbers of primordial follicles per section declined. (F) Total numbers of large ovarian antral follicles were unaffected. The animal n value is presented in Table S1C (*p<0.05; **p<0.01, ***p<0.001).
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mechanisms involved in the targeting of these regions to produce a transgenerational change in DNA methylation may differ among the exposure groups. Another genomic feature investigated was the CpG density within the DMR identified. The frequency of CpG number per 100 bp for the DMR demonstrates the DMR identified for all exposures have an average CpG content of 4.9 CpG/100 bp with none above 15 CpG/100 bp, Figure 5. A small CpG cluster in a CpG desert appears to be a primary feature of the transgenerational DMR identified, and not shores or islands of CpG. Therefore, specific genomic features such as low CpG density, isolated CpG clusters, and the presence of a unique DNA sequence motif may be involved in facilitating the programming of these epigenetic sites (DMR) in the male germ line.

Discussion

The current study used pharmacologic doses of all the compounds and mixtures based on approximately 1% of the oral LD50 dose for most exposures (compounds), Table S1A. The objective was to determine if these exposures have the capacity to promote epigenetic transgenerational inheritance of a disease phenotype, and not to do risk assessment of the exposures. Now that the current study has established the transgenerational actions of these compounds, risk assessment toxicological studies involving dose curves of relevant environmental doses are needed. The phenotypes observed may vary with the dose as shown with the plastics in the current study. Since the F1 generation involves direct exposure and the F3 generation is germ line mediated transgenerationally, the phenotypes can differ between the generations. In addition to considering the mode of administration and dose, the critical window of exposure to promote the epigenetic transgenerational phenotype is gonadal sex determination, which for the human is 6–18 weeks of gestation. The gestating women in the first half of pregnancy would be the population most sensitive to exposures of environmentally induced epigenetic transgenerational inheritance.

The transgenerational disease phenotype investigated focused on pubertal onset and gonadal function. It was previously observed with vinclozolin induced transgenerational adult onset rat disease [2], the majority of disease developed between 6–12 months of age [6]. Therefore, additional adult onset diseases are anticipated as the animals age, but remain to be investigated. In regards to pubertal onset the plastics, low dose plastics, dioxin and hydrocarbon (jet fuel) exposures promoted an early (precocious) pubertal onset, Figure 1, with no transgenerational effects on male pubertal onset, Figure S6. In the majority of developed countries early pubertal onset in girls has increased significantly in the past several decades [25,28]. This precocious puberty can promote behavioral, mental and endocrine physiological effects in the female and increase the incidence of adult onset disease [28]. Previous studies have suggested environmental exposures of estrogenic endocrine disruptors may be in part the causal factor for this pubertal onset condition. The current study extends this hypothesis to not only consider the direct exposures of the female, but ancestral exposures of the previous generations. The potential that early pubertal onset may in part involve epigenetic transgenerational inheritance mechanisms now needs to be considered.

In considering gonadal function and fertility both the testis and ovary were investigated. The testis was found to have an increased spermatogenic cell apoptosis in the jet fuel hydrocarbon F3 lineage males, Figure 1. Previous observations with vinclozolin also showed a transgenerational spermatogenic cell apoptosis phenotype [2]. In many regions of the world human sperm numbers have declined [30] and male infertility has increased [31]. The potential that environmentally induced epigenetic transgenerational inheritance may be a factor in these disease conditions needs to be considered. In regards to ovarian function all the environmental exposures were found to promote a decline in total follicle numbers and specifically the primordial follicle pool size, Figure 1. The primordial follicle pool size is the ovarian reserve for oocyte (egg) production throughout reproductive life [39]. The primordial follicle pool develops early in fetal (human) or early postnatal (rodent) life and then can not increase, but declines with age. Human females enter menopause when the primordial follicle pool is exhausted. A premature loss of follicles promotes infertility and is termed premature ovarian failure (POF), which is associated in part with the dramatic increase in female infertility in many parts of the world [32]. Previously it was hypothesized that POF was primarily of genetic origin, but the current study suggests environmental exposures and epigenetic transgenerational inheritance may also be a significant factor in the disease etiology to increase female infertility and premature onset of menopause. The environmental induction of the pubertal, testis and ovarian transgenerational disease phenotypes suggests that environmental epigenetics and epigenetic transgenerational inheritance will be molecular factors to consider in these and other disease etiologies.

The environmental compounds and mixtures used in the current study are all reported to be major exposures for the general population and military personnel. The ability of epigenetics to be involved in the long term and transgenerational actions of these exposures needs to be further investigated. The current study documents the distinct actions of each exposure to promote a unique sperm epigenome alteration, Figure 2. Interestingly, these environmentally induced distinct epigenetic changes in differential DNA methylation regions (DMR) provide epigenetic biomarkers for ancestral environmental exposures. Each exposure had a distinct epigenetic signature that can be used as a biomarker. Although further research on individual animal variation, alterations in DMR in different cell types, and developmental effects on DMR are needed, the current study provides the proof of concept that epigenetic biomarkers for environmental exposures exist.

In addition to the identification of these ancestral epigenetic biomarkers in sperm, genomic features were identified that provide insight into why these sites may become permanently reprogrammed. A DNA sequence motif previously identified and termed "Environmentally Induced DNA Methylation Region 1 (EDM1)" [5] was found to be associated with a high percentage of the promoter regions of the hydrocarbon and dioxin exposure groups. Similar observations were previously made in examining the vinclozolin induced DMR in transgenerational sperm [5]. Interestingly, the plastics and pesticide exposure groups DMR did not have the presence of the EDM1 motif above background random promoter levels. Therefore, distinct molecular mechanisms may be involved in promoting the sensitivity of transgenerationally programmed DMR. This may include an alternate DNA sequence motif to be elucidated, or a more stochastic mechanism to be considered. The other genomic feature identified involved

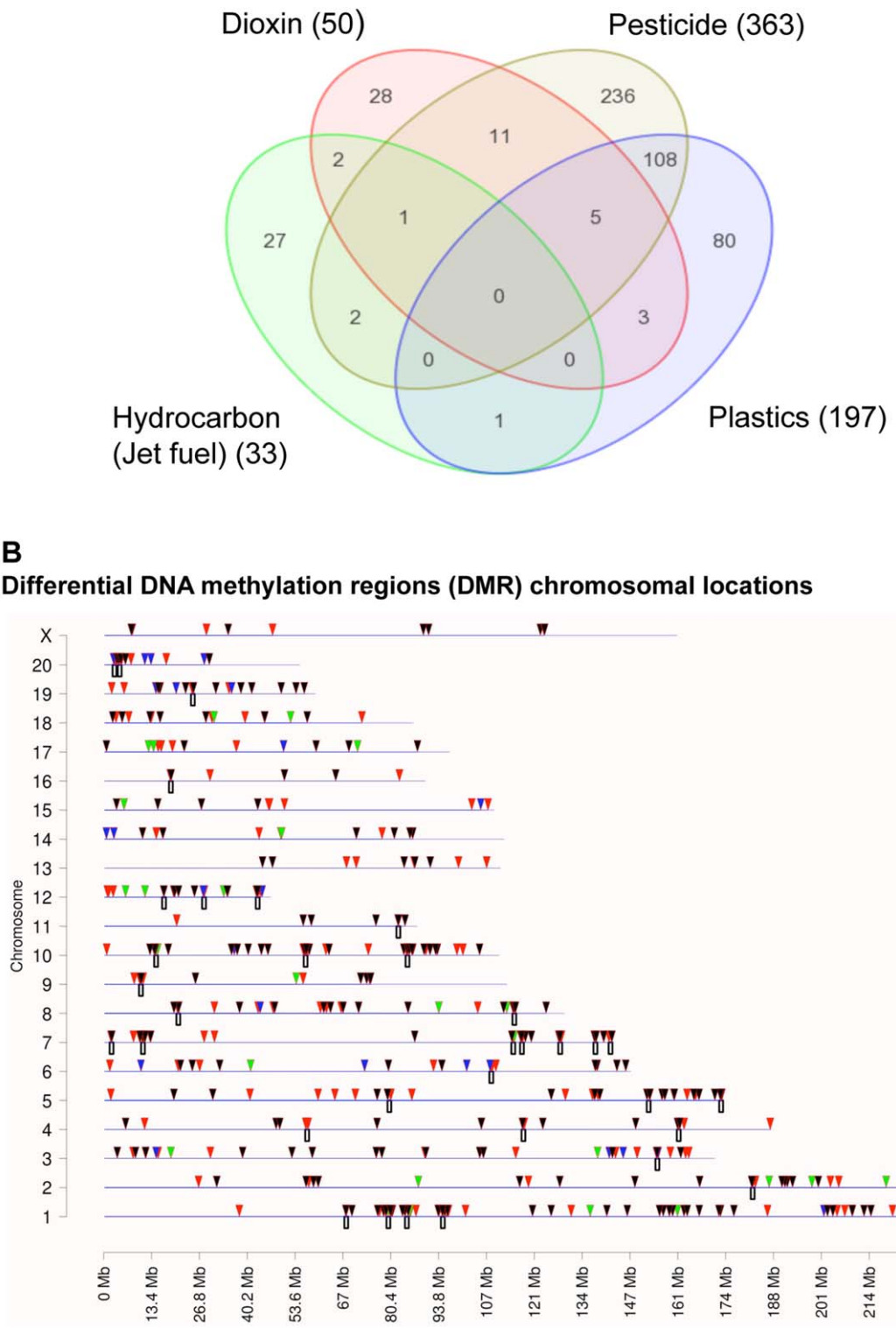

Figure 2. The transgenerational DMR associated with each exposure group identified. (A) Venn diagram of exposure DMR lists of F3 generation rat genes with differential DNA methylation due to *in vivo* exposure of F0-generation gestating female with Dioxin, Pesticide, Plastics or Hydrocarbons/Jet fuel. (B) Chromosomal location of each exposure group DMR are indicated with red arrow (plastics), green arrow (dioxin), blue arrow (hydrocarbon) and black arrow (pesticide). The chromosome number and size are indicated. The box below the line indicates DMR cluster in 2–5 megabase regions with statistical significance ($p<0.05$).
doi:10.1371/journal.pone.0031901.g002

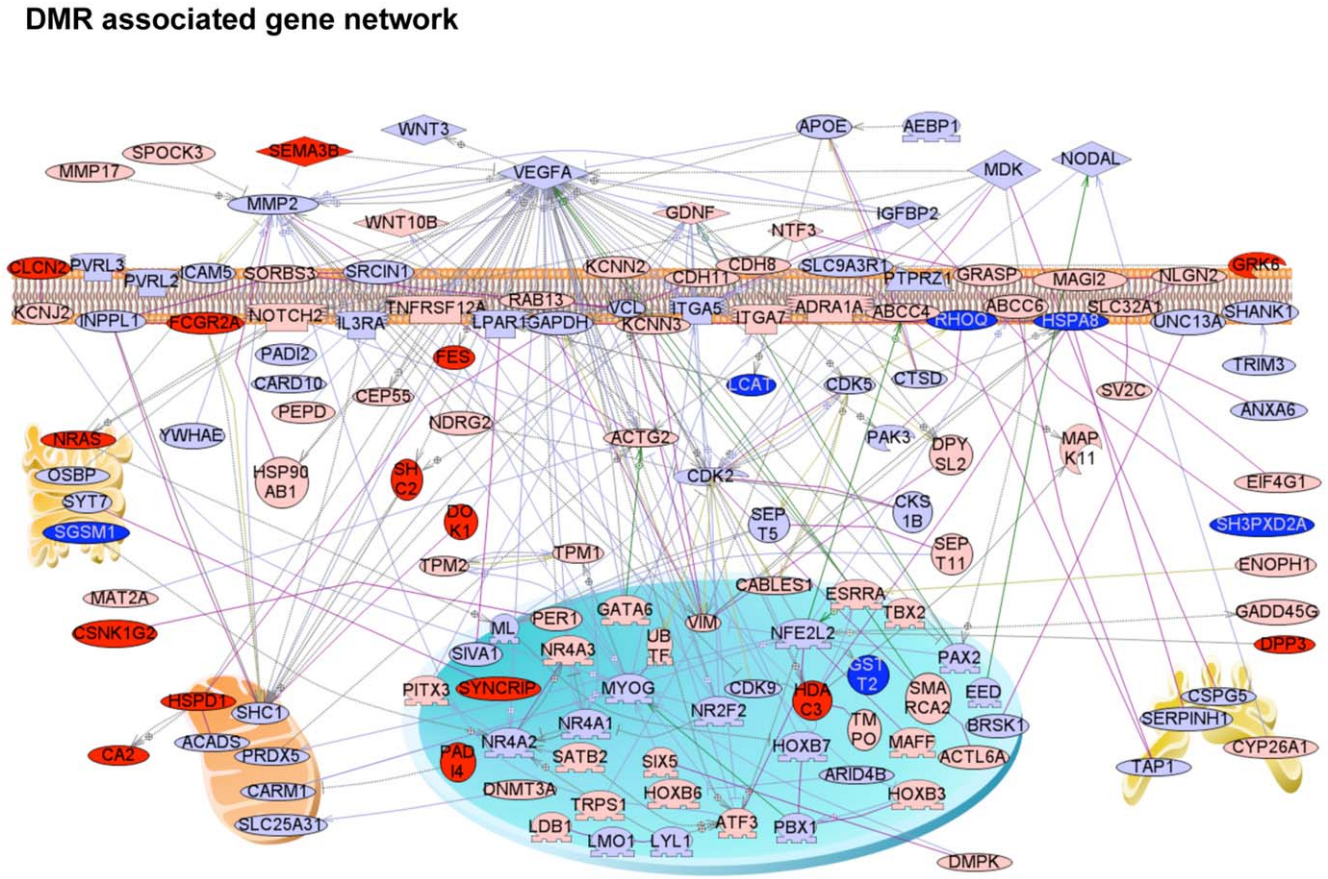

Figure 3. Direct connection gene sub-network for combined genes with transgenerational DMR associated exposures for Dioxin (red shapes), Pesticide (light blue shapes), Plastics (pink shapes) or Hydrocarbons/Jet fuel (dark blue shapes) indicated. Only 140 directly connected genes out of 499 unique genes associated with the combined lists are shown. Node shapes code: oval and circle – protein; diamond – ligand; circle/oval on tripod platform – transcription factor; ice cream cone – receptor; crescent – kinase or protein kinase; irregular polygon – phosphatase. Arrows with plus sign show positive regulation/activation, arrows with minus sign – negative regulation/inhibition; grey arrows represent regulation, lilac – expression, purple – binding, green – promoter binding, and yellow/olive – protein modification.
doi:10.1371/journal.pone.0031901.g003

the CpG content or density associated with all the DMRs identified for all exposures. The previous dogma is that epigenetic modifications in CpG islands or shores with highest CpG density are critical. The DMRs identified had what is considered a low range CpG density [40] with an average of 8 CpG/100 bp content and no DMR with a CpG density greater than 15 CpG/100 bp, Figure 5. Therefore, the DMR appear to have small clusters of CpG in a CpG desert, as previously described [41]. Evolutionarily CpG deserts develop due to the high mutation rate of CpG sites. The maintenance of small CpG clusters in these deserts may suggest a conserved critical epigenetic regulatory site. These genomic features are speculated to have a role in how the DMR become permanently programmed and promote epigenetic transgenerational inheritance. The current study focuses on a genome wide analysis of promoters. Further investigation of genome wide effects and the role of these genomic features is now needed to provide further insights into the molecular elements of epigenetic transgenerational inheritance.

The transmission of epigenetic information between generations in the absence of any direct environmental exposures is defined as epigenetic transgenerational inheritance [1,2,3]. Therefore, in the case of exposure of a gestating female, only after the F3 generation can epigenetic transgenerational inheritance be considered [1]. The previous observations that vinclozolin and methyoxychlor induced epigenetic transgenerational inheritance [2] developed the question of compound specificity. The current study indicates different environmental compounds and mixtures with very different effects on signal transduction processes involved can all promote epigenetic transgenerational phenotypes. Therefore, the specific compound or signaling event does not appear critical, but instead any agent that can modify the normal development and differentiation of the primordial germ cell during gonadal sex determination [1,3] can impact epigenetic programming and promote transgenerational inheritance. Although the majority of exposures will influence somatic cells and disease or phenotypes in the individual exposed, those actions that promote epigenetic transgenerational inheritance may have additional significant biological impacts. This includes providing a molecular mechanism for environmental toxicology, disease etiology, early life basis of adult onset disease [1,3] and evolutionary biology [42]. The availability of ancestral environmental epigenetic biomarkers is anticipated to significantly facilitate the research in these areas of science.

Materials and Methods

Animal studies

All experimental protocols for the procedures with rats were pre-approved by the Washington State University Animal Care

A

Gene Symbol	Gene name	Gene ID	Region changed	MeDIP-qPCR exposure/control ratio			
				Plastics	Dioxin	Pesticide	Jet Fuel
Carm1	Coactivator-associated arginine methyltransferase 1	363026	chr8:20650587-20651612			3.491126	
Dmpk or Six5	Dystrophia myotonica-protein kinase or SIX homeobox 5	308405 or 308406	chr1:78450272-78451687	3.710558		2.519094	
Fgf15	Fibroblast growth factor 15	170582	chr1:205323456-205324556	25.69776			
Flg	Filaggrin	24641	chr2:186309317-186310200		3.096977	4.735184	
Hoxb6	Homeo box B6	497986	chr10:85032294-85033194	2.108708			
Hspd1	Heat shock protein 1 (chaperonin)	63868	chr9:53896237-53896837		0.695904		
Irx2	Iroquois homeobox 2	306657	chr17:746309-746989			2.053513	
Nras	Neuroblastoma ras oncogene	24605	chr2:198292829-198293429		7.986455	11.12386	
Ntng1	Netrin G1	295382	chr2:205805922-205806522	0.148596			
Prrt1	Proline-rich transmembrane protein 1	406167	chr20:4220107-4221198			3.885713	
Rhoq	Ras homolog gene family, member Q	85428	chr6:10413845-10414445				3.141808
Satb2	SATB homeobox 2	501145	chr9:55824749-55825838			0.132503	
Sema3b	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	363142	chr8:112852022-112852622		2.477172		2.480198
Shc2	SHC (Src homology 2 domain containing) transforming protein 2	314612	chr7:11584014-11584614		2.080849		
Tbx2	T-box 2	303398	chr10:74084425-74085225	7.887618			
Vom2r69	Vomeronasal 2 receptor, 69	289433	chr14:740492-741794				0.517274

B

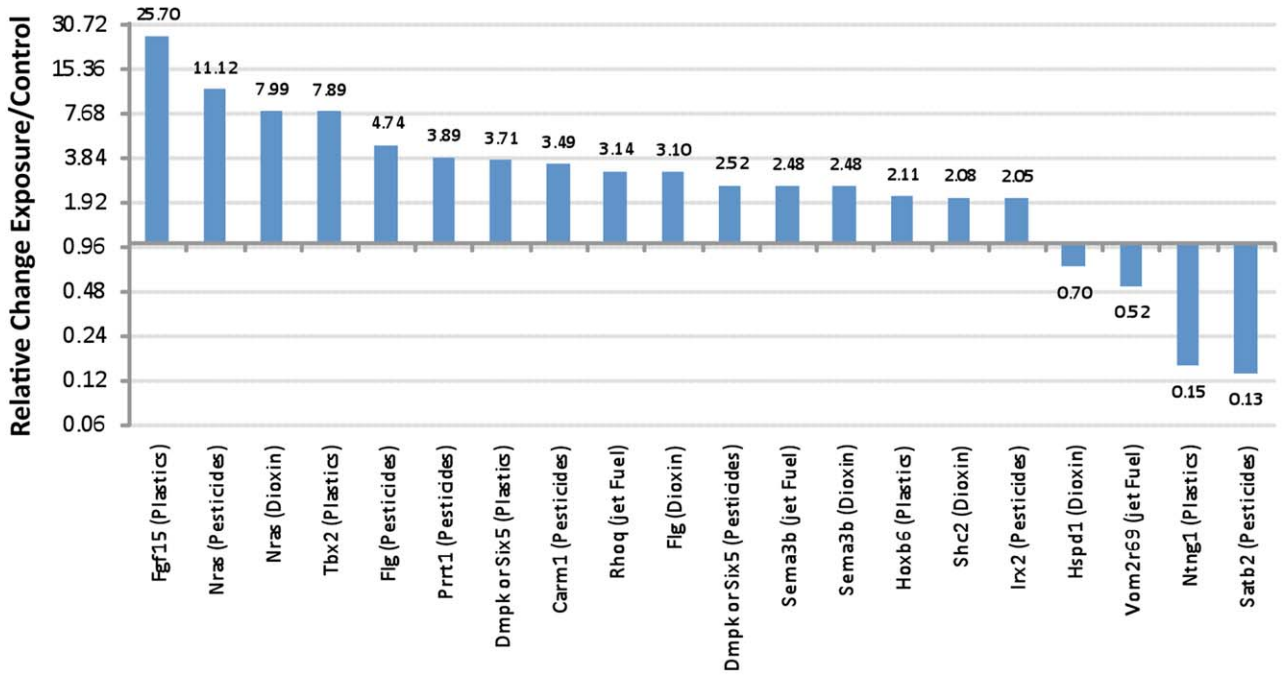

Figure 4. The MeDIP-qPCR analysis of (A) selected DMR for each exposure was used to confirm MeDIP-Chip analysis and (B) relative change (exposure/control) ratio presented for each DMR. All changes shown are statistically significant between control and exposure ($p<0.05$).
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and Use Committee (IACUC approval # 02568-026). The University Department of Environmental Health and Safety approved all the protocols for the use of hazardous chemicals in this experiment. Sprague Dawley SD female and male rats of an outbred strain (Harlan) at about 70 and 100 days of age were maintained in ventilated (up to 50 air exchanges/hour) isolator

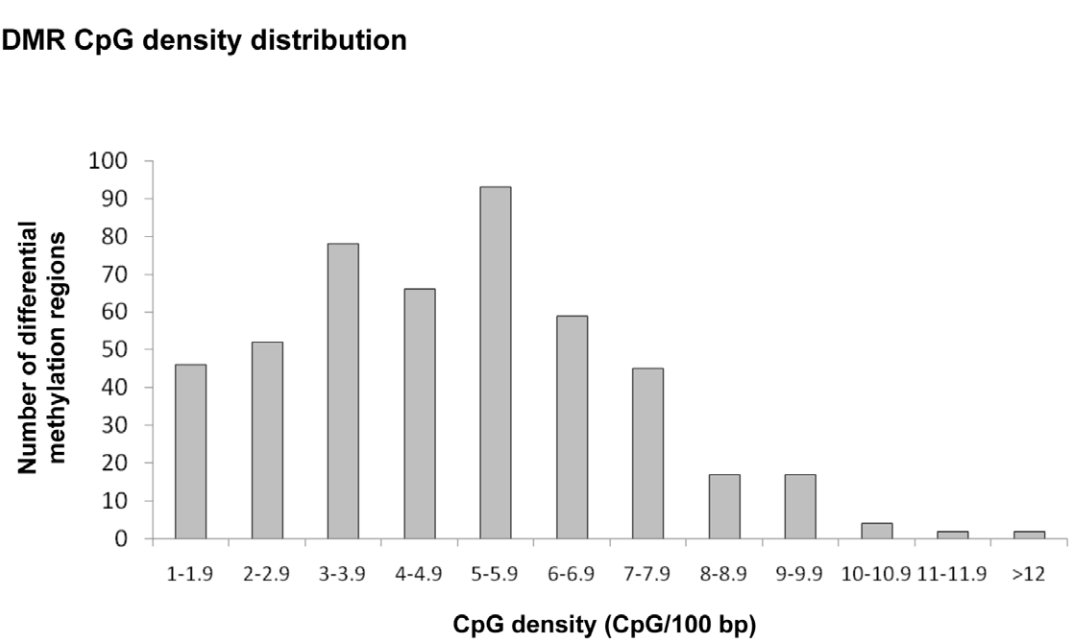

Figure 5. Differential DNA methylated region (DMR) CPG density distribution. The CpG density (CpG/100 bp) associated with all exposure DMR are presented with number of DMR on y axis and density (CpG per 100 bp) on x axis.
doi:10.1371/journal.pone.0031901.g005

cages (cages with dimensions of 10 ¾″ W×19 ¼″ D×10 ¾″ H, 143 square inch floor space, fitted in Micro-vent 36-cage rat racks; Allentown Inc., Allentown, NJ) containing Aspen Sani chips (pinewood shavings from Harlan) as bedding, and a 14 h light: 10 h dark regimen, at a temperature of 70 F and humidity of 25% to 35%. The mean light intensity in the animal rooms ranged from 22 to 26 ft-candles. Rats were fed ad lib with standard rat diet (8640 Teklad 22/5 Rodent Diet; Harlan) and ad lib tap water for drinking. During the procedures, rats were held in an animal transfer station (AniGard 6VF, The Baker Company, Sanford, ME) that provided an air velocity of about 0.5 inch.

At proestrus as determined by daily vaginal smears, the female rats, (90 days) were pair-mated with male rats (120 days). On the next day, the females were separated and their vaginal smears were examined microscopically and if they were sperm-positive (day 0) the rats were tentatively considered pregnant and then weighed with a digital animal weighing balance to monitor increases in body weight. Vaginal smears were continued for monitoring diestrus status in these rats until day 7. On embryonic day 7 (E-7) these females were weighed to determine if there was a significant increase in (greater than about 10 g) body weight, to confirm pregnancy in sperm-positive females. These pregnant rats were then given daily intraperitoneal injections of any one of the following single chemicals or mixtures with an equal volume of sesame oil (Sigma) on days E-8 through E-14 of gestation [43]. Treatment groups were Control, Pesticide (Permethrin+DEET), Plastics (Bisphenol-A, DBP and DEHP), Dioxin (TCDD), and Jet Fuel (JP8 hydrocarbon). The pregnant female rats treated with various mixtures were designated as the F0 generation. When there was a drop in the litter size and the sex ratio of pups in F1 generation of Plastics group, another treatment group was included with only half the dose of Bisphenol-A, DBP and DEHP and this group was designated 'Low Dose Plastics' group. Doses, percent of oral LD50, and sources of chemicals for the compounds are given in Table S1A.

Breeding for F1, F2, and F3 generations, weaning measures and puberty checks

The offspring of the F0 generation were the F1 generation. Likewise F2 and F3 generation offspring were generated. The breeding used males and females from the same lineage (control or exposure), but did not use any sibling or cousin crosses to avoid inbreeding artifacts. These rats were weaned from their mothers at 21 days of age. At weaning, the following weaning traits were measured; litter size, sex ratio, weaning weight (in grams), and anogenital index (AGI). Anogenital distance (AGD), was measured with a caliper that had an accuracy of $1/100^{th}$ of a mm. Males have a significantly higher AGD than that of females. Weaning weights of rats were measured by a digital balance. AGI was computed as the AGD in mm (from the ventral edge of the anal opening to the caudal edge of the genital opening) per gram of body weight at weaning. Starting at the age of 30 days for females and 35 days for males, puberty checks were performed. These checks were performed on a daily basis until puberty in each rat was confirmed. Onset of puberty for females was indicated by a clear vaginal opening, and for males it was indicated when the glans penis was able to fully extend free of the preputial fold (balano-preputial separation) [37] (Figure S6).

Dissection of rats for tissue collection

Both female and male rats of F1, F2 and F3 generation at 90–120 days of age were euthanized by CO_2 inhalation and cervical dislocation for dissection, collection and examination of tissues including testis, epididymis, and ovary. Body and tissue weights were measured at dissections. Blood samples were collected, allowed to clot, centrifuged and serum samples stored for hormone assays. Tissues were fixed in Bouin's solution (Sigma) and 70% ethanol, then processed for paraffin embedding by standard procedures for histopathology examination. Five-micrometer sections were made and were either unstained or stained with H & E stain.

TUNEL cell death assay

Testis sections were examined by Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (In situ cell death detection kit, Fluorescein, Roche Diagnostics, Mannheim, Germany) as per the manufacturer's protocols. The sections were deparaffinized in xylene, rehydrated through descending series of ethyl alcohols, deionized water and 1× PBS buffer. The sections were deproteinized by incubation at 37°C in 250 ml of 1× PBS buffer containing 150 µl of Fungal Proteinase K (20 mg/ml; Invitrogen, Carlsbad, CA) and washed in 1× PBS buffer. About 20–25 µl of the enzyme-label solution mix was applied to testis sections. Slides were incubated at 37°C for 90 min, washed in fresh 1× PBS buffer for 10 min, mounted with GVA mount and kept at 4°C until examination. Testis sections were examined in a fluorescent microscope in dark to count the number of brightly fluorescing germ cells that are apoptotic.

Ovarian analysis

Evaluation of adult ovaries: Ovaries taken from rats at the time of sacrifice were fixed, paraffin embedded and sectioned at 5 µm thickness. Every 30th section was collected and hematoxylin/eosin stained. The three stained sections (150 µm apart) through the central portion of the ovary with the largest cross-section were evaluated for number of primordial follicles, developing pre-antral follicles, small antral follicles, large antral follicles, small cystic structures and large cysts. The mean number of each evaluated structure per section was calculated across the three sections. Follicles had to be non-atretic and have the oocyte nucleus visible in the section in order to be counted. Primordial follicles had an oocyte surrounded by a single layer of either squamous or both squamous and cuboidal granulosa cells [44]. Developing pre-antral follicles had one or more complete layers of cuboidal granulosa cells. Small antral follicles had a fluid-filled antrum and a maximum diameter of 51 µm measured across the outermost granulosa cell layer. Large antral follicles had a diameter greater than 51 µm.

Sperm DNA isolation and methylated DNA immunoprecipitation (MeDIP)

Sperm heads were separated from tails through sonication following previously described protocol (without protease inhibitors) [45] and then purified using a series of washes and centrifugations [46] from a total of nine F3 generation rats per treatment lineage that were 120 days of age. DNA extraction on the purified sperm heads was performed as previously described [5]. Equal concentrations of DNA from individual sperm samples were then used to produce pools of DNA material. Three DNA pools were produced in total per treatment, which contained the same amount of sperm DNA from three animals. Therefore a total of 45 animals were used for building three DNA pools per treatment for the 4 experimental groups plus controls. These DNA pools were then used for methylated DNA immunoprecipitation (MeDIP). MeDIP was performed as follows: 6 µg of genomic DNA was subjected to series of three 20 pulse sonications at 20% amplitude and the appropriate fragment size (200–1000 ng) was verified through 2% agarose gels; the sonicated genomic DNA was resuspended in 350 ul TE and denaturated for 10 min at 95°C and then immediately placed on ice for 5 min; 100 ul of 5× IP buffer (50 mM Na-phosphate pH 7, 700 mM NaCl, 0.25% Triton X-100) was added to the sonicated and denatured DNA. An overnight incubation of the DNA was performed with 5 ug of antibody anti-5-methylCytidine monoclonal from Diagenode S.A (Denville, NJ) at 4°C on a rotating platform. Protein A/G beads from Santa Cruz (Santa Cruz, CA) were prewashed on PBS-BSA 0.1% and resuspended in 40 ul 1× IP buffer. Beads were then added to the DNA-antibody complex and incubated 2 h at 4°C on a rotating platform. Beads bound to DNA-antibody complex were washed 3 times with 1 ml 1× IP buffer; washes included incubation for 5 min at 4°C on a rotating platform and then centrifugation at 6000 rpm for 2 min. Beads-DNA-antibody complex were then resuspended in 250 ul digestion buffer (50 mM Tris HCl pH 8, 10 mM EDTA, 0.5% SDS) and 3.5 ul of proteinase K (20 mg/ml) was added to each sample and then incubated overnight at 55°C on a rotating platform. DNA purification was performed first with phenol and then with chloroform:isoamyl alcohol. Two washes were then performed with 70% ethanol, 1 M NaCl and glycogen. MeDIP selected DNA was then resuspended in 30 ul TE buffer.

Tilling array MeDIP-Chip analysis

Roche Nimblegen's Rat DNA Methylation 3×720 K CpG Island Plus RefSeq Promoter Array was used, which contains three identical sub-arrays, with 720,000 probes per sub-array, scanning a total of 15,287 promoters (3,880 bp upstream and 970 bp downstream from transcription start site). Probe sizes range from 50–75 mer in length with the median probe spacing of 100 bp. Three different comparative (MeDIP vs MeDIP) hybridizations experiments were performed for each experimental group versus control, each encompassing DNA samples from 6 animals (3 treatment and 3 control groups) and 3 sub-arrays. MeDIP DNA samples from experimental groups were labeled with Cy3 and MeDIP DNA samples from the control group were labeled with Cy5.

Bioinformatic and statistic analyses of Chip data

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

Following normalization each probe within each array was subjected to a smoothing procedure, whereby the probe's normalized M values were replaced with the median value of all probe normalized M values across all arrays within a 600 bp window. If the number of probes present in the window was less than 3, no value was assigned to that probe. Each probe's A values were likewise smoothed using the same procedure. Following normalization and smoothing each probe's M value represents the median intensity difference between vinclozolin lineage and control lineage of a 600 bp window. Significance was assigned to probe differences between lineage and generation samples by calculating the median value of the intensity differences as compared to a normal distribution scaled to the experimental mean and standard deviation of the normalized M. A Z-score and P-value were computed for each probe from that distribution. The statistical analysis was performed in pairs of comparative IP hybridizations between treatment lineage (T) and control lineage (C) (e.g. T1-C1 and T2-C2; T1-C1 and T3-C3; T2-C2 and T3-C3). In order to assure the reproducibility of the candidates

obtained, only the candidates showing significant changes in every one of the paired comparisons were chosen as having a significant change in DNA methylation between each of the experimental group and controls. This is a very stringent approach to select for changes, since it only considers repeated changes in all paired analysis.

Clustered Regions of interest were then determined by combining consecutive probes within 600 bases of each other, and based on whether their mean M values were positive or negative, with significance p-values less than 10^{-5}. The statistically significant differential DNA methylated regions were identified and P-value associated with each region presented. Each region of interest was then annotated for gene and CpG content. This list was further reduced to those regions with an average intensity value exceeding 9.5 (log scale) and a CpG density $\geq$1 CpG/100 bp.

MeDIP-qPCR confirmation

The MeDIP-Chip differential DNA methylation sites identified were further tested with a quantitative PCR analysis [47,48]. Real time qPCR quantification of each significant region obtained from the array was performed on MeDIP samples and the values were normalized to the DNA concentration of MeDIP samples measured by picogreen. These qPCR assays were optimized and performed by the Genomics Core Laboratory at the University of Arizona, Tucson, AZ. Three technical replicates of Real Time qPCR reactions were performed for each one of three different MeDIPs per experimental group. Each MeDIP was from pools of sperm DNA samples from three animals. Ct values were obtained and the relative presence of specific DNA amplicons was calculated between control and exposure groups through the equation 'relative change = $2^{-\Delta Ct}$'. Statistical analysis between control and exposure groups was performed with student's t-test and changes with $p<0.05$ were considered significant. The level of DNA in the pool is a weighted average of all individuals, as previously described, [49].

Statistical analysis

For statistical analysis, all the data on weaning traits and onset of puberty were averaged for each litter. These averages were used as input in the program GraphPad© Prism 5 statistical analysis program. One-way ANOVA or t-test were used to determine if the data on puberty, number of apoptotic germ cells, number of ovarian follicles from the individual treatment groups differ from those of Control groups with a probability of significance, $p = 0.05$.

Supporting Information

Figure S1 Weaning traits including litter size and sex ratio were measured in three generations of rat progeny derived from pregnant F0 females exposed to environmental compounds (Pesticide, Plastics, Dioxin and Jet Fuel). Litter size and sex ratio were reduced only in Plastics group in F1 generation rats (* $p<0.05$; ** $p<0.01$).
(PDF)

Figure S2 Weaning weight measured in three generations of rat offspring derived from pregnant F0 females exposed to environmental compounds (Pesticide, Plastics, Dioxin and Jet Fuel). Weaning weight increased only in Pesticide group in F2 generation rats.
(PDF)

Figure S3 Anogenital indexes (AGI) were computed based on anogenital distance and weaning weights in three generations of rat offspring derived from pregnant F0 females exposed to environmental compounds (Pesticide, Plastics, Dioxin and Jet Fuel). AGI was unaffected in both female and male rats of F1 generations. AGI was reduced in Pesticide and Plastics groups of F2 female rats while it increased in LD Plastics F2 female rats. AGI declined in Pesticide group of F2 male rats while it increased in LD Plastics F2 male rats (* $p<0.05$; ** $p<0.01$).
(PDF)

Figure S4 Onset of puberty in female and male rats were investigated in three generations of rat offspring derived from pregnant F0 females exposed to environmental compounds (Pesticide, Plastics, Dioxin and Jet Fuel). Data from the first two generations are shown. (Puberty data of F3 generation rats are presented in Fig. 1). In the F1 generation, a delayed onset of puberty was recorded in female rats of Plastics group, and male rats of Plastics and Jet Fuel groups. In the F2 generation, an early onset of puberty was found for females rats of Plastics, LD Plastics, Dioxin and Jet Fuel groups and for the male rats of Plastics and Dioxin groups (* $p<0.05$; ** $p<0.01$; *** $p<0.001$).
(PDF)

Figure S5 Serum hormone concentrations were measured in the third generation rat offspring derived from pregnant F0 females exposed to environmental compounds (Pesticide, Plastics, Dioxin and Jet Fuel)(* $p<0.05$; ** $p<0.01$). (A) Serum testosterone concentrations in male rats were severely reduced in Plastics, Dioxin and Jet Fuel groups. (B) Serum progesterone concentrations were unaffected in female rats (C) Serum LH concentrations were unaltered in male rats (D) Serum LH concentrations were not changed in female rats.
(PDF)

Figure S6 Onset of puberty was identified by (A) the opening of vaginal orifice in female rats and (B) the separation of glans penis from the prepuce in male rats.
(PDF)

Table S1 Doses and Sources of Chemicals used.
(PDF)

Table S2 List of rat sperm differential methylation regions (DMR).
(PDF)

Table S3 Pathways influenced by genes associated with DMR.
(PDF)

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Author Contributions

Conceived and designed the experiments: MKS. Performed the experiments: MM CG RT MMH. Analyzed the data: MKS MM CG RT MMH. Wrote the paper: MKS MM CG.

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Environmentally Induced Epigenetic Transgenerational Inheritance of Altered Sertoli Cell Transcriptome and Epigenome: Molecular Etiology of Male Infertility

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Abstract

Environmental toxicants have been shown to induce the epigenetic transgenerational inheritance of adult onset disease, including testis disease and male infertility. The current study was designed to determine the impact of an altered sperm epigenome on the subsequent development of an adult somatic cell (Sertoli cell) that influences the onset of a specific disease (male infertility). A gestating female rat (F0 generation) was exposed to the agriculture fungicide vinclozolin during gonadal sex determination and then the subsequent F3 generation progeny used for the isolation of Sertoli cells and assessment of testis disease. As previously observed, enhanced spermatogenic cell apoptosis was observed. The Sertoli cells provide the physical and nutritional support for the spermatogenic cells. Over 400 genes were differentially expressed in the F3 generation control versus vinclozolin lineage Sertoli cells. A number of specific cellular pathways were identified to be transgenerationally altered. One of the key metabolic processes affected was pyruvate/lactate production that is directly linked to spermatogenic cell viability. The Sertoli cell epigenome was also altered with over 100 promoter differential DNA methylation regions (DMR) modified. The genomic features and overlap with the sperm DMR were investigated. Observations demonstrate that the transgenerational sperm epigenetic alterations subsequently alters the development of a specific somatic cell (Sertoli cell) epigenome and transcriptome that correlates with adult onset disease (male infertility). The environmentally induced epigenetic transgenerational inheritance of testis disease appears to be a component of the molecular etiology of male infertility.

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Introduction

Environmentally induced epigenetic transgenerational inheritance of adult onset disease [1] can be promoted by factors such as toxicants [2,3] or nutrition [4,5,6,7]. Environmental chemicals shown to promote transgenerational disease include the fungicide vinclozolin [2,8], plastics (bisphenol A (BPA) and phthalates DEHP and DBP) [3,9,10], pesticides (methoxychlor and permethrin) [2,3], dioxin [3,11], and hydrocarbons [3]. A number of transgenerational diseases/abnormalities have been shown to be induced such as testis disease [2,9,12], prostate disease [13,14], kidney disease [7,14], ovarian disease [3,15], reproductive tract abnormalities [14], brain and behavior abnormalities [10,16,17], and immune abnormalities [14]. Environmentally induced transgenerational phenomena have been observed in plants [18], flies [19,20], worms [21,22], rodents [2,11], and humans [23,24]. The current study was designed to investigate the actions of a specific toxicant (vinclozolin) to promote a transgenerational alteration in a somatic cell (Sertoli) that correlates to the induction of disease in the tissue (testis).

Transgenerational phenotypes involve the germline transmission of epigenetic alterations (e.g. DNA methylation) in the absence of any direct environmental exposures [1,25]. Environmental exposures during fetal gonadal sex determination modifies the epigenetic (DNA methylation) programming of the germline to induce permanently programmed differential DNA methylation regions (DMR) that then transmit an altered epigenome to the subsequent generation [1,26]. Normal primordial germ cell development in the gonad requires the erasure and re-methylation of the DNA to promote the development of a male (sperm) versus female (egg) germline [26,27,28]. The somatic cells and tissues derived from this epigenetically altered germline will promote all somatic cells to develop a modified epigenome and transcriptome [29]. Each tissue will develop an organ specific transgenerational transcriptome [29,30] that is associated with the disease/abnormality of the tissue [29]. An example provided is the vinclozolin induced transgenerational ovarian disease that correlates with an altered granulosa cell epigenome and transcriptome associated with the development of polycystic ovarian disease [15]. This provides insights into the molecular etiology of disease development within the tissue.

The testis is the site of spermatogenesis that occurs within seminiferous tubules composed of Sertoli cells and an adjacent basal layer of mesenchymal peritubular cells [31,32]. The interstitial tissue between tubules is composed of Leydig cells, the site of testosterone production, and testicular macrophages [33,34]. All these somatic cells cooperate in testicular function to

support germ cell development and production [31,32]. The most critical cell for the support of the developing spermatogenic cells is the Sertoli cell that provides the physical support, formation of the blood testis barrier, and nutritional factors needed for spermatogenesis [32]. The Sertoli cells synthesize a number of transport binding proteins (e.g. transferrin) to carry essential factors (e.g. iron) to the developing germ cells [32]. In addition, the Sertoli cells produce pyruvate/lactate that is used as the primary energy metabolite by the germ cells that are sequestered within the blood testis barrier and not able to acquire glucose [35,36].

Testis disease is primarily associated with abnormal spermatogenesis and reduced sperm counts leading to male infertility [37,38]. This can be the result of developmental defects such as cryptorchidism or hypospadias [39]. A number of studies have suggested environmental exposures promote testis abnormalities and disease [3,12,40]. This includes endocrine disruptors and environmental chemicals. A number of histopathologies have been associated with testis disease and termed testicular dysgenesis syndrome [38,39,40]. One of the primary abnormalities is increased spermatogenic cell apoptosis resulting in reduced sperm numbers [41,42]. The male infertility associated with testis disease has increased dramatically over the past decades and now affects over 10% of the human male population [43]. Although abnormal endocrinology and environmental factors have been associated with testis disease [44], the specific molecular etiologies remain to be elucidated.

Previous studies have demonstrated that exposure of a gestating female to the agriculturally used fungicide vinclozolin [2] during gonadal sex determination promotes the epigenetic transgenerational inheritance of adult onset testis disease [2,12,14,45]. In the F3 generation 90% of the males had a transgenerational spermatogenic cell defect of enhanced apoptosis and reduced sperm count and motility [2,12]. The objective of the current study was to utilize this model to elucidate components of the molecular etiology of male infertility. Since the Sertoli cell is the primary somatic cell that supports the development of the spermatogenic cells, the role of the Sertoli cell in mediating the transgenerational testis disease is investigated. Observations demonstrate that the vinclozolin induced epigenetic transgenerational inheritance of spermatogenic cell defects and testis disease is associated with a transgenerational alteration in the Sertoli cell epigenome and transcriptome.

Results

Transgenerational Spermatogenic Cell Abnormality

The experimental design involved the exposure of a gestating female (F0 generation) Sprague-Dawley rat to vinclozolin (100 mg/kg/day) during days 8–14 (E8–14) of fetal development [2,12]. The F1 generation progeny were bred to obtain the F2 generation and the F2 bred to obtain the F3 generation [2,12]. No sibling or cousin breeding was used in order to avoid any inbreeding artifacts. Corresponding control (vehicle DMSO) lineage F1, F2 and F3 generations were obtained to compare to the vinclozolin lineage males. Some of the direct exposure F1 generation and transgenerational F3 generation control and vinclozolin lineage males were aged to 1 year and testes collected to examine the number of apoptotic spermatogenic cells as previously described [2,12]. The F1 and F3 generation vinclozolin lineage males had an increased number of apoptotic spermatogenic cells ($p<0.05$), Figure 1. As previously described [2,12], the vinclozolin lineage animals had spermatogenic cell defects that correlated to testis disease and male infertility [14].

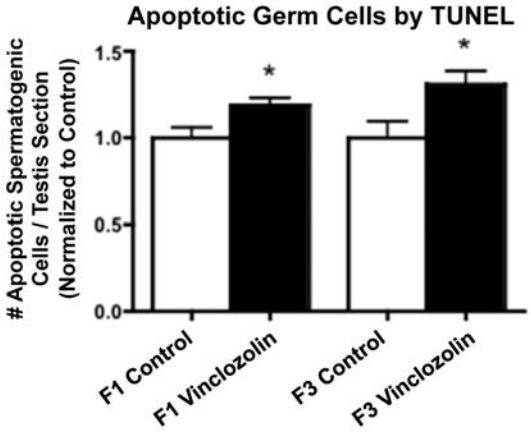

Figure 1. Spermatogenic cell apoptosis in 1-year-old males. Relative apoptotic spermatogenic cell number per testis section is presented. F1 and F3 generation control and vinclozolin lineage testis were examined with the mean ± SEM presented and asterisks (*) indicating a statistically significant difference ($p<0.05$).
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Another set of F3 generation males from the control and vinclozolin lineages were aged to 20 days of postnatal development and testes collected to isolate purified populations of Sertoli cells. At this age of development no spermatogenic cell apoptosis has been observed, so avoid disease artifacts, and the highest purity cell preparations are obtained [2,12]. The isolated Sertoli cell populations from three different groups of animals and cell isolates were used to obtain Sertoli cell RNA and DNA from F3 generation control and vinclozolin lineages.

Sertoli Cell Transgenerational Transcriptome

The Sertoli cell RNA was used in a microarray analysis and the quality of the RNA and array monitored. The comparison of the microarrays demonstrated nearly identical profiles on the arrays, Supplemental Figure S1. No batch effects were observed due to cell or RNA isolation dates. The differential gene expression between the control and vinclozolin lineage F3 generation Sertoli cell arrays was determined with a statistical difference ($p<0.05$), fold change (>1.2 fold) and mean difference (>10) as cut off limits, described in the Methods. The comparison identified 417 differentially expressed genes in the F3 generation Sertoli cell control versus vinclozolin comparison, Supplemental Table S1. The differentially expressed genes involved 198 genes with up-regulation and 219 genes with down-regulation, Figure 2. The functional gene categories of transcription, metabolism, signaling and development were predominant.

A cellular signaling and process pathway analysis was performed as previously described [46]. Analysis of the 417 differentially expressed genes identified 22 pathways with variable impact factors and statistically significant over-representation, Table 1. One of the top pathways most relevant to the Sertoli cell was the pyruvate/lactate metabolism pathway, Figure 3. A number of the key enzymes in pyruvate and lactate metabolism were modified that influence pyruvate and lactate production by the Sertoli cell. These are subsequently a required energy source for the spermatogenic cells. The key enzymes that are all significantly down regulated (>2 fold) were hydroxyacyl-glutathione hydrolase (*Hagh*), pyruvate dehydrogenase beta (*Pdhb*), lactate dehydrogenase A like bb (*Ldnalbb*), and dihydrollipoxamideS-acetyltransferase (*Dlat*), Figure 3. Other prominent pathways affected, Table 1, were the proteasome, nucleotide excision repair, RNA transport, p53

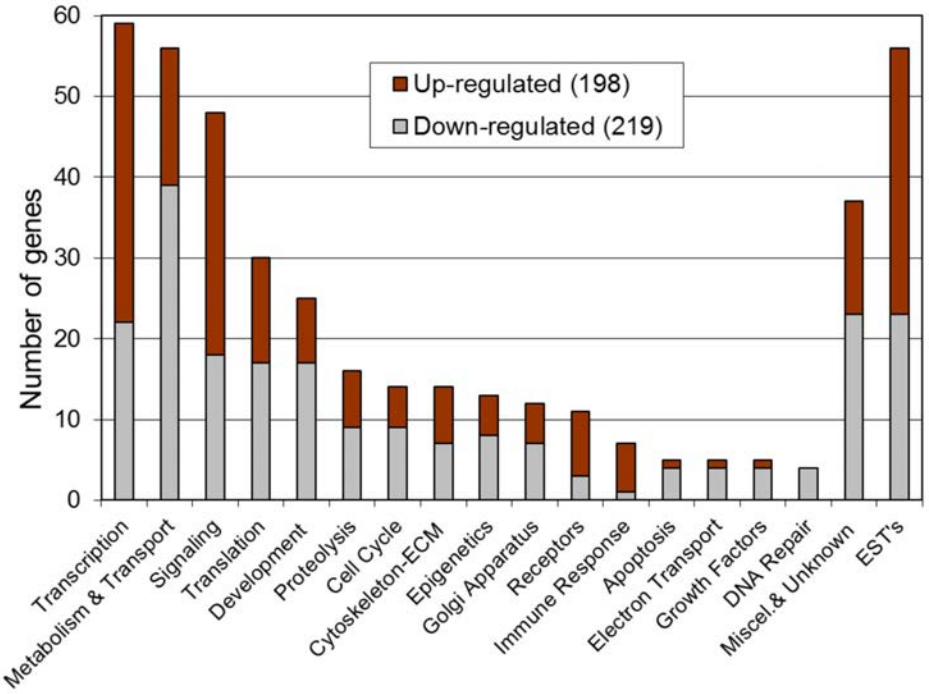

Figure 2. Gene functional categories in F3 generation vinclozolin lineage Sertoli cells differentially expressed gene (417 genes). The number of genes associated with the different functional categories are presented for up-regulated (black bar) or down-regulated (gray bar).
doi:10.1371/journal.pone.0059922.g002

signaling, and mTOR signaling (Supplemental Figure S2). The pathway class most affected was Genetic Information Processing, Table 1.

A gene network analysis was performed on the 417 differentially expressed genes with an unbiased literature evaluation protocol using the Pathway Studio software. The down or up regulated genes associated and cellular localization are shown, Figure 4. The most highly interconnected genes were the *Igf1r*, *Jak2*, *Hsp90aa1*, *Hif1a* and *Ccne1*. As identified in the gene functional categories, Figure 2, a number of cellular processes are influenced by the gene network identified.

The differentially regulated genes were mapped to the genome and shown in Figure 5. All chromosomes contained differentially expressed genes. The potential over-representation of gene clusters in specific chromosomal regions was examined as previously described [30]. Potential 2–5 megabase regions were examined for statistical over-representation of differentially expressed genes. These gene clusters are identified in Figure 5 and will be correlated to the epigenetic analysis described below.

Sertoli Cell Transgenerational Epigenome

The F3 generation control and vinclozolin lineage Sertoli cell DNA was used in a methylated DNA immunoprecipitation (MeDIP) and genome wide promoter tiling array (Chip) analysis (MeDIP-Chip) [8] as described in the Methods. A comparative hybridization of the F3 generation control and vinclozolin lineage Sertoli cell MeDIP samples identified differential DNA methylation regions (DMR), as previously described [8]. The MeDIP-Chip analysis identified 101 DMR and the chromosomal locations of the DMR are shown in Figure 5 and Supplemental Table S2. All chromosomes contained DMR. Interestingly, none of the Sertoli cell DMR identified were in common with the previously identified sperm DMR [8]. In the analysis of sperm DMR previously reported [8], two genomic features were identified. The first was a consensus DNA sequence localization in the ~600 bp region of the DMR termed "environmental induced DNA methylation motif 1" (EDM1) [8]. This DNA sequence motif is a >20 bp sequence motif associated with 68.8% of the DMR identified in sperm [3,8], as described in the Methods. The EDM1 motif was found in 7.1% of the Sertoli cell DMR identified, which was not statistically different from a computer generated random promoter region set with a 16.1% incidence of occurrence. Therefore, the EDM1 motif genomic feature found in sperm DMR was not associated with the Sertoli cell DMR. The second genomic feature previously identified in sperm DMR was a low density CpG content of less than 10 CpG/100 bp [3,8]. The number of CpG/100 bp is generally between 1–4 in the sperm DMR. The Sertoli cell DMR were also found to contain a low density of CpG (<10 CpG/100 bp), Figure 6. The majority of DMR had 1 or 2 CpG/100 bp. No DMR was found to have a CpG density greater than 10.6 CpG/100 bp. Therefore, the Sertoli cell DMR are similar to the Sperm DMR in that a CpG "desert" of low density CpG is a genomic feature involved [3,8].

The MeDIP-Chip DMR data was confirmed with a select set of Sertoli cell DMR using an MeDIP-quantitative PCR (QPCR) analysis. The selected 26 DMR sites with 7 confirmations using the MeDIP-QPCR analysis are presented in Supplemental Figure S3. This analysis confirmed the MeDIP-Chip data for these DMR. A technical limitation to this analysis is that only <150 bp region within the ~600–800 bp DMR can be examined, such that false negatives are common due to the inability to interrogate the entire DMR. Although the MeDIP-Chip data for a number of DMR were confirmed, a better technology is needed for future studies.

A correlation of Sertoli cell differentially expressed genes with the DMR identified only two differentially expressed genes that also had a corresponding promoter DMR (Pdrx5 and Pole3), Supplemental Table S3. Therefore, the majority of differentially expressed genes did not contain a DMR for potential direct

Table 1. Associated Functional Pathway Categories.

Functional Pathway Category	Pathway	Altered Genes in Pathway	Total Genes in Pathway	Impact Factor
	Oxidative phosphorylation	13	207	NA
Metabolism	Pyruvate metabolism	4	73	NA
	Glycerophospholipid metabolism	4	82	NA
	Proteasome	5	51	7.5
	Nucleotide excision repair	4	43	5.8
Genetic Information Processing	RNA transport	10	134	NA
	Protein processing in endoplasmic reticulum	7	137	NA
	Spliceosome	6	120	NA
	mTOR signaling pathway	4	54	5.9
	Phosphatidylinositol signaling system	4	70	18.9
Signal Transduction	ErbB signaling pathway	4	83	3.8
	Jak-STAT signaling pathway	5	136	4.5
	MAPK signaling pathway	7	253	1.8
	p53 signaling pathway	5	67	4.8
Cellular Processes	Cell cycle	8	117	6.1
	Oocyte meiosis	5	109	NA
	Focal adhesion	4	187	3.2
Organismal Systems/ Immune System	Fc epsilon RI signaling pathway	4	70	4.2
	Chemokine signaling pathway	4	189	NA
	Progesterone-mediated oocyte maturation	5	88	NA
Organismal Systems/ Endocrine System	GnRH signaling pathway	4	91	2.8
	Insulin signaling pathway	4	130	2.6

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regulation of gene expression. Previously, we identified the potential presence of "epigenetic control regions" (ECR) that contain a statistically significant over-representation of differential expressed genes [30]. Gene clusters within 2–5 megabase regions that contain a statistically over-representation of differentially expressed genes are shown in Figure 5 and Supplemental Table S4. Six clusters had a DMR present within a 2 megabase window, Figure 5 and Supplemental Table S3. These regions may act as an ECR to regulate gene expression in the region as previously described [30]. An example of one potential ECR is presented in Figure 7. This ECR contained two DMR (*Rnase1a* and *Kctdll*) and had 10 differentially expressed genes within the approximately 5 megabase region shown. Therefore, 61 genes in the 16 potential DMR may be regulated as an ECR.

A correlation of potential distal gene expression identified 31 DMR that were within 2 megabase of 38 differentially regulated genes, Supplemental Table S3. Although the potential mechanism involved are unclear, distal regulation through mechanisms such as non-coding RNA have been demonstrated [30]. A final correlation used an unbiased literature based analysis to identify indirect gene interactions between the DMR and differentially expressed genes. The DMR associated genes that have been shown to interact with the Sertoli cell differentially expressed genes are shown in Figure 8. A potential correlation is shown between 25 DMR and 21 differentially expressed genes. Although a limited number of genes had direct regulation, a large number of the differentially expressed genes correlate to potential ECR, distal regulation sites or indirect gene interactions, Supplemental Table S3 and S4.

The 101 DMR associated genes and the 417 Sertoli cell differentially regulated genes were used to identify correlations with genes previously associated with male infertility. This analysis used a literature based procedure in the Pathway Studio software and results are shown in Figure 9. The correlation demonstrated one DMR and eight differentially expressed genes had a direct relationship with previously associated male infertility genes. Observations provide additional insights into the molecular etiology of male infertility.

Discussion

The ability of environmental toxicants such as vinclozolin have previously been shown to promote the epigenetic transgenerational inheritance of adult onset disease such as male infertility [2,12,14]. Epigenetic transgenerational phenomena require the germ line transmission of a permanently modified epigenome (e.g. epimutation) [1,26]. How this modified germline epigenome is translated into later life adult onset disease was investigated in the current study with a focus on testis biology and disease. Observations confirmed the ability of vinclozolin to promote F3 generation spermatogenic cell apoptosis, that has previously been correlated to adult onset male infertility [2,12]. This transgenerational model system was used to evaluate the molecular etiology of male infertility. The Sertoli cell is the essential somatic cell known to support spermatogenesis [31,32] and abnormal Sertoli cell

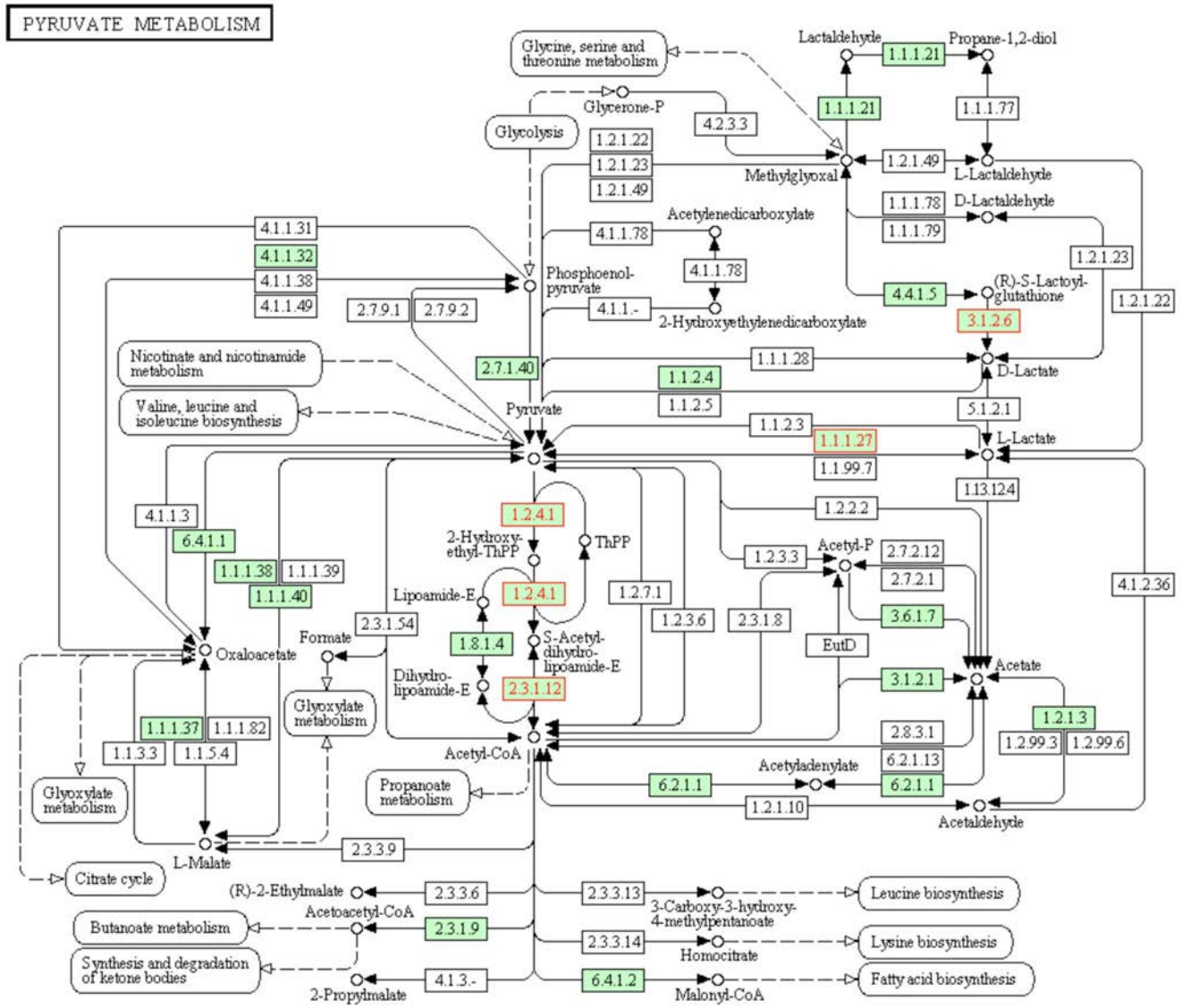

Figure 3. Pyruvate and lactate metabolic pathway with colored genes having significantly different altered expression in the F3 generation vinclozolin lineage Sertoli cells.
doi:10.1371/journal.pone.0059922.g003

function has been shown to correlate to testis disease [38,39,40]. Therefore, the Sertoli cell was the focus of the current study to assess how a germline epimutation may translate to male infertility.

The Sertoli cell transgenerational transcriptome induced in the F3 generation vinclozolin lineage identified 417 differentially expressed genes. All somatic cell types derived from the gamete having germline epimutations will have a transgenerational alteration in the transcriptome, as previously shown for 11 different male and female tissues [30] and granulosa cells [15]. Each tissue and somatic cell was found to have a unique transgenerational transcriptome. The Sertoli cell transcriptome identified in the current study supports the concept of transgenerational alteration of individual somatic cell types. Cell types or tissues sensitive to this alteration in the transcriptome will be susceptible to develop disease [1,29]. The vinclozolin lineage Sertoli cell transgenerational transcriptome was found to have both up and down regulated genes with predominant cellular pathways and gene functional categories affected. A gene network analysis identified specific genes that influence a number of different signaling pathways and processes.

Considering the role of Sertoli cells in the maintenance of spermatogenic cell development, a critical pathway was identified that directly correlates with germ cell viability. Sertoli cells acquire glucose on their basal surface and convert this to pyruvate and lactate that is then secreted and provided to germ cells, sequestered within the blood-testis barrier, as a primary energy source [35,36]. Previous studies have shown abnormal pyruvate or lactate production or transport promotes the degeneration of spermatogenic cells through the process of apoptosis [36,47,48,49]. Therefore, the transgenerational alteration in pyruvate or lactate production by the Sertoli cells provides a direct mechanism for the induction of spermatogenic cell apoptosis observed in the F3 generation vinclozolin lineage males. Although other differentially expressed genes and cellular processes are likely involved, the abnormal pyruvate or lactate production directly

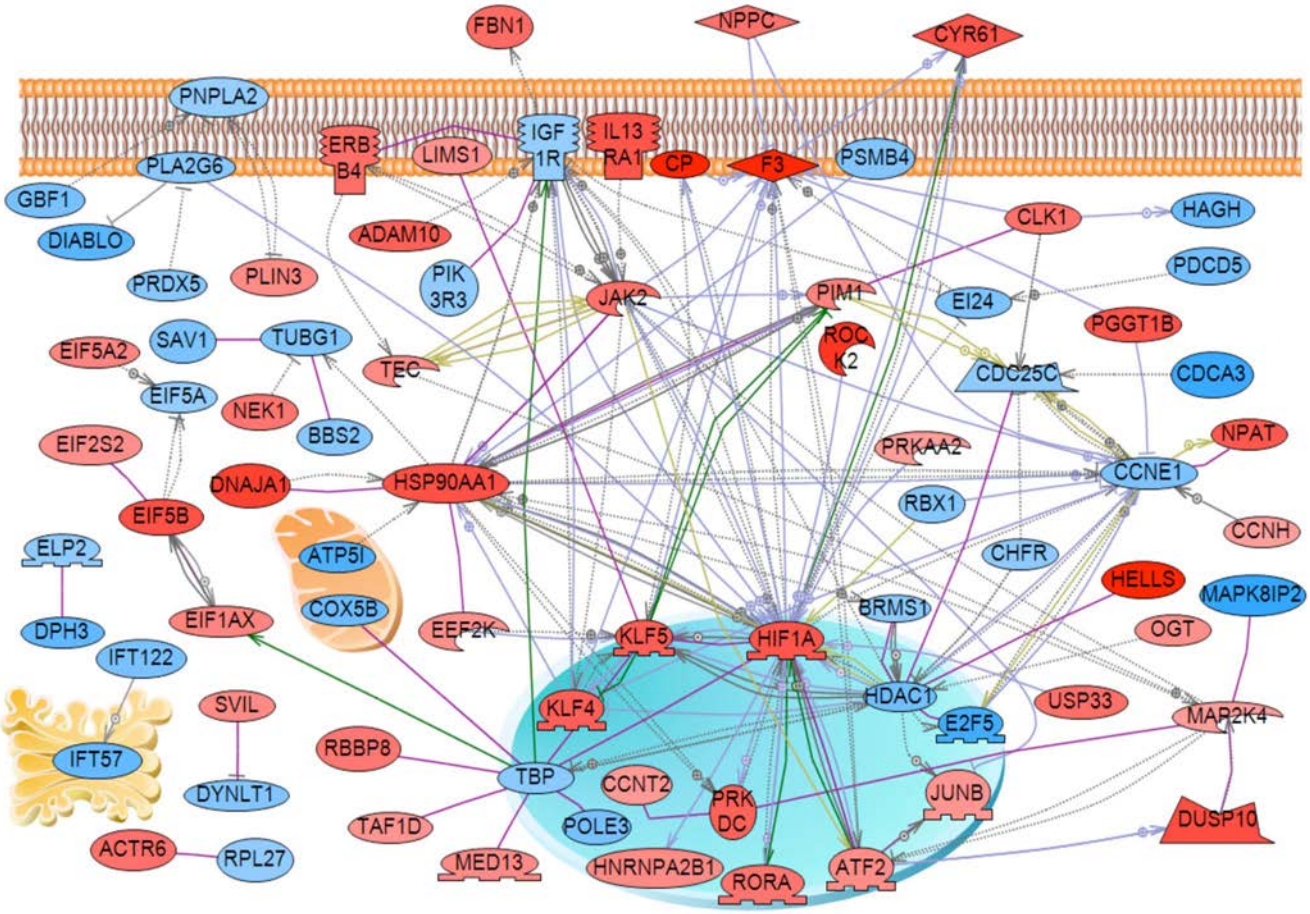

Figure 4. Gene network of Sertoli cell differentially expressed genes. Genes having direct connectivity are presented with cellular localizations indicated.
doi:10.1371/journal.pone.0059922.g004

correlates with the transgenerational adult onset male infertility observed [2,12].

The F3 generation vinclozolin lineage Sertoli cells also had an altered transgenerational epigenome. The genome-wide promoter analysis identified 101 differential DNA methylation regions (DMR) that previously have been termed epimutations [1,8,29]. The Sertoli cell transgenerational DMR were localized on the genome and genomic features analyzed. Interestingly, the previously identified EDM1 DNA sequence motif shown to be associated with the sperm DMR [8], was not associated with the Sertoli cell DMR. Observations suggest different molecular mechanisms may be involved in the germline versus somatic cell DMR generation and function. In contrast, the low density of CpG (<10 CpG/100 bp) identified in the sperm DMR was also observed in the Sertoli cell DMR. As previously discussed [3,8], these "CpG deserts" may have evolved as critical regulatory epigenetic sites. The high mutation rates observed in CpG sites [50] suggests evolutionarily over time that regions of the genome develop low density CpG (e.g. CpG deserts). In the event a CpG cluster is regulatory in these deserts then evolutionary pressure maintains the presence of the clusters in these desert regions. Observations suggest that this same genomic feature observed in sperm of a low density <10 CpG/100 bp in the 600–800 bp DMR also exists in somatic cells (Sertoli cells). Although this CpG desert genomic feature was similar, the specific DMR in sperm were found to have no overlap with the Sertoli cell DMR identified. Therefore, the DMR in somatic cells are likely unique to the cell type and generated from a specific cascade of epigenetic alterations derived from the germline and early embryo.

A correlation of the transgenerational Sertoli cell transcriptome with the epigenome identified potential distinct regulatory mechanisms. Interestingly, only two genes (Prdx5 and Pole3) had a direct correlation to suggest the potential for the DMR to directly regulate the promoter and expression of the adjacent gene. Therefore, the majority of the altered transcriptome and DMR act through different mechanisms. Distal regulation was considered and approximately 10% of the differentially expressed genes were found within 2 megabases of a DMR. Such distal regulation could involve mechanisms such as non-coding RNA [30,51]. Perhaps the most interesting correlation is the role of epigenetic control regions (ECR) [30]. Previously we demonstrated that differentially expressed genes can be statistically over-represented and localized in gene clusters on the genome [30]. When an epigenetic regulatory site such as a DMR is also localized in the 2–5 megabase gene cluster then the region may be regulated through an ECR. The initial epigenetic regulatory regions identified were imprinting control regions (ICR) [51,52]. A defined DNA methylation region was found to regulate a non-coding RNA that distally for several megabase regulated the expression of multiple genes [51]. The ICR are likely a subset of a large number of such ECR that allow for a limited number of epigenetic regulatory sites to influence the expression of a large number of genes [30]. The

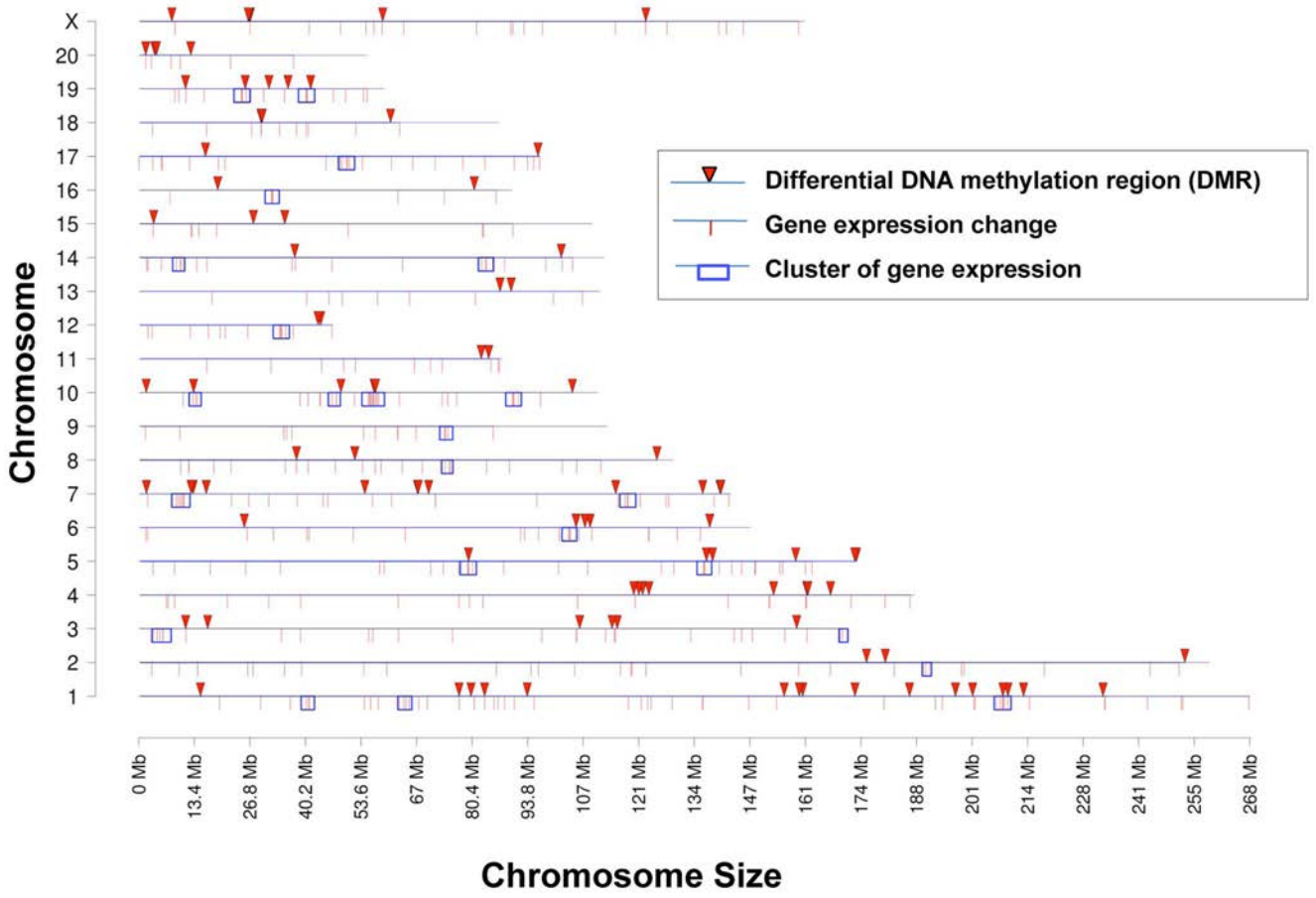

Figure 5. Chromosomal localization of differential DNA methylation regions (DMR), differential expressed genes, and clusters of gene expression. The chromosomal number and relative size are presented. The DMR (arrow), gene expression changes (line) and over-represented clusters of gene expression (box) are indicated.
doi:10.1371/journal.pone.0059922.g005

F3 generation Sertoli cell differentially expressed genes clustered into 16 different potential regions and a number of these directly correlated to the location of DMR identified. A large number of genes were regulated within these potential ECR. Therefore, a combination of direct regulation, distal regulation, and ECR are likely involved in the Sertoli cell transgenerational transcriptome identified. In addition, indirect regulatory links between DMR associated genes and the differentially expressed genes were identified. The epigenetic regulation of somatic cell gene expression and role of epigenetic transgenerational inheritance needs to be further elucidated.

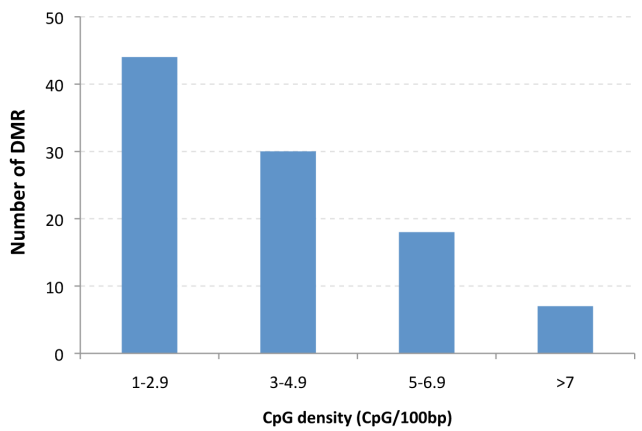

Figure 6. CpG density of the F3 generation vinclozolin lineage Sertoli cell DMR. The number of DMR and CpG density (CpG/100 bp) range are presented.
doi:10.1371/journal.pone.0059922.g006

The current study used a transient exposure during the developmental period of germ cell epigenetic programming and gonadal sex determination [1,2,3]. The disease observed in the F1 generation offspring are due to direct exposure of the fetal somatic cells and not to a germline mediated transgenerational mechanism observed in the F3 generation. Although a comparison of the F1 and F3 generation control versus vinclozolin lineage Sertoli cells would be interesting, there is no anticipated correlations due to the distinct molecular mechanism involved. Similarly, direct exposure of an adult male can alter the epigenetic programming in spermatogenic cells to subsequently effect the disease in the F1 generation offspring [1], but these direct exposures have not previously been shown to promote transgenerational effects to subsequent generations. Therefore, the current study focused on the F3 generation to investigate the epigenetic transgenerational inheritance of disease mechanisms. Future studies to investigate the differences with the direct exposure fetal or adult male effects on the F1 generation will be interesting, but have distinct mechanism with the observations presented in the current study.

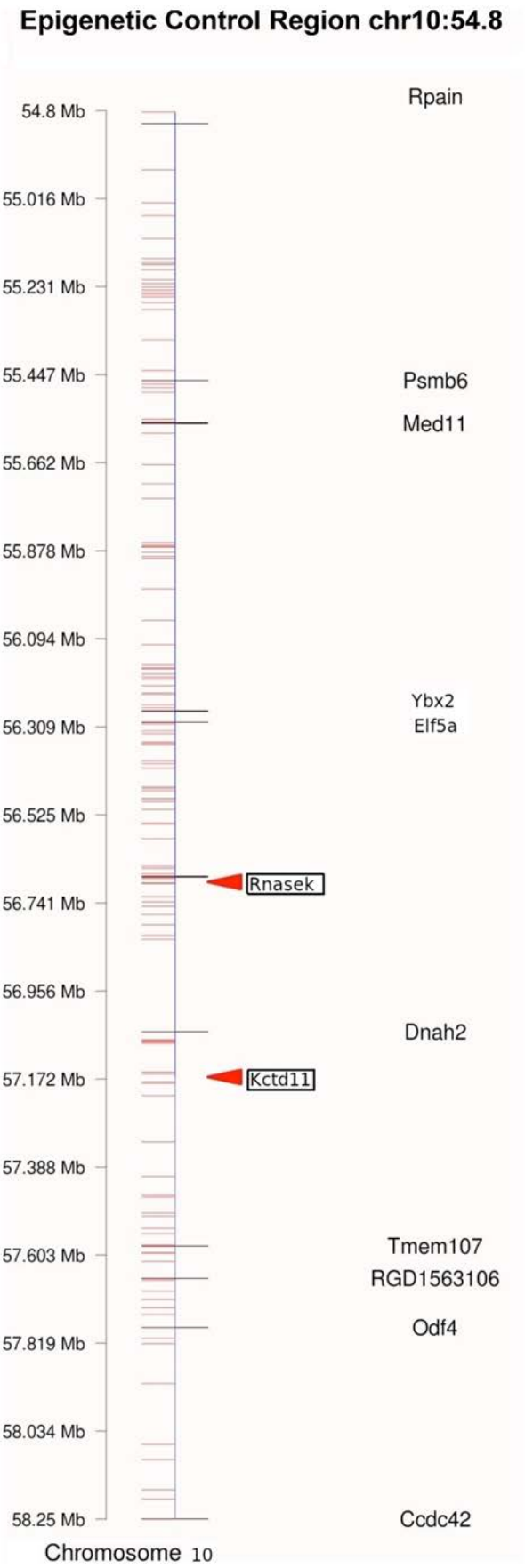

Figure 7. Example of an epigenetic control region (ECR) on chromosome 10. The chromosomal location in megabases is presented with all genes (horizontal lines) and regulated genes (gene symbols) listed. The DMR are listed in arrow heads.
doi:10.1371/journal.pone.0059922.g007

Male infertility in the human population has now increased to affect over 10% of the population [43]. A number of studies suggest environmental factors such as toxicants likely have a significant role in the etiology of male infertility [53,54]. Direct exposure toxicity of some of these agents for testis function has been observed [2,3], but how such exposures may promote later life disease has not been clarified. The current study used a rat model involving an environmental toxicant (vinclozolin) induced epigenetic transgenerational inheritance of testis disease, that appears in the vast majority of F3 generation males, to study the molecular etiology of male infertility. Observations demonstrate a transgenerational effect on the Sertoli cell transcriptome and epigenome that impacts critical cellular processes involved in testis function. The pyruvate/lactate pathway is critical for spermatogenesis and the transgenerational Sertoli cell transcriptome suggests that abnormal pyruvate/lactate production could directly promote the spermatogenic cell apoptosis observed. Further studies are needed to directly test this mechanism, but previous

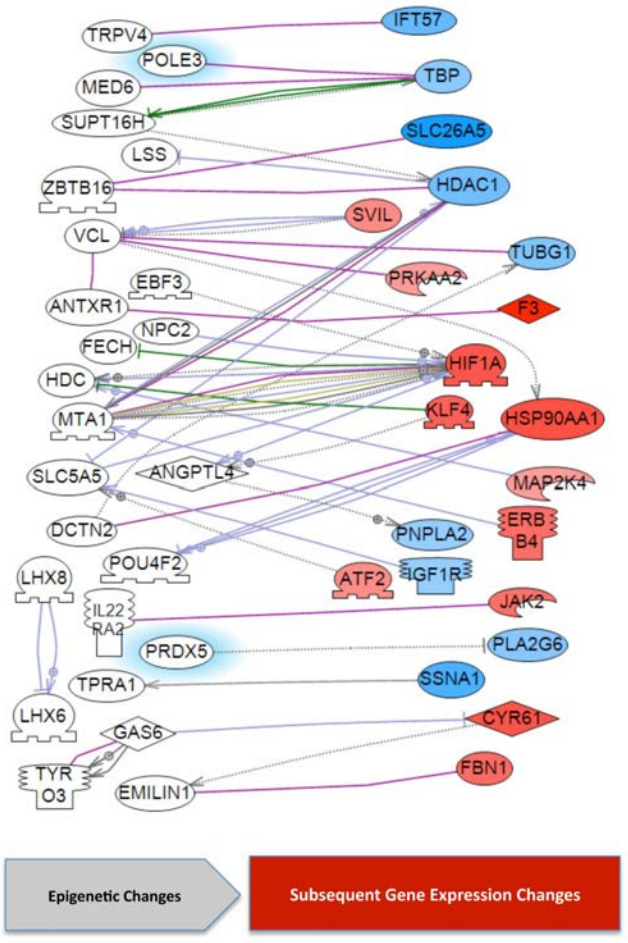

Figure 8. Gene interactions between DMR and differentially expressed genes in F3 generation vinclozolin lineage Sertoli cells. The white genes are DMR associated genes and the colored genes differentially expressed genes.
doi:10.1371/journal.pone.0059922.g008

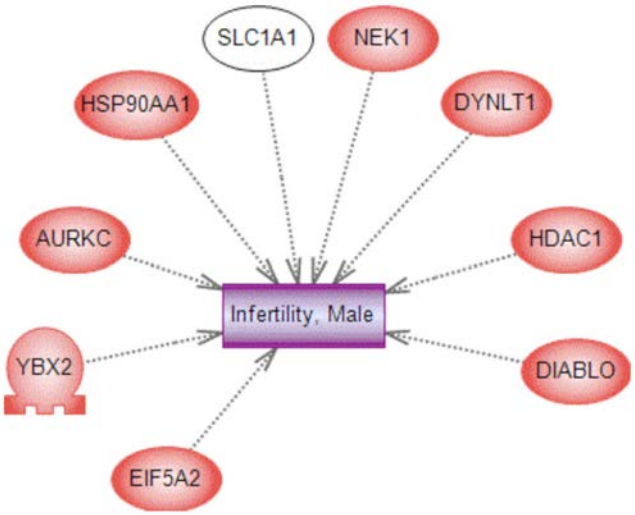

Figure 9. DMR and differentially expressed genes that correlate with male infertility/testis disease. The genes identified in the literature associated with male infertility and testis disease that correlate with F3 generation vinclozolin lineage DMR and differentially expressed genes are presented.
doi:10.1371/journal.pone.0059922.g009

literature supports the critical role of pyruvate/lactate in spermatogenic cell viability. A correlation of the transgenerational Sertoli cell DMR and differentially expressed genes with previously identified genes associated with male infertility [55,56] identified nine correlated genes. Therefore, in addition to the abnormal pyruvate/lactate metabolism the abnormal expression of these genes is known to be associated with male infertility.

The molecular etiology of male fertility identified suggests environmental toxicant exposure of a gestating female at the critical period of gonadal sex determination promotes an abnormal programming of the germ line epigenome (DNA methylation) that is transmitted transgenerationally to subsequent generations and promotes adult onset testis disease. This is correlated to abnormal Sertoli cell function and reduction in pyruvate/lactate production, as well as other critical gene expression abnormalities, to promote spermatogenic cell apoptosis and male infertility. The degree environmental induced epigenetic transgenerational inheritance of testis disease is associated with human male infertility now needs to be assessed. The rat model used clearly demonstrates that these exposures and molecular events are associated with the high incidence of male infertility transgenerationally. Combined observations clearly establish the role of transgenerational alterations in a somatic cells transcriptome and epigenome as a likely component of the etiology of environmentally induced epigenetic transgenerational inheritance of adult onset disease.

Materials and Methods

Animals and Treatments

Hsd:Sprague Dawley®™SD®™ female and male rats of an outbred strain (Harlan) were maintained in ventilated (up to 50 air exchanges/hour) isolator cages (cages with dimensions of 10 ¾" W×19 ¼ "D×10 ¾" H, 143 square inch floor space, fitted in Micro-vent 36-cage rat racks; Allentown Inc., Allentown, NJ) containing Aspen Sani chips (pinewood shavings from Harlan) as bedding, on a 14 h light: 10 h dark regimen, at a temperature of 70 F and humidity of 25% to 35%. Rats were fed ad libitum with standard rat diet (8640 Teklad 22/5 Rodent Diet; Harlan) and *ad libitum* tap water for drinking. At pro-estrus as determined by daily vaginal smears, the female rats (90 days of age) were pair-mated with male rats (120 days). On the next day, the pairs were separated and vaginal smears were examined microscopically. If sperm were detected (day 0) the rats were tentatively considered pregnant. Vaginal smears were continued for monitoring diestrus status in these rats until day 7. Pregnant rats for the treatment group (six different gestating females for each group) were given daily intraperitoneal injections of vinclozolin (100 mg/kg BW/d; Chem Service, West Chester, PA) and an equal volume of sesame oil (Sigma) on days E8 through E14 of gestation; Vinclozolin was dissolved in DMSO (Sigma). Pregnant rats for the control group were given daily intraperitoneal injections of DMSO (100 ul/kg BW/d) and an equal volume of sesame oil (Sigma) on days E8 through E14 of gestation [45]. The pregnant female rats treated with vinclozolin were designated as the F0 generation. All experimental protocols for the procedures with rats were pre-approved by the Washington State University Animal Care and Use Committee (IACUC approval # 02568-029).

Breeding for F1, F2, and F3 Generations

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

Measurement of Testicular Apoptotic Cells by TUNEL Analysis

Testis sections were examined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (*in situ* cell death detection kit, Fluorescein, Roche Diagnostics, Mannheim, Germany) as per the manufacturer's protocols. Sections were deparaffinized and rehydrated through alcohol series. They were deproteinized by Proteinase K (20 mg/ml; Invitrogen, Carlsbad, CA), washed with PBS and then 25 µl of the enzyme-label solution mix was applied and incubated at 37°C for 90 min. After PBS washes, slides were mounted and kept at 4°C until examination with a fluorescent microscope using dark field. Both testis sections of each slide were microscopically examined to identify and to count apoptotic germ cells by the bright fluorescence.

Sertoli Cell Preparation

Sertoli cells were isolated from the testes of 20-day-old rats (P20) using a sequential enzymatic digestion procedure previously described [57]. This pubertal period allows the optimum purity cells prior to disease onset. Three pools of P20 Sertoli cells were produced per treatment, with each pool containing cells from 2 to 6 animals. In brief, decapsulated testes were minced with razor blades and then tissue fragments were digested with trypsin (1.5 mg/ml, Life Technologies, Gaithersburg, MD) to remove the interstitial cells. This was followed by incubation with collagenase (1 mg/ml type I, Sigma) for removal of peritubular cells and then hyaluronidase (1 mg/ml, Sigma) for removal of germ cells. The purity of the Sertoli cell preparations were determined by immunohistochemistry to be >98% [57]. Final Sertoli cell pellets

were then resuspended in 1 ml Trizol™ (Invitrogen) for further RNA and DNA extractions.

RNA Extraction and Microarray Transcriptome Analysis

Messenger RNA was isolated using the Trizol™ (Invitrogen) method as per the manufacturer protocol. Messenger RNA was independently extracted from 3 pools of Sertoli cells (i.e. 3 biological replicas) per treatment. The mRNA processing and hybridizations were performed at the Genomics Core Laboratory, Center for Reproductive Biology, Washington State University, Pullman, WA using standard Affymetrix reagents and protocol. Briefly, mRNA was transcribed into cDNA with random primers, then cRNA was transcribed from the cDNA, and from that, single-stranded sense DNA was synthesized which was fragmented and labeled with biotin. Biotin-labeled fragmented ssDNA was then hybridized to the Rat Gene 1.0 ST microarrays containing more than 27,000 transcripts (Affymetrix, Santa Clara, CA, USA). Hybridized chips were scanned on an Affymetrix Scanner 3000. CEL files containing raw data were then pre-processed and analyzed with Partek Genomic Suite 6.5 beta software (Partek Incorporated, St. Louis, MO) using an RMA and GC-content adjusted algorithm (Supplemental Figure S1). The signals from an average of 28 different probes for each transcript were compared to give a single value. Two-way ANOVA was performed between the Sertoli cell transcriptomes from vinclozolin-lineage and controls. One factor of variation was treatment and the other was batch effect. Corrections were made for Sertoli cell preparation date batch effect by the Partek software according to the Methods of Moments [58]. The selection of the gene expression change was based on the expression change between vinclozolin and control lineage Sertoli cells limited to p-values <0.05, expression fold change >1.2, and the mean difference between vinclozolin and control un-logged signals >10. A higher stringency cut off (>1.5 fold change) was not utilized since many biological effects are observed with 20% alterations in gene expression so a more genome wide view of the transcriptome is identified with the stringency utilized. CEL files from this study have been deposited with the NCBI gene expression and hybridization array data repository (GEO, http://www.ncbi.nlm.nih.gov/geo, GEO # pending) and can be also accessed through www.skinner.wsu.edu. For gene annotation, the Affymetrix annotation file RaGene1_0stv1.na31.rn4.transcript.csv was used unless otherwise specified.

Pathway and Gene Network Analysis

Known functional relationships among the F3 generation differentially expressed genes were identified using the KEGG pathways from the University of Kyoto (Japan) Encyclopedia for Genes and Genome website (http://www.genome.jp/kegg/) and Pathway Express (http://vortex.cs.wayne.edu) [59]. Functional relationships among the F3 generation differentially expressed genes and genes with changes in DNA methylation were interrogated using Pathway Studio software (Ariadne, Genomics Inc. Rockville MD), using an unbiased, automated survey of published scientific literature (Global Literature Analysis). This analysis identifies functional relations among genes, such as direct binding, up-regulation or down-regulation and also builds sub-networks of genes and cellular processes based on their interconnections.

DNA Extraction and Methylated DNA Immunoprecipitation (MeDIP)

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

Tilling Array and MeDIP-Chip Bioinformatic and Statistical Analyses

Roche Nimblegen's Rat DNA Methylation 3×720 K CpG Island Plus RefSeq Promoter Array was used, which contains three identical sub-arrays, with 713,670 probes per sub-array, scanning a total of 15,287 promoters (3,880 bp upstream and 970 bp downstream from transcription start site). Probe sizes range from 50–75 mer in length with the median probe spacing of 100 bp. Three different comparative (amplified MeDIP vs. amplified MeDIP) hybridizations experiments included in three sub-arrays were performed by Nimblegen. Each comparative hybridization experiment contained one biological replicate of Sertoli cell Whole Genome Amplified-MeDIP-DNA sample from each lineage-treatment. Samples from experimental groups were labeled with Cy3 and MeDIP DNA samples from the control groups were labeled with Cy5. For each comparative hybridization experiment, raw data from both the Cy3 and Cy5 channels were imported into R (R Development Core Team (2010), R: A language for statistical computing, R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL http://www.R-project.org), checked for quality and converted to MA values (M = Cy5-Cy3; A = (Cy5+Cy3)/2). The following normalization procedure was conducted. Within each array, probes were separated into groups by GC content and each group was separately normalized,

between Cy3 and Cy5 using the loess normalization procedure. This allowed for GC groups to receive a normalization curve specific to that group. After each array had its CG groups normalized within the array, the arrays were then normalized across arrays using the A quantile normalization procedure. Following normalization each probe within each array was normalized and M values were replaced with the median value of all probe normalized M values across all arrays within a 600 bp window. If the number of probes present in the window was less than 3, no value was assigned to that probe. Each probe's A values were likewise normalized using the same procedure. Following normalization each probe's M value represents the median intensity difference between vinclozolin generation and control generation of a 600 bp window. Significance ($p<10^{-5}$) was assigned to probe differences between treatment-generation samples and control generation samples by calculating the median value of the intensity differences as compared to a normal distribution scaled to the experimental mean and standard deviation of the normalized M. A Z-score and P-value were computed for each probe from that distribution. The statistical analysis was performed in pairs of comparative IP hybridizations between treatment-lineage (T) and control-lineage (C). T1-C1 and T2-C2 gave 715 sites; T1-C1 and T3-C3 gave 633 sites; T2-C2 and T3-C3 gave 807 sites. In order to assure the reproducibility of the candidates obtained, only the candidates showing significant changes in all three of the paired comparisons were chosen as having a significant change in DNA methylation between the experimental group and controls. This is a very stringent approach to select for changes, since it only considers those changes repeated in all paired analyses.

Clustered Regions of interest were then determined by combining consecutive probes within 600 bases of each other, and based on whether their mean M values were positive or negative, with significance p-values less than 10^{-5}. The statistically significant differential DNA methylated regions were identified and p-value associated with each region presented. Each region of interest was then annotated for gene and CpG content. This list was further reduced to those regions with an average intensity value exceeding 9.5 (log scale) and a CpG density $\geq$1 CpG/100 bp. The web-based tool FIMO (Found Individual Motifs Occurrences) was used for determining the incidence of motifs in sets of sequences [60].

Chromosomal Location of Gene Expression Clusters

An R-code was developed to find chromosomal locations of ECRs (Figure 8). A 2 megabase sliding window with 50,000 bases interval was used to find the associated genes in each window. Then a Z-test statistical analysis with $p<0.05$ was used on these windows to find the ones with over-representation of differentially expressed genes. The consecutive windows with over-represented genes were merged together to form clusters of genes which we named ECR regions. Typical ECR regions ranged from 2–5 megabase.

Supporting Information

Figure S1 Microarray histograms for each array and box plot for The F3 generation control and vinclozolin lineage Sertoli cell samples. Array data was pre-processed with RMA and GC-content adjusted algorithm in Partek GS program. The y-axis presents the new hybridization signal and box plots the mean ± SEM.
(PDF)

Figure S2 Cellular signaling and process pathways impacted by differentially expressed genes from KEGG (see Methods). a) Proteosome, b) Nucleotide excision repair, c) MTOR signaling pathway, d) RNA transport.
(PDF)

Figure S3 Quantitative PCR of F3 generation Sertoli cell MeDIP for selected genes. The fold change (2^-deltaC+) between the control and vinclozolin lineage Sertoli cell MeDIP samples is presented with the black bars indicating samples with a significant difference ($p<0.05$).
(PDF)

Table S1 Differentially expressed genes from F3 generation vinclozolin lineage Sertoli cells as compared to control lineage cells (416 genes & ESTs).
(PDF)

Table S2 Differential DNA methylation regions (DMR) in F3 generation vinclozolin lineage Sertoli cells.
(PDF)

Table S3 Relations between vinclozolin-induced transgenerational DMR and expression changes in Sertoli cells.
(PDF)

Table S4 Clusters of transgenerational changes in gene expression and their relation to DMR in vinclozolin-lineage F3 Sertoli cells.
(PDF)

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Author Contributions

Edited the manuscript: MKS CGB MS MH EN. Conceived and designed the experiments: MKS. Performed the experiments: CGB MS MH EN. Analyzed the data: MKS CGB MS MH EN. Wrote the paper: MKS CGB.

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Environmentally Induced Epigenetic Transgenerational Inheritance of Ovarian Disease

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Abstract

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

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Introduction

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

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

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

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

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

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

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

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

Results

Transgenerational Ovarian Disease

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

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

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

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

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

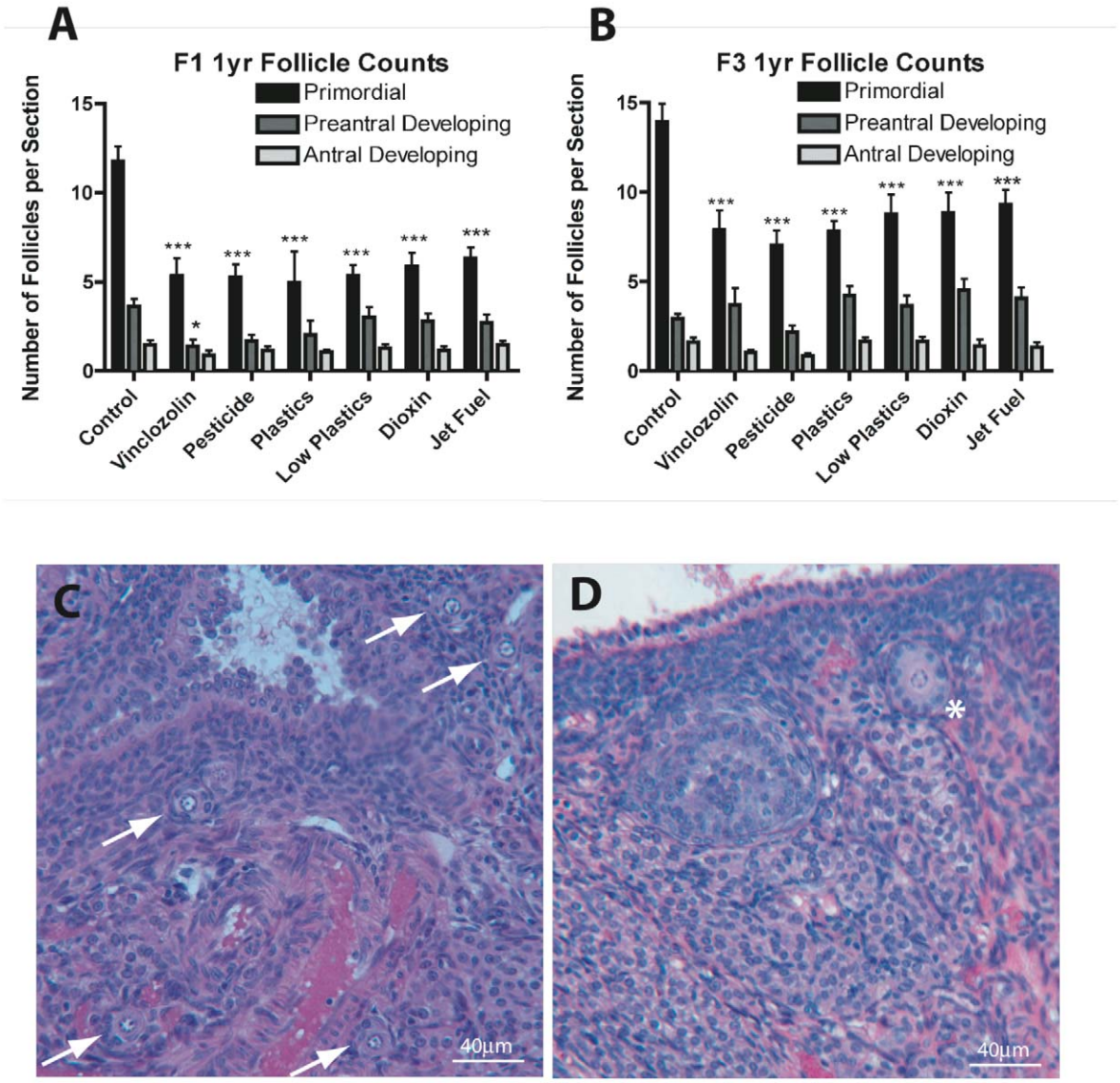

Figure 1. Follicle numbers and development. A) Number of primordial, preantral, and antral follicles per section in F1 generation ovaries. N = 9 animals per treatment group. B) Number of follicles per section in F3 generation ovaries. N = 9 animals per treatment group. Asterisks indicate groups significantly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

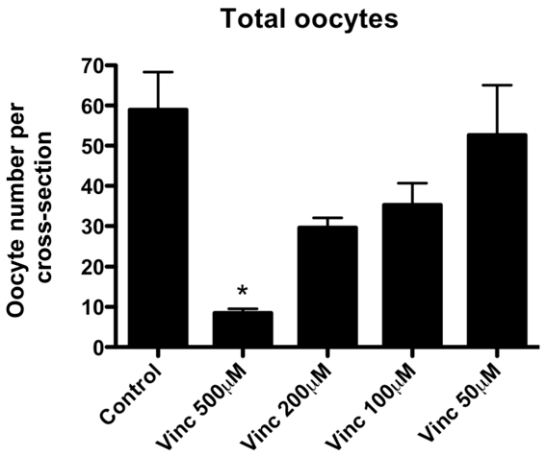

Figure 2. Number of oocytes per ovarian cross-section in ovaries taken from 4-day old rats and cultured whole for 10 days in the presence of different concentrations of vinclozolin. Data are from five different experiments performed in replicate. Asterisks indicate groups significantly (*$p \leq 0.05$)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

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

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

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

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

Transgenerational Granulosa Cell Transcriptome

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

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

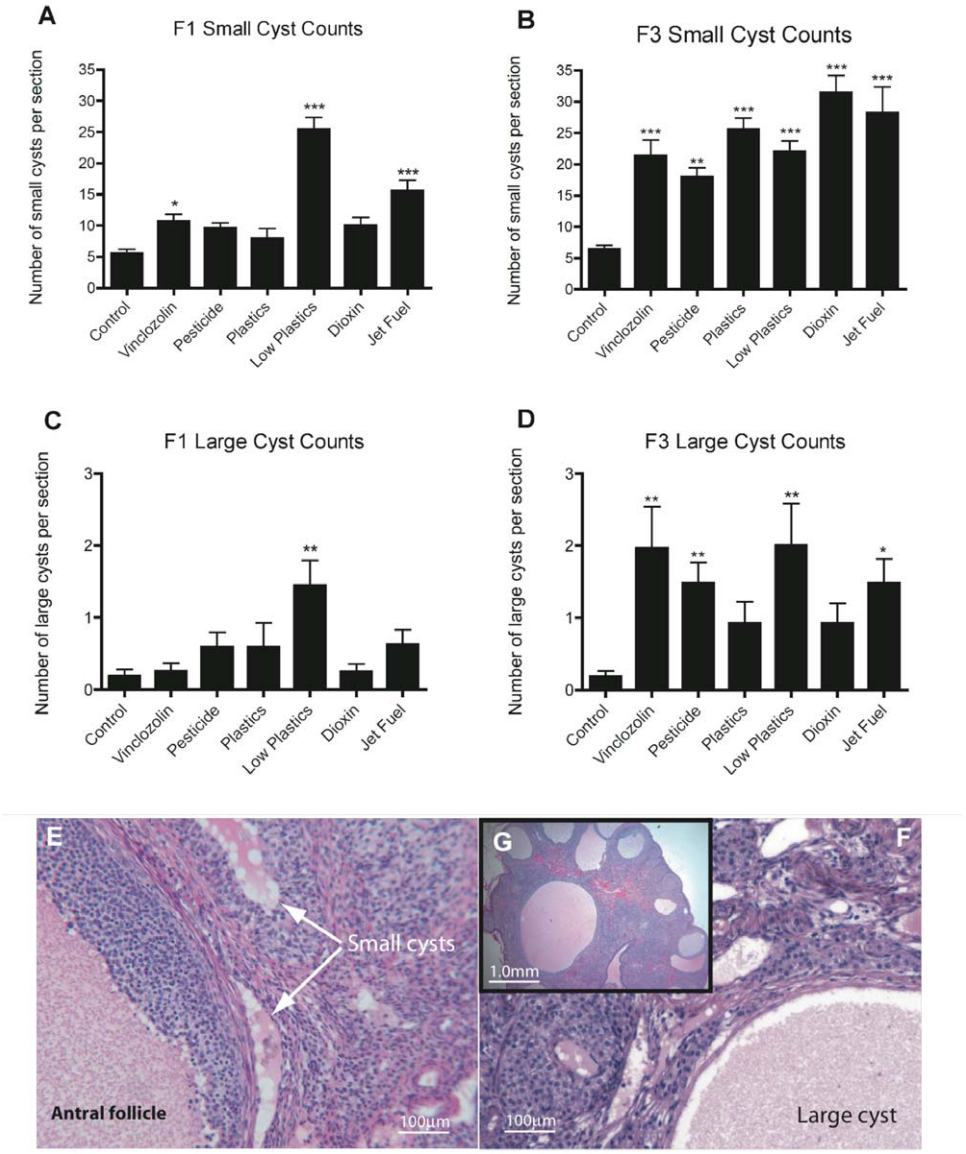

Figure 3. Ovarian cysts. Number of small (50–250 µm) cysts per section in F1 (A) and F3 (B) generation ovaries. Number of large (>250 µm) cysts per section in F1 (C) and F3 (D) generation ovaries. N = 9 animals per treatment group. Asterisks indicate groups significantly (*p≤0.05, **p≤0.01, ***p≤0.005) different than controls by ANOVA followed by Dunnet's post-hoc test. E) H and E stained section of F3 ovary showing small cysts. F) H and E stained F3 ovary showing a large cyst. G) Expanded view of small and large cysts.
doi:10.1371/journal.pone.0036129.g003

tionships among the F3 generation differentially expressed genes identified. An unbiased literature based network analysis was performed as described in Methods to determine which genes are functionally linked with respect to binding, signaling or regulation. This created a gene network of direct connections as shown in Figure 6. Some genes show significant functional connections to others, such as ESR1, MMP2 and CXCL12. Such highly connected genes may play important regulatory roles in these F3 generation granulosa cells and in the development of ovarian disease states. Therefore, a transgenerational change in the granulosa cell transcriptome was identified that may be in part a causal factor in the molecular etiology of the transgenerational ovarian disease. Further analysis of the 523 genes with transgenerational alterations in gene expression identified previously known genes involved in ovarian disease and more specifically polycystic ovarian disease. A total of 30 genes were found to be related to ovarian disease and 5 directly related to PCO disease, Figure 7. Therefore, genes known to have a relationship with PCO and ovarian disease were shown to have altered expression. The potential role of the transgenerational change in the granulosa cell epigenome to promote this transcriptome alteration is described below.

Transgenerational Granulosa Cell Epigenome

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

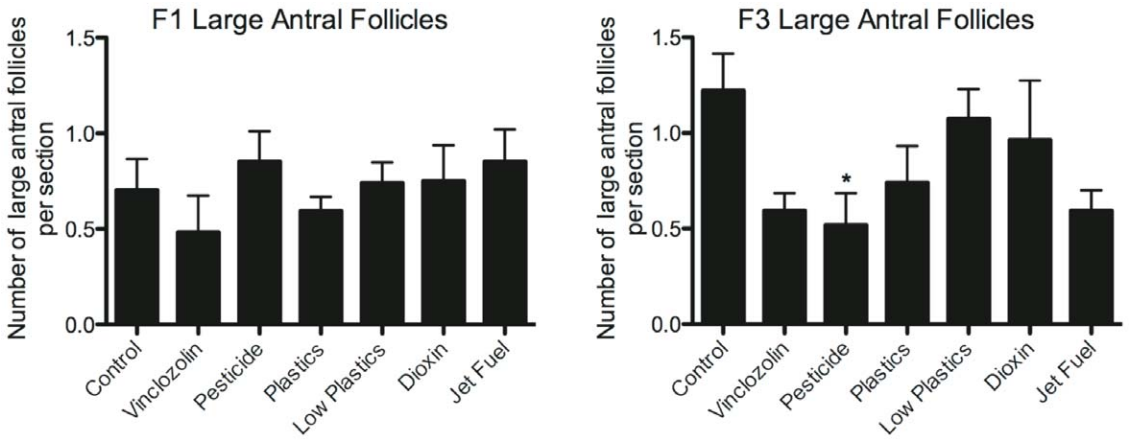

Figure 4. Large antral follicles. A) Number of large antral follicles per section in F1 generation ovaries. B) Number of large antral follicles per section in F3 generation ovaries. N = 9 animals per treatment group. Asterisks indicate groups significantly (*$p \leq 0.05$) different than controls by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

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

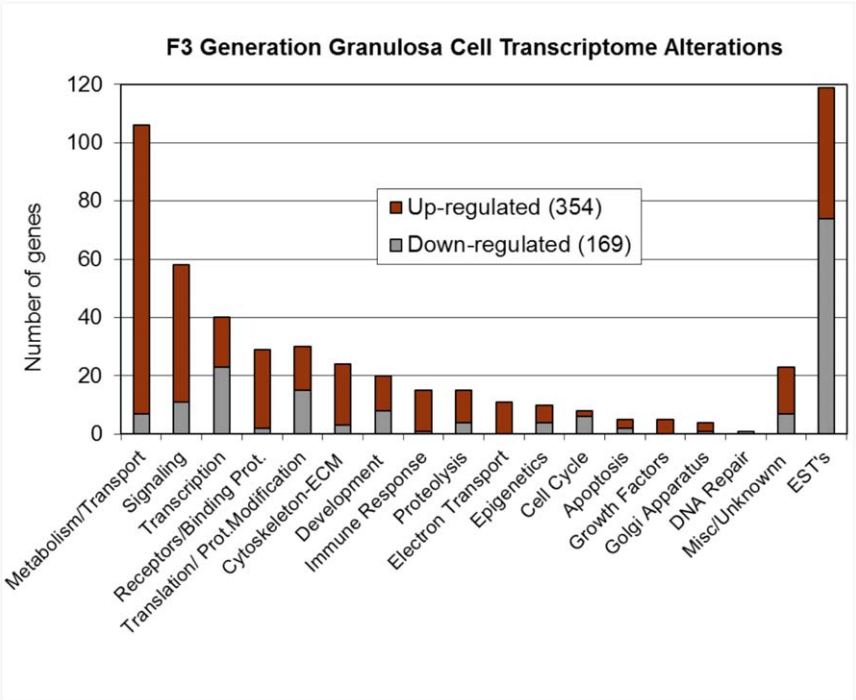

Figure 5. Number of genes with mRNA expression levels significantly different between Control and Vinclozolin-lineage F3 generation granulosa cells. Genes are placed into functional categories.
doi:10.1371/journal.pone.0036129.g005

Table 1. Physiological Pathway Enrichment.

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

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

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

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

Discussion

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

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

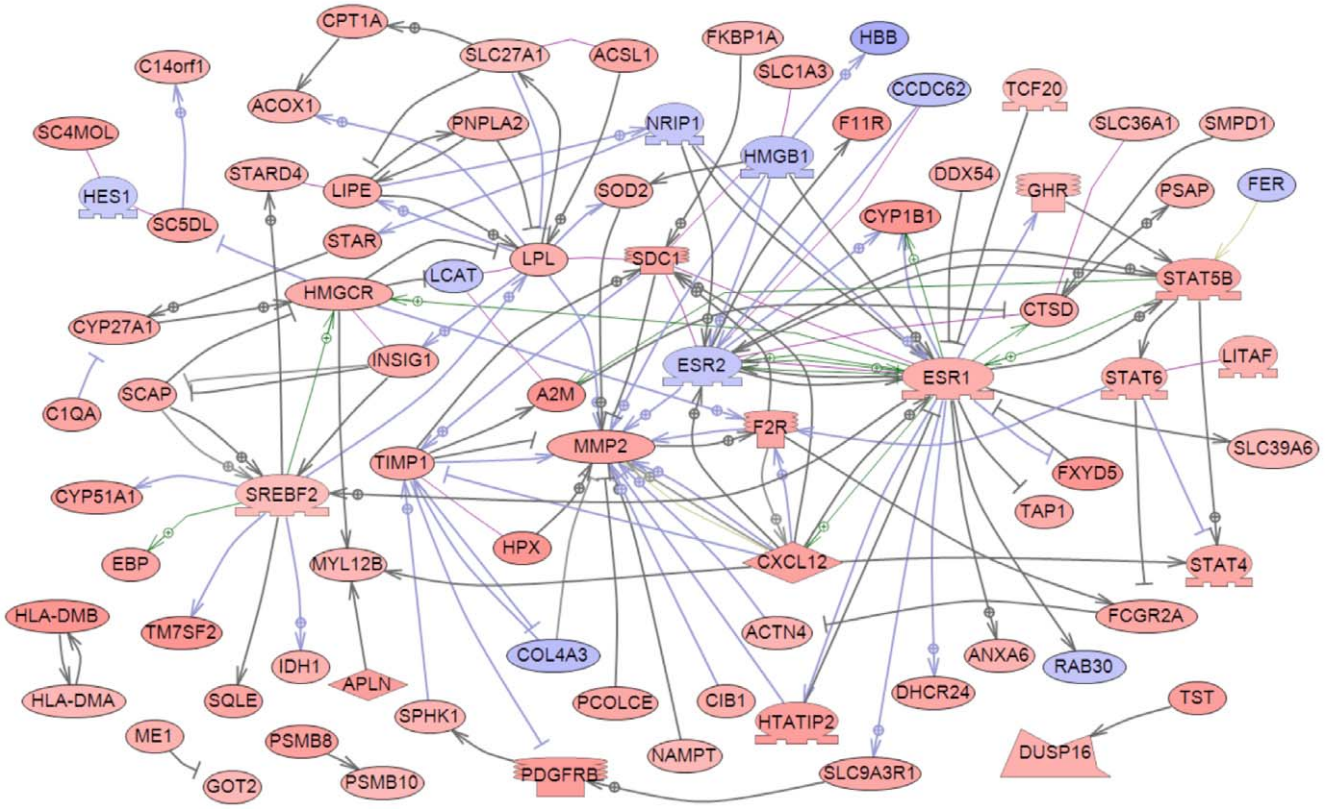

Figure 6. Gene network of known relationships among those genes found to be differentially expressed in Control compared to Vinclozolin-lineage F3 generation granulosa cells. Network is derived from an un-biased search of literature using Pathway Studio™. Node shapes code: oval and circle – protein;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

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

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

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

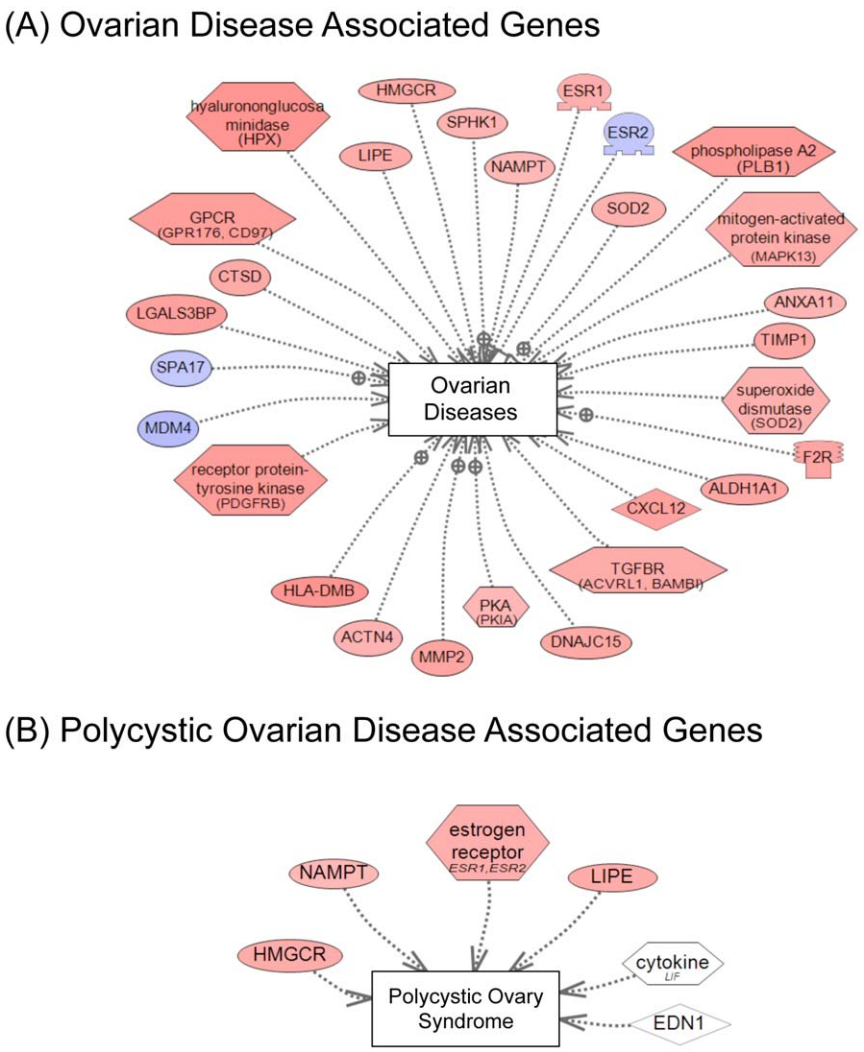

Figure 7. Ovarian diseases associated differentially expressed genes between F3 generation vinclozolin and control lineage granulosa cell. Sub-networks were identified using global literature analysis with Pathway Studio™. Node and arrow code is the same as for Figure 6. **A:** General ovarian diseases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

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

Molecular Etiology of Transgenerational Adult Onset Ovarian Disease

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

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

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

Table 2. Cont.

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

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

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

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

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

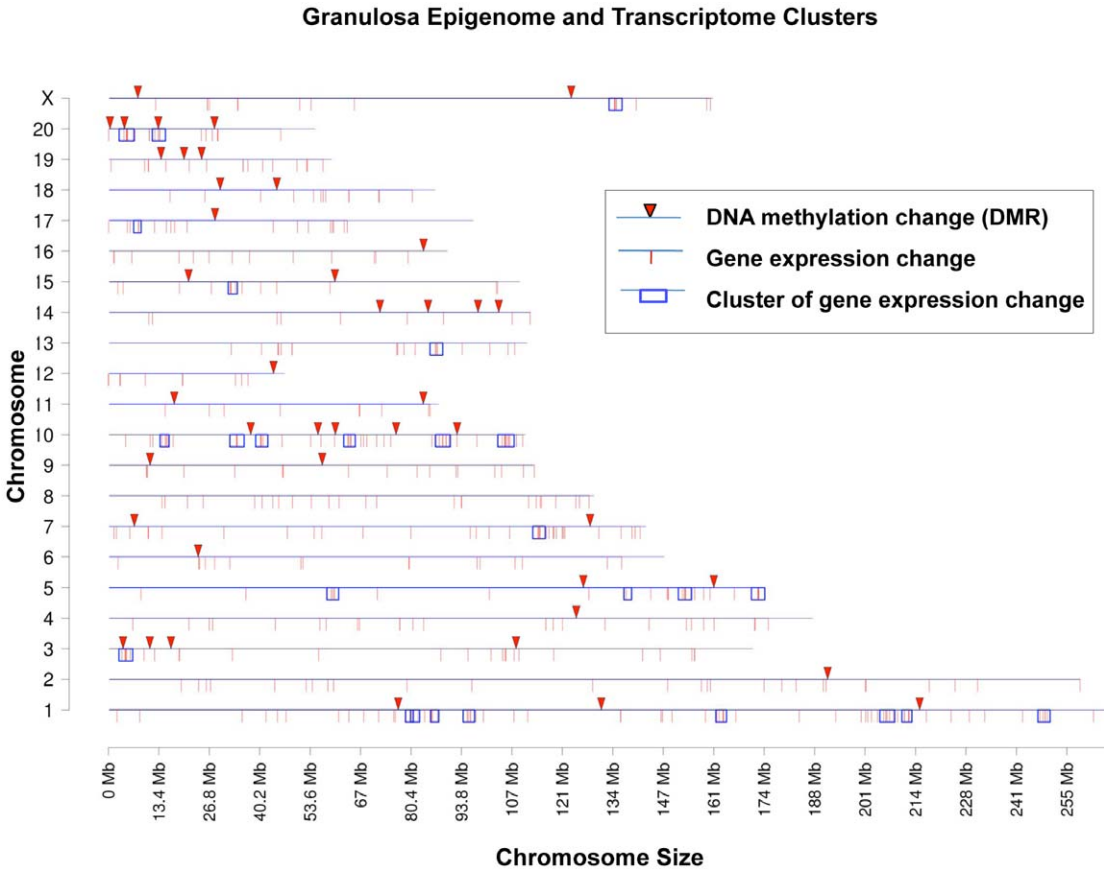

Figure 8. Chromosomal plot showing regions with vinclozolin-induced transgenerational changes in granulosa cells. Differential DNA methylation regions are displayed as inverted red triangles, changes in gene expression are displayed as red ticks, and a significant gene cluster of these genes with changed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

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

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

Summary

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

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

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

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

Methods

Animals and treatments

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

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

Breeding for F1, F2, and F3 generations

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

Evaluation of adult ovaries

Ovaries taken from rats at the time of sacrifice (one year of age) were fixed in Bouin's solution, paraffin embedded and sectioned at 5 μm thickness. Every 30th section was collected and hematoxylin/eosin stained. The three stained sections (150 μm apart) through the central portion of the ovary with the largest cross-section were evaluated microscopically for number of primordial follicles, developing pre-antral follicles, small antral follicles, large antral follicles, small cystic structures and large cysts. The mean number of each evaluated structure per section was calculated across the three sections. Follicles had to be non-atretic and have the oocyte nucleus visible in the section in order to be counted. Atretic follicles have granulosa cells or oocytes with pyknotic nuclei, an uneven or reduced layer of granulosa cells, and/or an uneven and less distinct basement membrane. Primordial follicles are in an arrested state and have an oocyte surrounded by a single layer of either squamous or both squamous and cuboidal granulosa cells [100,101]. Normally a few primordial follicles at a time will undergo primordial to primary follicle transition and become developing follicles. Developing pre-antral follicles had one or more complete layers of cuboidal granulosa cells. Small antral follicles had a fluid-filled antrum and a maximum diameter of 510 μm measured across the outermost granulosa cell layer. Large antral follicles had a diameter greater than 510 μm. Large antral follicles may eventually ovulate. Cysts were defined as fluid-filled structures of a specified size that were not filled with red blood cells and which were not follicular antra. A single layer of cells may line cysts. Small cysts were 50–250 μm in diameter measured from the inner cellular boundary across the longest axis. Large cysts were greater than 250 μm in diameter.

Neonatal rat ovary culture

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

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

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

Super-ovulation and collection of granulosa cells

F3 generation rats from both vinclozolin-treated and control lineages were treated with Pregnant Mare Serum Gonadotropin (Sigma cat, St. Louis, MO)(30 IU PMSG injected IP) at five to six months of age. Two days later animals were sacrificed and ovaries removed. The ovarian bursa and its adherent fat was removed from each ovary and the ovaries processed for granulosa cell collection [104]. The ovaries were suspended in the base medium used for all procedures was Ham's F-12 (Thermo Scientific). Following sequential 30 minute incubations at 37 °C in 6 mM EGTA in F-12 (to decrease Ca^{2+} - mediated cell adhesion) and then 0.5 M sucrose in F-12 (to increase osmotic pressure within follicles), ovaries were returned to F-12. Granulosa cells were released into the medium from antral follicles using 30-gauge needles and gentle pressure. Oocytes were removed by aspiration under a dissecting microscope. Granulosa cells from each rat were collected into 1.5 mL tubes, allowed to settle for 10 minutes and supernatant removed. 1.0 mL of Trizol^{TM} (Invitrogen) was added to each sample, and then samples were stored at -70° until the time of RNA and DNA isolation.

Microarray transcriptome analysis

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

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

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

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

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

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

Methylated DNA immunoprecipitation (MeDIP)

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

Tiling Array MeDIP-Chip Analysis

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

Bioinformatic and Statistical Analyses of Chip Data

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

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

Clustered Regions of interest were then determined by combining consecutive probes within 600 bases of each other, and based on whether their mean M values were positive or negative, with significance p-values less than 10^{-5}. The statistically significant differential DNA methylated regions were identified and P-value associated with each region presented. Each region of interest was then annotated for gene and CpG content. This list was further reduced to those regions with an average intensity value exceeding 9.5 (log scale) and a CpG density $\geq$1 CpG/100 bp.

Statistical Analysis for ovarian morphological data

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

Supporting Information

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

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

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

Table S2 Differential expressed gene clusters.
(PDF)

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

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Author Contributions

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

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