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Environmental Epigenetics

Primary Papers

1. Manikkam M, et al., (2012) Plos One 7(2):e31901.
2. Skinner MK, et al., (2012) Genome Biology 3;13(10):R91.
3. Skinner MK et al., (2015) Epigenetics 10:762-771.

Discussion

Student 30 – Ref #1 above

- What are the transgenerational phenotypes?
- How can the epigenetic biomarkers of exposure be used?
- What does the transgenerational actions of multiple environmental exposures suggest?

Student 31 – Ref #2 above

- What is the experimental design?
- What is the significance of a tissue specific transgenerational transcriptome?
- Why is an Epigenetic Control Region significant?

Student 32 – Ref #3 above

- What epigenetic and genetic technologies and alterations were investigated?
- How did epigenetic change promote genetic mutations and genome instability?
- What impact can environmental epigenetics have on genetics and inheritance?

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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.

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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

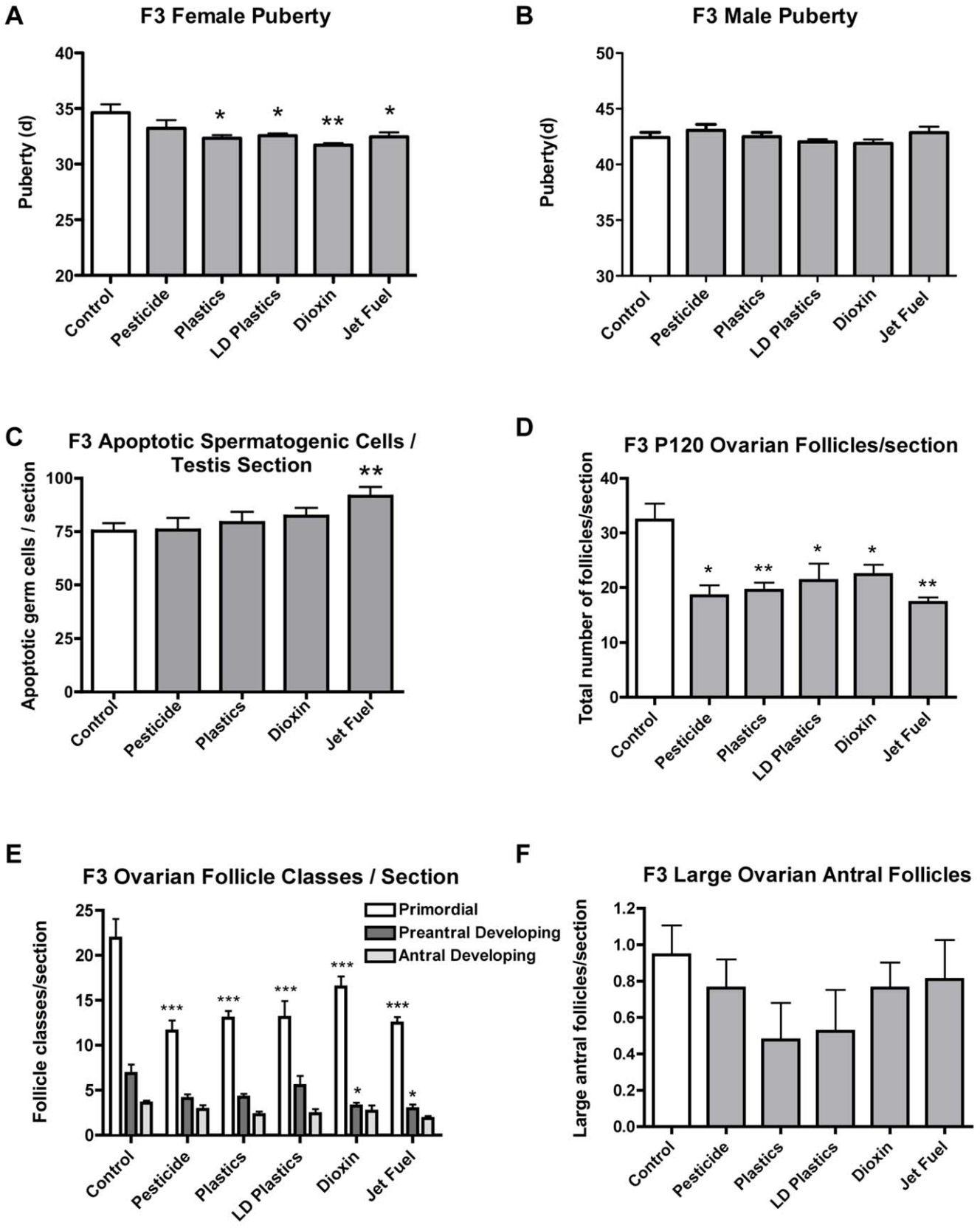

Figure 1. Ancestral (F0 generation female) exposures to environmental compounds promote transgenerational diseases, altering onset of puberty, testicular spermatogenic function and ovarian follicular development in F3 generation rat progeny. (A) Onset of female puberty was advanced from exposures to 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

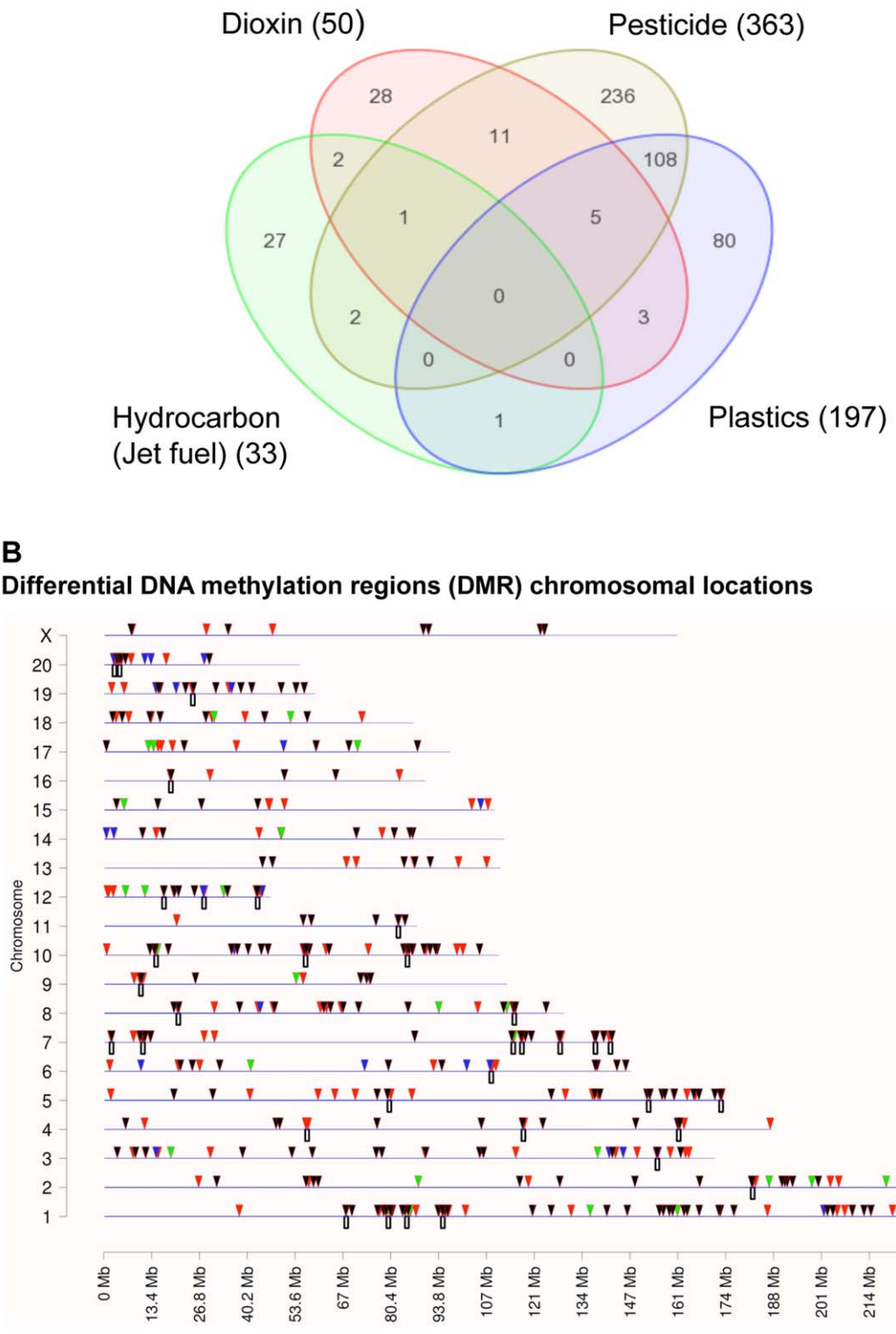

Figure 2. The transgenerational DMR associated with each exposure group identified. (A) Venn diagram of exposure DMR lists of F3 generation rat genes with differential DNA methylation due to *in vivo* exposure of F0-generation gestating female with Dioxin, Pesticide, Plastics or Hydrocarbons/Jet fuel. (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 ($p<0.05$).
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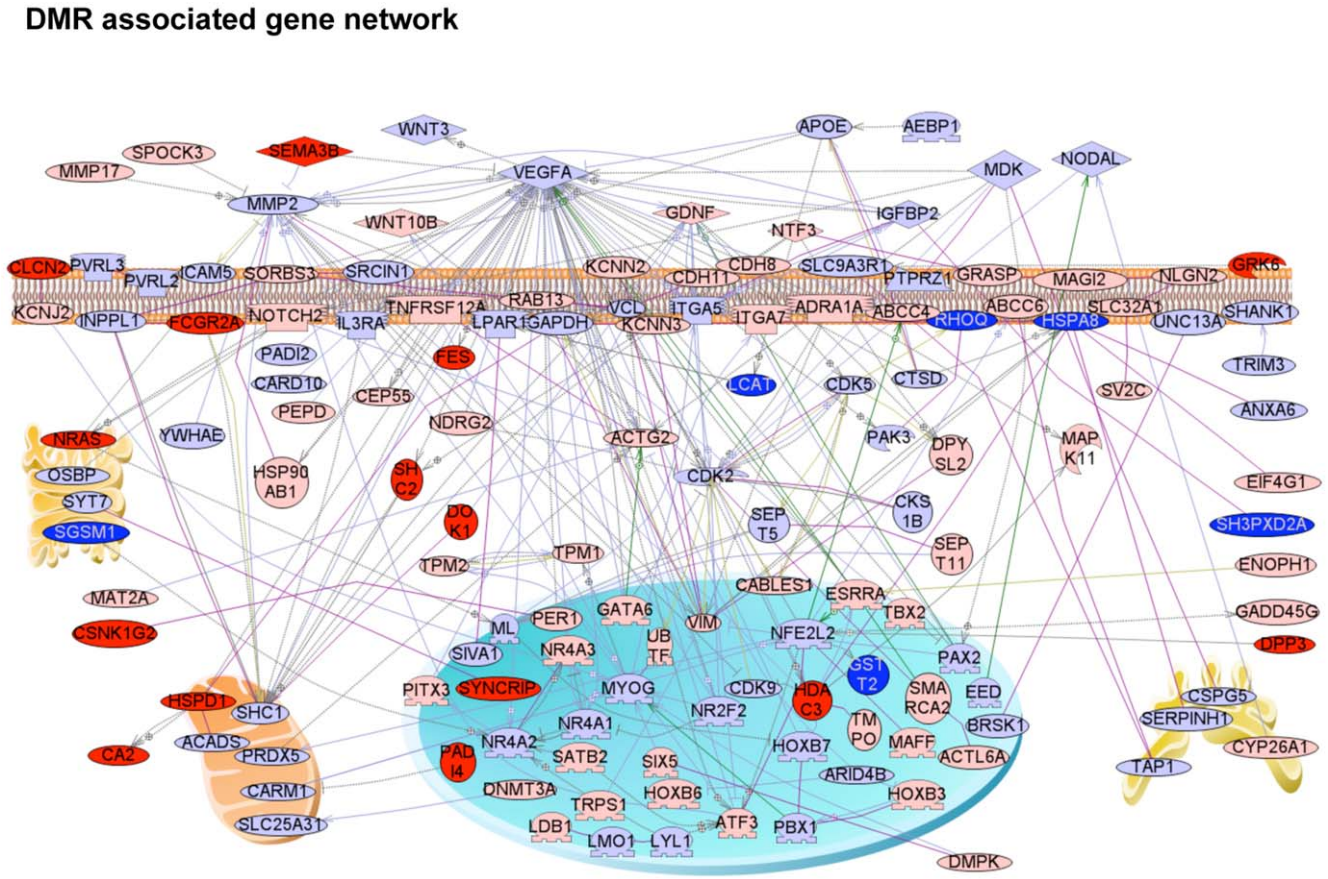

Figure 3. Direct connection gene sub-network for combined genes with transgenerational DMR associated exposures for Dioxin (red shapes), Pesticide (light blue shapes), Plastics (pink shapes) or Hydrocarbons/Jet fuel (dark blue shapes) indicated. Only 140 directly connected genes out of 499 unique genes 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

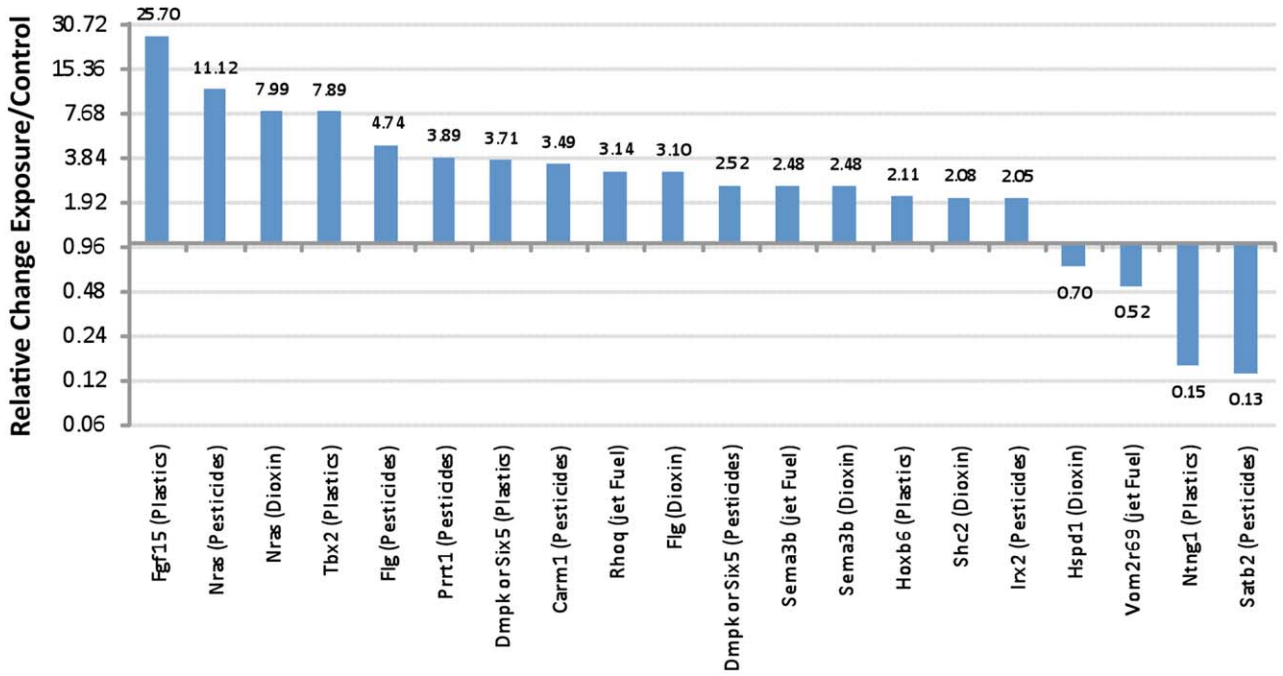

Figure 4. The MeDIP-qPCR analysis of (A) selected DMR for each exposure was used to confirm MeDIP-Chip analysis and (B) relative change (exposure/control) ratio presented for each DMR. All changes shown are statistically significant between control and exposure ($p<0.05$).
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and 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

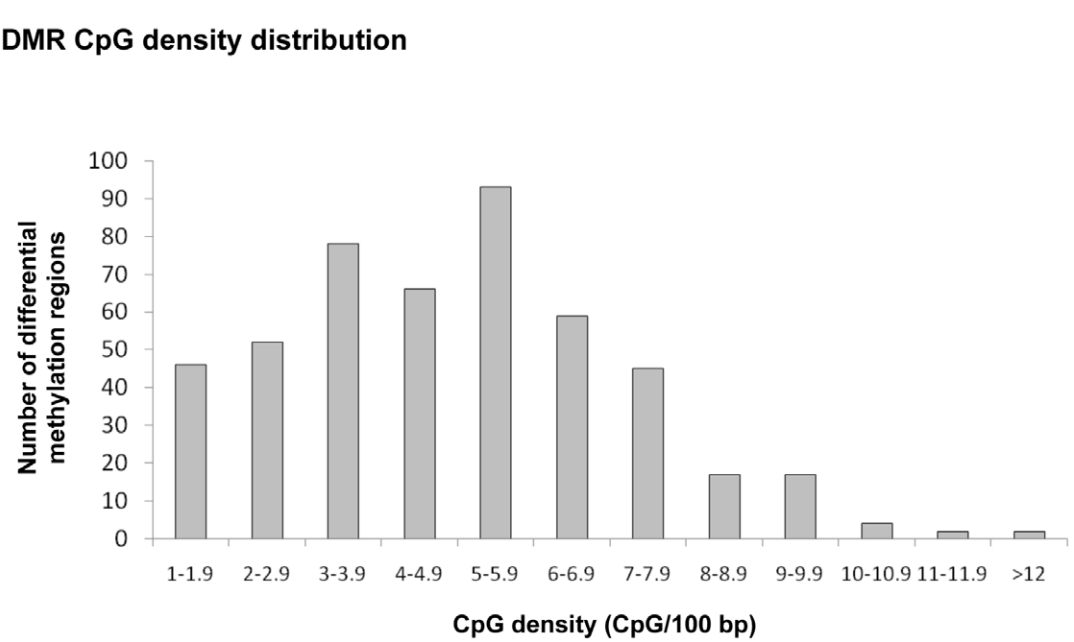

Figure 5. Differential DNA methylated region (DMR) CPG density distribution. The CpG density (CpG/100 bp) associated with all exposure DMR are presented with number of DMR on y axis and density (CpG per 100 bp) on x axis.
doi:10.1371/journal.pone.0031901.g005

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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Epigenetic transgenerational inheritance of somatic transcriptomes and epigenetic control regions

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abstract

Background: Environmentally induced epigenetic transgenerational inheritance of adult onset disease involves a variety of phenotypic changes, suggesting a general alteration in genome activity.

Results: Investigation of different tissue transcriptomes in male and female F3 generation vinclozolin versus control lineage rats demonstrated all tissues examined had transgenerational transcriptomes. The microarrays from 11 different tissues were compared with a gene bionetwork analysis. Although each tissue transgenerational transcriptome was unique, common cellular pathways and processes were identified between the tissues. A cluster analysis identified gene modules with coordinated gene expression and each had unique gene networks regulating tissue-specific gene expression and function. A large number of statistically significant over-represented clusters of genes were identified in the genome for both males and females. These gene clusters ranged from 2-5 megabases in size, and a number of them corresponded to the epimutations previously identified in sperm that transmit the epigenetic transgenerational inheritance of disease phenotypes.

Conclusions: Combined observations demonstrate that all tissues derived from the epigenetically altered germ line develop transgenerational transcriptomes unique to the tissue, but common epigenetic control regions in the genome may coordinately regulate these tissue-specific transcriptomes. This systems biology approach provides insight into the molecular mechanisms involved in the epigenetic transgenerational inheritance of a variety of adult onset disease phenotypes.

Background

Epigenetic transgenerational inheritance involves the germ line transmission of epigenetic marks between generations that alter genome activity and phenotype [1-3]. Environmental factors (for example, toxicants or nutrition) at a critical time during fetal gonadal sex-determination have been shown to alter DNA methylation programming of the germ line to promote the presence of imprinted-like sites that can be transmitted through the sperm to subsequent generations [1,4]. Animals derived from a germ line with an altered epigenome have been shown to develop adult-onset disease or abnormalities such as spermatogenic cell defects, mammary tumors, prostate disease, kidney disease, immune abnormalities and ovarian defects [5-7]. The epigenetic transgenerational inheritance of such abnormal phenotypes has been shown to develop in F1 to F4 generations after environmental exposure of only an individual F0 generation gestating female [1]. Recently, we have found a variety of environmental toxicants (plastics, pesticides, dioxin (TCDD), hydrocarbons, and vinclozolin) can promote the epigenetic transgenerational inheritance of adult-onset disease phenotypes [8]. Similar observations of epigenetic transgenerational inheritance of altered phenotypes have been shown in worms [9], flies [10], plants [11], rodents [1,5] and humans [12]. Environmentally induced epigenetic transgenerational inheritance provides an additional mechanism to consider in disease etiology and areas of biology such as evolution [2,13]. The current study was designed to provide insights into how a male germ line with an altered epigenome can transmit a variety of altered disease states and phenotypes.

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During migration down the genital ridge to colonize the fetal gonad, the primordial germ cells undergo an erasure of DNA methylation to allow a pluripotent state for the stem cell; then, at the onset of gonadal sex determination, DNA re-methylation is initiated in a sex-specific manner to generate the male or female germ line [2,14,15]. The germ line re-methylation is completed later in gonadal development. This developmental period in the mammal is the most sensitive to environmental insults for altering the epigenome (DNA methylation) of the male germ line [1,2,16]. After fertilization the paternal and maternal alleles are demethylated to, in part, develop the pluripotent state of the embryonic stem cells; re-methylation of these is then initiated at the blastula stage of embryonic development [2,14]. A set of imprinted genes escapes this demethylation to allow a specific DNA methylation pattern to be maintained and transferred between generations [17,18]. The ability of environmentally induced epigenetic transgenerational inheritance to transmit specific epigenetic changes between generations suggests the germ line epimutations act similarly to imprinted-like sites that, although they undergo developmental programming, develop a permanently programmed DNA methylation pattern [2,4]. Observations suggest environmentally induced epigenetic transgenerational inheritance involves the development of programmed epimutations in the germ line (sperm) that then escape the de-methylation after fertilization to transmit an altered epigenome between generations.

After fertilization the gametes transmit their genetics and epigenetics into the developing embryo and subsequently to all somatic cell types derived from the embryo. The altered sperm epigenome can then promote a cascade of altered epigenetic and genetic transcriptome changes into the developing cell types and tissues [19]. Therefore, the speculation is that all cells and tissues will have an altered transcriptome. These altered transcriptomes would appear throughout development to generate an adult tissue or cell type with an altered differentiated state associated with this transgenerational transcriptome [16,19]. Previously, epigenetic transgenerational inheritance of an altered testis transcriptome [20] and ovarian granulosa cell transcriptome [7] has been observed. Although some tissues may be resistant to dramatic alterations in physiology due to these transcriptome changes, other tissues that are sensitive will have an increased susceptibility to develop disease [2,7,16,20]. The current study was designed to investigate the epigenetic transgenerational inheritance of transcriptomes in a variety of different tissues and investigate potential gene bionetworks involved.

Gene expression of a specific cell type or tissue goes through a continuous cascade of changes from a stem cell through development to a stable adult differentiated state [7]. Similarly, the epigenome goes through a cascade of developmental changes to reach a stable epigenome in the adult associated with specific cell types [19]. The genetic and epigenetic components interact throughout development to promote the developmental and subsequent adult state of differentiation [16]. The classic paradigm for the regulation of gene expression involves the ability to alter promoter activity to regulate the expression of the adjacent gene. The epigenome plays an important role in this mechanism through histone modifications that fine tune the expression of the adjacent gene [21]. In contrast to histones, DNA methylation can be distal and not correlated with promoter regions, yet appears to regulate genome activity [22,23]. Although major alterations in DNA methylation of promoters clearly can alter gene expression, distal regulatory sites also have an important role in gene regulation [22,24]. One of the best examples of such a mechanism involves imprinted genes such as *H19* and *IGF2* [17]. The DNA methylation region of the imprinted gene in the promoter of the adjacent gene regulates allele-specific gene regulation for a wide number of genes. An additional role for these epigenetic DNA methylation sites can also be to influence distal gene expression through an imprinting control region (ICR) [23].

The ICR for *IGF2* and *H19* [17,25] has been shown to act through long non-coding RNA (lncRNA) and distally for over a megabase in either direction to regulate the expression of multiple genes [26,27]. Therefore, an epigenetic DNA methylation region can regulate the expression of a number of distal genes [17,28]. Similar observations have also been made in plant systems [29,30]. The speculation is made that a large family of epigenetic sites will have the ability to regulate the expression of multiple genes distally. These regions we term 'epigenetic control regions' (ECRs). The ICR previously identified will likely be a subset of a larger family of such regions not required to have an imprinted gene characteristic, but use a variety of mechanisms from non-coding RNA to chromatin structural changes. The current study was designed to identify the potential presence of such ECRs in the epigenetic transgenerational inheritance model investigated. The existence of such ECRs can help explain how subtle changes in the epigenome may have dramatic effects on the transcriptome of a cell type or tissue.

Environmentally induced epigenetic transgenerational inheritance of adult-onset disease and phenotypic variation [2] involves the germ line transmission of an imprinted-like epigenome (for example, DNA methylation) [4] that subsequently affects the transcriptomes of all cell types and tissues throughout the life of the individual derived from that germ line. The current study identifies transgenerational transcriptomes in all the tissues investigated in both female and male progeny. A systems biology approach was used to investigate the molecular and

cellular pathways and processes common to the epigenetic transgenerational inheritance of the tissue transcriptomes identified. Gene bionetwork analysis was used to identify underlying gene networks that may provide insight into the epigenetic control of the differential gene expression. Combined observations identified potential ECRs that help explain, in part, how a tissue-specific transgenerational transcriptome was generated and how a subtle alteration in the germ line epigenome may promote adult onset disease phenotypes.

Results

Transgenerational transcriptomes

The experimental design involved developing F3 generation Harlan Sprague Dawley rat control and vinclozolin lineage male and female adult animals as previously described [1,5]. The F0 generation gestating females were transiently exposed to vinclozolin or vehicle (DMSO) control during embryonic day 8 to 14 (E8 to E14) and then F1 generation offspring bred to produce the F2 generation followed by production of the F3 generation as described in the Materials and methods. No sibling or cousin breedings were used to avoid any inbreeding artifacts. Animals were aged to 4 months and then sacrificed to collect from males the testis, seminal vesicle, prostate, liver, kidney and heart; and from females the ovary, uterus, liver, kidney and heart. A total of six different control and six different vinclozolin F3 generation lineage animals, each one from different litters, were used and microarrays ran on each tissue using three pools of two animals each. A total of 66 microarrays were run on F3 generation control and vinclozolin lineage male and female rat tissues. The microarray data were obtained and compared for quality control as shown in Additional file 1. All microarrays within a tissue set compared well with no outliers, so all were used in subsequent data analysis. A comparison of control lineage and vinclozolin lineage tissues was made to identify the differentially expressed genes consistent between all animals and microarrays with a minimum of a 1.2-fold change in expression and mean difference of raw signal >10 as previously described [31]. As outlined in the Materials and methods, since a 20% alteration in gene expression can have cellular and biological impacts, particularly for transcription factors, the gene expression used a 1.2-fold cutoff that had a statistical difference rather than minimize the list with a more stringent cutoff value. The mean difference cutoff was used to eliminate background level signal expression changes. Differential gene expression with a statistical significance of $P < 0.05$ was used to identify the differentially expressed gene sets for each tissue; these are termed the 'signature list'. These less stringent criteria led to a relatively larger number of genes for the subsequent network analysis that can further filter out noisy signal using advanced soft thresholding techniques. The signature lists for all tissues are presented in Additional file 5 and genes categorized functionally. A summary of the signature list gene sets is presented in Figure 1.

The general overlap of genes between the tissues and between males and females is shown in Figure 1. These differentially expressed genes in the various tissues represent transgenerational transcriptomes in the F3 generation. No predominant overlap with large numbers of differentially exposed genes were found between the different tissues and between male and female lists (Figure 1). A specific comparison of genes between the tissues for male and female is presented in Figure 2. Venn diagrams show the majority of differentially expressed genes are tissue-specific with negligible overlap among all tissues. Therefore, each tissue had a predominantly unique transgenerational transcriptome and negligible overlap was observed between male and female tissues.

The specific differentially expressed genes were placed in Gene Ontology (GO) functional categories from Affimetrix annotations and similar trends were found among the different tissue signature lists and between the male and female lists. Therefore, no specific functional categories were predominant in any of the individual lists and no major differences exist. The categories are shown in Figure 3 for all tissues. Further analysis of specific cellular pathways and processes determined the number of genes associated with the various tissue signature lists. A list of those pathways containing the highest number of genes altered within the pathway or process for the top 30 is provided in Table 1. A more extensive list of differentially expressed genes correlating to specific pathways and processes is provided in Additional file 6. Observations demonstrate no predominant pathways or cellular processes were associated with the various signature lists. In contrast, a relatively large number of pathways and processes were influenced by all the tissue signature lists (Figure 1).

Gene bionetwork analysis

Gene networks were investigated using a previously described bionetwork analysis method [31] that utilizes all the array data to examine coordinated gene expression and connectivity between specific genes [32,33]. Initially, cluster analysis of the differential gene expression lists was used to identify gene modules, which were then used to identify gene networks and functional categories. The connectivity index (k.in) for individual genes is shown in Additional file 5 and the number of connections for each gene with a cluster coefficient for male and female list comparisons is shown in Additional file 2. A cluster analysis was performed on the combined male tissue signature lists, the combined female tissue signature lists and a

Sex.Tissue	Signature Lists	Increased	Decreased	F.Heart	F.Kidn	F.Liver	F.Ovary	F.Uterus	All F-lists	M.Heart	M.Kidn	M.Liver	M.Prostate	M.SV	M.Testis	All M-lists
	Number of Pathways			108	79	29	80	138	142	79	151	141	191	90	99	152
F.Heart	**406**	336	70		38	14	8	5	**108**	47	67	70	147	48	45	152
F.Kidn	**150**	83	67	9		12	28	54	**79**	26	52	53	62	38	27	152
F.Liver	**99**	36	63	5	0		10	18	**29**	13	19	17	20	12	14	152
F.Ovary	**305**	211	94	13	5	1		56	**80**	34	51	51	66	42	33	152
F.Uterus	**279**	138	141	7	2	3	14		**138**	60	92	89	115	67	59	152
All F-lists	**1298**	865	433	**406**	**151**	**99**	**305**	**55**		66	107	107	138	73	67	152
M.Heart	**172**	57	115	6	3	4	3	5	19		55	56	147	36	43	**79**
M.Kidn	**725**	385	340	64	20	6	23	12	117	16		84	149	61	55	**151**
M.Liver	**266**	166	100	7	6	2	6	9	27	5	7		150	60	47	**141**
M.Prostate	**1112**	276	836	24	14	3	25	26	90	3	45	14		72	70	**191**
M.SV	**274**	105	169	12	3	2	6	8	29	1	20	6	20		36	**90**
M.Testis	**552**	203	349	8	17	5	14	1	45	4	21	5	34	8		**99**
All M-lists	**3046**	1196	1850	109	58	19	61	55	285	**172**	**725**	**266**	**1112**	**274**	**552**	

Pathway overlap

Genes overlap

Figure 1 Number of differentially expressed genes and pathways that overlap between signature lists. The total number of genes or pathways for a signature list is shown in bold and only pathways with three or more affected genes are counted. F, female; M, male; SV, seminal vesicle.

combination of all female and male signature lists (Figure 4). Gene modules were identified that involved coordinated gene expression and connectivity between the genes assessed. The modules are shown in colors on the axes, with white indicating no connectivity and red highest connectivity (Figure 4). The heat diagram identified modules as boxed gene sets and assigned them a specific color. The combined male and female cluster analysis demonstrates strong modularity (Figure 4c), but the sexually dimorphic transgenerational transcriptomes identified in Figure 2 suggest that sex-specific cluster analysis and modules will be more informative, and these were used in all subsequent analyses. A list of sex-specific modules and represented gene sets are shown in Table 2. Identification of co-expressed gene modules is actually a process to enhance the signal by filtering out noisy candidates using advanced soft thresholding and network techniques. To access the robustness of the approach with respect to different cutoffs for detecting differentially expressed genes, we also constructed additional male and female co-expression networks based on a more stringent mean difference cutoff of a 1.5-fold change in gene expression. The 1.5-fold networks have a smaller number of modules than their counterparts, but all the modules from the 1.5-fold networks all significantly overlapped (Fisher's exact test P-values < 1.6e-7) with the modules identified in the previous networks based on a mean difference cutoff of 1.2-fold change in gene expression.

The correlation of the gene modules with cellular pathways and processes is shown in Additional file 7. A relatively even distribution is observed for the various pathways with no significant over-representation. As observed with the tissue signature lists, similar pathways with the largest numbers of genes affected are represented (Additional file 7). Therefore, no predominant cellular pathway or process was observed within the gene modules identified.

Gene network analysis was performed to potentially identify the distinct or common connections between the various tissue signature lists and gene modules identified. A direct connection indicates a functional and/or binding interaction between genes while indirect connections indicate the association of a gene with a cellular process or function. This analysis used the literature-based Pathway Studio software described in the Materials and methods. Analysis of the female gene modules identified only one module (turquoise) that had a direct connection network (Additional file 3A). The gene network analysis of the male modules found that the yellow, brown and turquoise modules have direct connections (Additional file 3). None of the other female or male modules had direct connection gene networks. Therefore, no specific gene networks were

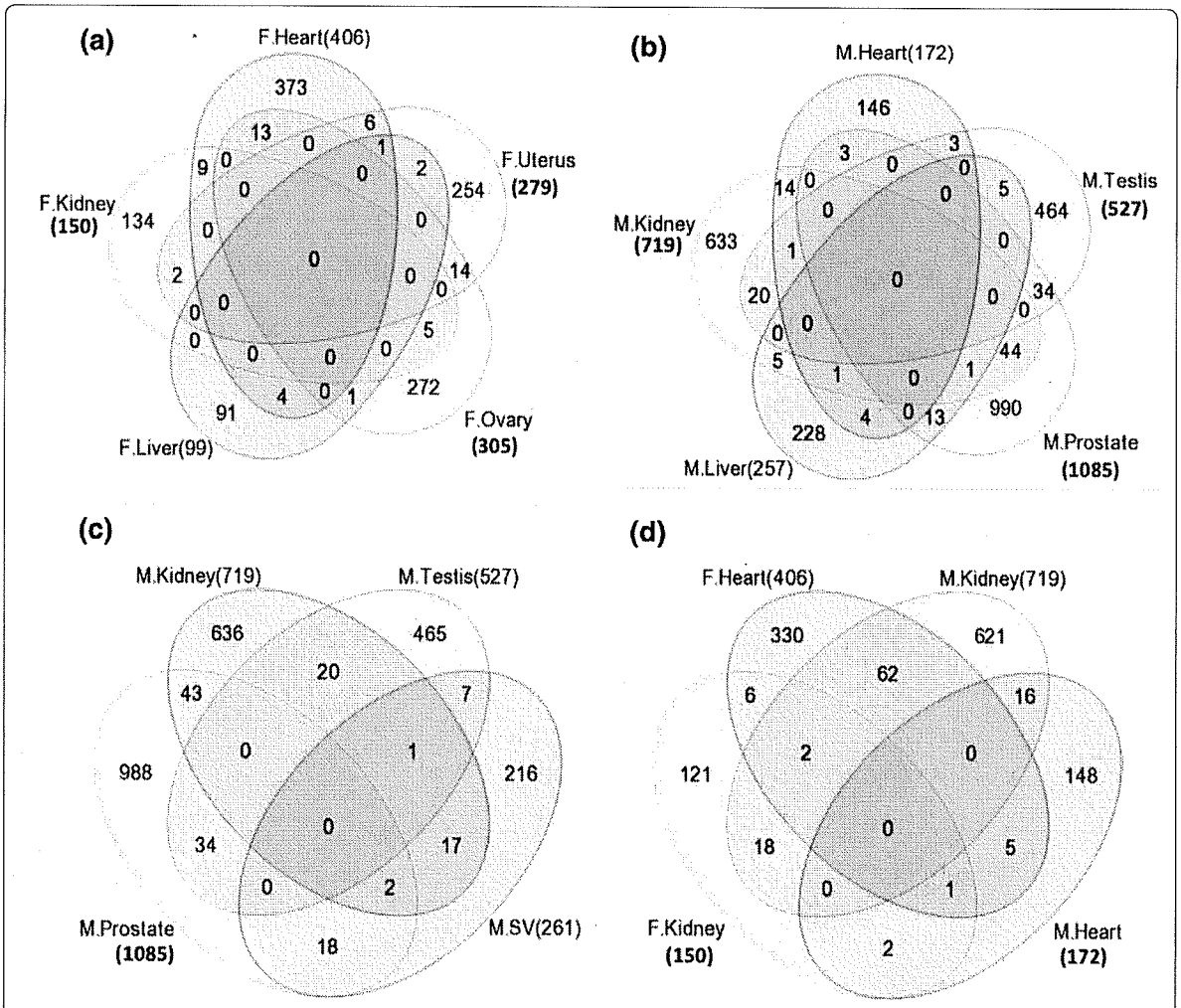

Figure 2 Venn diagrams of male and female tissue signature lists of F3 generation vinclozolin lineage differentially expressed genes. **(a)** Female (F) heart, kidney, liver, uterus, and ovary. **(b)** Male (M) heart, kidney, liver, testis, and prostate. **(c)** Male kidney, testis, seminal vesicle (SV), and prostate. **(d)** Female heart and kidney and male heart and kidney. Numbers in brackets are the total number of genes in the signature list.

common between the gene modules. The possibility that the tissue signature lists of differentially expressed genes may contain gene networks was also investigated. The majority of tissue signature lists confirmed the direct connection gene networks (Additional file 4). Analysis of the individual tissue gene networks did not show any major overlap or common regulatory gene sets within the different gene networks. Therefore, each tissue acquires a different and unique gene network that is also distinct between the sexes (sexually dimorphic; Additional file 4).

The cluster analysis (Figure 4) identified gene modules with genes with coordinated gene regulation and a connectivity index (k.in) was identified (Additional files 2 and 5). The top 10% of genes from each module with the highest connectivity index were combined for male (258 total genes) and female (75 total genes) gene modules, and gene networks identified for the male and female gene sets (Figure 5). The combined female gene module top 10% connectivity gene network identified only five directly connected genes as critical components of the network. This indicates the general lack of an underlying gene network in the female tissue modules. The combined male gene module network identified over 30 directly connected genes as critical components (Figure 5b). Although the tissue-specific gene networks are different and unique (Additional file 4), a combined gene network of the most highly connected and critical genes in the gene modules was identified for the male. Although a common gene network among the various tissues does not appear to be involved in the epigenetic transgenerational inheritance

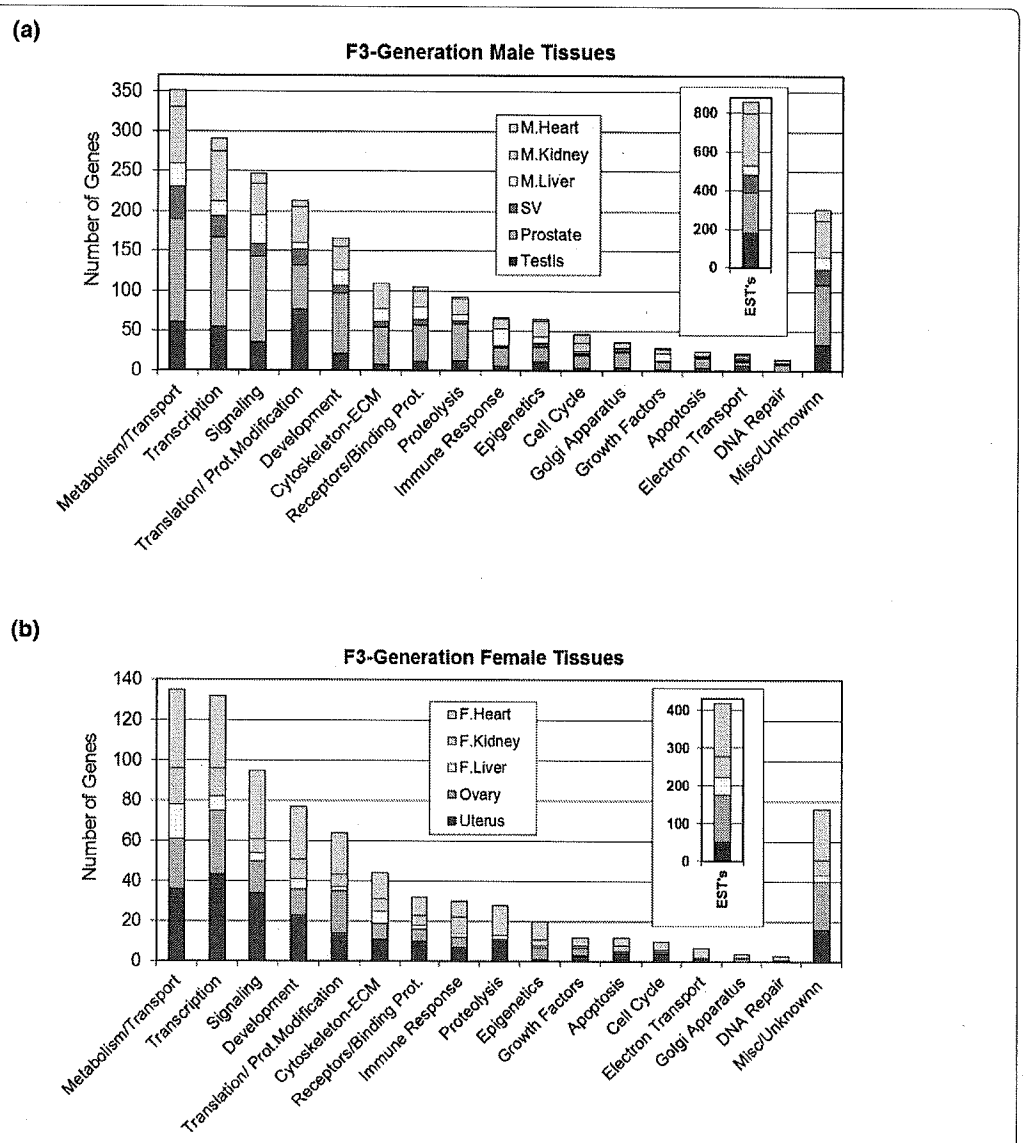

Figure 3 Number of genes differentially expressed in F3 generation vinclozolin lineage tissues and their distribution among main functional categories. **(a)** Male (M) heart, kidney, liver, testis, seminal vesicle (SV), and prostate. **(b)** Female (F) heart, kidney, liver, uterus, and ovary. ECM, extracellular matrix.

mechanism, a network involving the most connected genes between the tissues was identified for the male (Figure 5). Observations suggest additional molecular mechanisms may be involved.

Epigenetic control regions

The total number of all differentially expressed genes in the tissue signature lists was 1,298 for female and 3,046 for male (Figure 1). The possibility that the chromosomal location of these genes may identify potential regulatory sites was investigated. All the genes for the female and male were mapped to their chromosomal locations and then a sliding window of 2 Mb was used to determine the regions with a statistically significant (Z test, $P < 0.05$) over-representation of regulated genes (Figure 6a,b). The analysis identified gene clusters in regions 2 to 5 Mb in size on nearly all chromosomes that have a statistically significant over-representation of regulated genes (Table 3). Several ECRs are up to 10 Mb, which we suspect involves adjacent ECRs. As these regions were associated with the epigenetic transgenerational inheritance of these tissue-specific transcriptomes, we termed them 'epigenetic control regions'.

Table 1 Pathway enrichment for 11 male and female rat tissue signature lists

	11 male and female tissues	Sex and tissue												
		Male							Female					
		6 tissues	Heart	Kidney	Liver	Prostate	SV	Testis	5 tissues	Heart	Kidney	Liver	Ovary	Uterus
Pathway name														
Number of genes in input list	4059	3046	172	725	266	1112	274	552	1298	408	151	99	305	279
Total number of affected pathways	230	224	79	151	141	191	90	99	191	108	79	29	80	138
Pathways in cancer	45	32	3	7	6	13	1	2	17	6	3	1	3	4
Protein processing in endoplasmic reticulum	39	38	3	4	1	22	5	4	3	1			1	1
HTLV-I infection	39	31		8	3	14	3	2	10	4	2		1	3
RNA transport	39	31	1	8		17	3	6	11	4		1	2	4
Transcriptional misregulation in cancers	31	26	1	8	6	7	4	3	12	6	3	1		3
Herpes simplex infection	30	23	2	10	3	7	2	1	9	3	1		2	3
Lysosome	29	27	1	5	4	16	2	2	5	1			1	3
Ribosome	29	27	2	5		1		20	4	1	1	2		
Endocytosis	29	26	1	4	8	11	1	3	5	2				3
Phagosome	28	27	1	6	6	12		2	7		3			4
MAPK signaling pathway	28	21	1	7	3	7	1	2	8	3			1	4
Spliceosome	27	17		8	1	1	2	7	12	6	1		1	5
Regulation of actin cytoskeleton	26	24	1	6	6	9		3	5	3		1		1
Alzheimer's disease	26	22	2	4		7		10	5				2	3
Huntington's disease	26	22	1	6		2		14	5	1		1	2	1
Purine metabolism	26	22	1	6	2	4	2	6	7	2				5
Focal adhesion	26	21	2	2	7	8		3	9	3		2		4
Chemokine signaling pathway	24	23	3	4	6	9	1	1	4				2	2
Pyrimidine metabolism	24	20	1	6	1	6	1	5	6	1		1	1	3
Tuberculosis	24	20		6	6	9	1		9	1	1			7
Influenza A	23	21	1	5	2	11	1	3	5	1	2			2
Oxidative phosphorylation	23	20	1	4		5		11	5		1		1	3
Leukocyte transendothelial migration	22	18	2	1	7	8		1	6	4	1			1
Cytokine-cytokine receptor interaction	21	19	1	6	7	6			4	3				1
Osteoclast differentiation	21	18	1	2	6	10	1		5	1			1	3
Cell adhesion molecules	20	14	2	3	4	6	1		7	3	1		1	2
Insulin signaling pathway	19	13		2	3	6	2		6	2				4
mRNA surveillance pathway	19	13	1	4		5	2	4	8	4			2	2

HTLV, human T-lymphotropic virus; MAPK, mitogen-activated protein kinase; SV, seminal vesicle.

The specific ECRs are presented in Figure 7 for the female and male combined signature lists. A comparison of the female and male tissue ECRs demonstrated many were in common. The common and sex-specific ECRs are shown in Figure 7. The number of differentially regulated genes associated with these ECRs ranged from 5 to 70 (Table 3). Selected ECRs from the male and female were mapped to demonstrate the differentially expressed genes in the ECRs

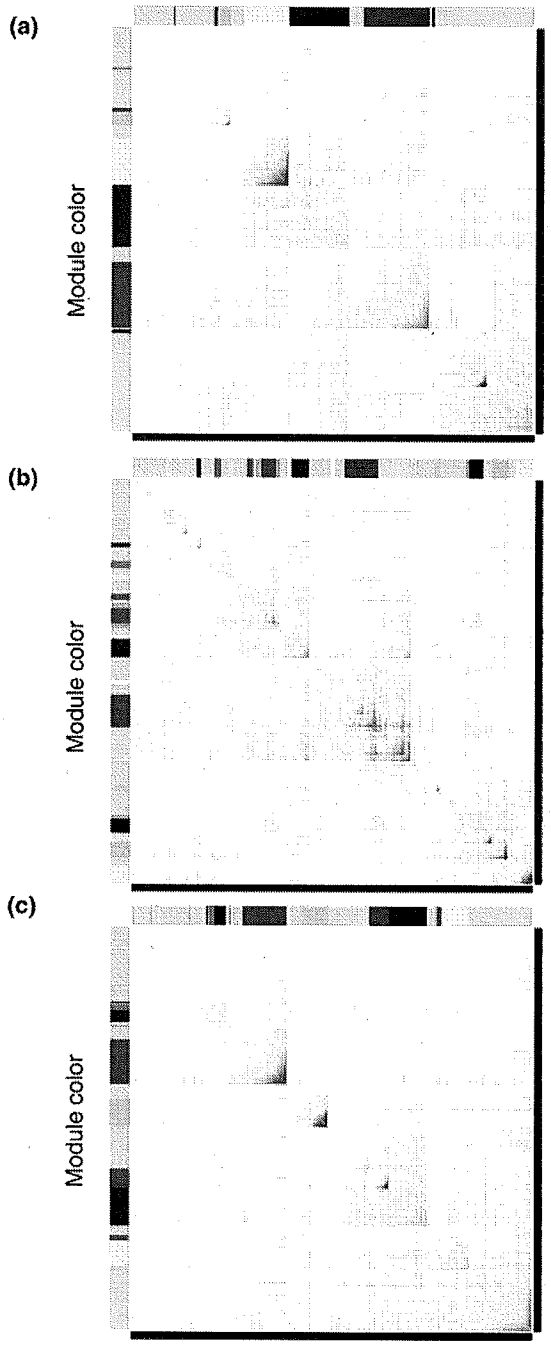

Figure 4 Gene bionetwork cluster analysis of 11 male and female tissues with corresponding gene modules. Topological overlap matrixes of the gene co-expression network consisting of genes differentially expressed in 11 tissues of the F3 vinclozolin lineage compared to F3 control lineage animals. Genes in the rows and columns are sorted by an agglomerative hierarchical clustering algorithm. The different shades of color signify the strength of the connections between the nodes (from white signifying not significantly correlated to red signifying highly significantly correlated). The topological overlap matrix strongly indicates highly interconnected subsets of genes (modules). Modules identified are colored along both column and row and are boxed. **(a)** Matrixes of the combined network for six male tissues. **(b)** Matrixes of the combined network for five female tissues. **(c)** Matrixes of the combined network for 11 male and female tissues.

Table 2 Overlap of male and female signature list genes with network modules

Signature list modules	Signature list	Tur	Blu	Brn	Red	Yel	Grn	Blk	Pink	Mag	Pur	Grn-yl	Tan	Sal	Cyn	M-blu	L cyn	Grey	L grn	L yl	Crl
Female		148	137	78	51	22	33	28	31	30	20	25	17	20	18	21	16	19	13	14	10
Heart	406	130	111	12	2	1	0	2	1	2	1	7	0	0	1	0	12	1	10	2	0
Kidney	151	1	13	0	5	4	1	2	0	22	1	10	0	1	17	2	0	0	0	0	0
Liver	99	2	6	3	0	0	0	1	0	1	0	5	0	0	0	2	2	0	1	0	0
Ovary	305	8	5	25	0	17	10	21	29	0	0	0	17	0	0	6	1	0	0	6	10
Uterus	279	7	0	38	44	0	14	2	1	0	18	2	0	19	0	8	1	18	0	3	0
All female lists, unique	1,298																				
Male		1016	363	525	426	48	7	24	33	27											
Heart	172	5	32	35	21	3	0	0	3	1											
Kidney	725	86	199	114	84	8	3	2	21	12											
Liver	266	41	10	84	14	2	0	0	0	8											
Prostate	1,112	736	56	39	9	26	0	11	1	0											
SV	274	47	28	77	10	0	0	0	7	4											
Testis	552																				

Tur, turquoise; Blu, blue; Brn, brown; Yel, yellow; Grn, green; Blk, black; Mag, magenta; Pur, purple; Grn-yl, green-yellow; Sal, salmon; Cyn, cyan; M-blu, midnight blue; L cyn, light cyan; L grn, light green; L yl, light yellow; Crl, coral. SV, seminal vesicle.

(Figure 8). An ECR common between male and female in chromosome 10 is shown in Figure 8a. The ECRs may provide a coordinated mechanism to regulate a set of functionally related genes that are expressed in different tissues (Additional file 8). Therefore, a limited number of regulatory sites such as the identified ECRs could regulate tissue-specific and sexually dimorphic gene expression from similar regions. However, the current study was designed simply to identify the ECRs, and their functional role remains to be established. The genes within the male and female ECRs were used to generate gene networks. The female ECR-associated genes generated a network with connection to cellular differentiation, cellular acidification and endocytosis (Figure 9a). The male ECR-associated genes generated a network linked with a larger number of cellular processes (Figure 9b). Therefore, no predominant gene network or cellular process was associated with the identified ECRs.

Previously, the ICRs identified have been shown to be associated with lncRNAs. Similar distal regulation involving lncRNAs has also been shown in plants [29,30]. The rat genome lncRNAs have not been fully characterized [34], but 20 rat lncRNAs have been reported. The possibility that these known rat lncRNAs may correlate with the identified ECRs was investigated (Figures 7 and 8). Interestingly, over half the known rat lncRNAs did correlate with the male and female ECRs. A full list of all these lncRNAs is provided in Additional file 9. Although more extensive characterization of the rat lncRNAs is required, those few known rat lncRNAs did correlate strongly with the identified ECRs. The functional role of these lncRNAs within the ECRs remains to be elucidated.

Vinclozolin-induced sperm epimutations associated with epigenetic transgenerational inheritance of adult-onset disease phenotypes have been reported [4]. Comparison of the chromosomal locations of 21 F3 generation sperm epimutations with the identified ECRs showed that they are correlated. Although specific sperm epigenetic alterations and clustered gene expression may be functionally related, further research regarding the specific epigenetic modifications within the ECRs remains to be investigated.

Discussion

Environmentally induced epigenetic transgenerational inheritance of adult-onset disease requires an epigenetically modified germline to transmit an altered baseline epigenome between generations [1,2]. The current study utilized the commonly used agricultural fungicide vinclozolin [35], which has been shown to induce epigenetic transgenerational inheritance of disease [1,5] and permanently alter the sperm epigenome (DNA methylation) [4]. Vinclozolin has been shown to promote in F3 generation lineage animals a number of adult-onset diseases, including of testis, prostate, kidneys, the immune system, and behavior and cancer [5,36]. This high degree of a variety of adult-onset disease states suggests that baseline alteration of the sperm epigenome influences the subsequent development and function of most tissues and cell types [16]. Other factors shown to promote epigenetic transgenerational inheritance of disease include bisphenol A [8,37], dioxin [8,38], pesticides [1,8], hydrocarbons (jet fuel) [8] and nutrition [39,40]. Therefore, a number of environmental factors have been shown to promote epigenetic transgenerational inheritance of phenotypic variation and this occurs in most species [2]. The current study was

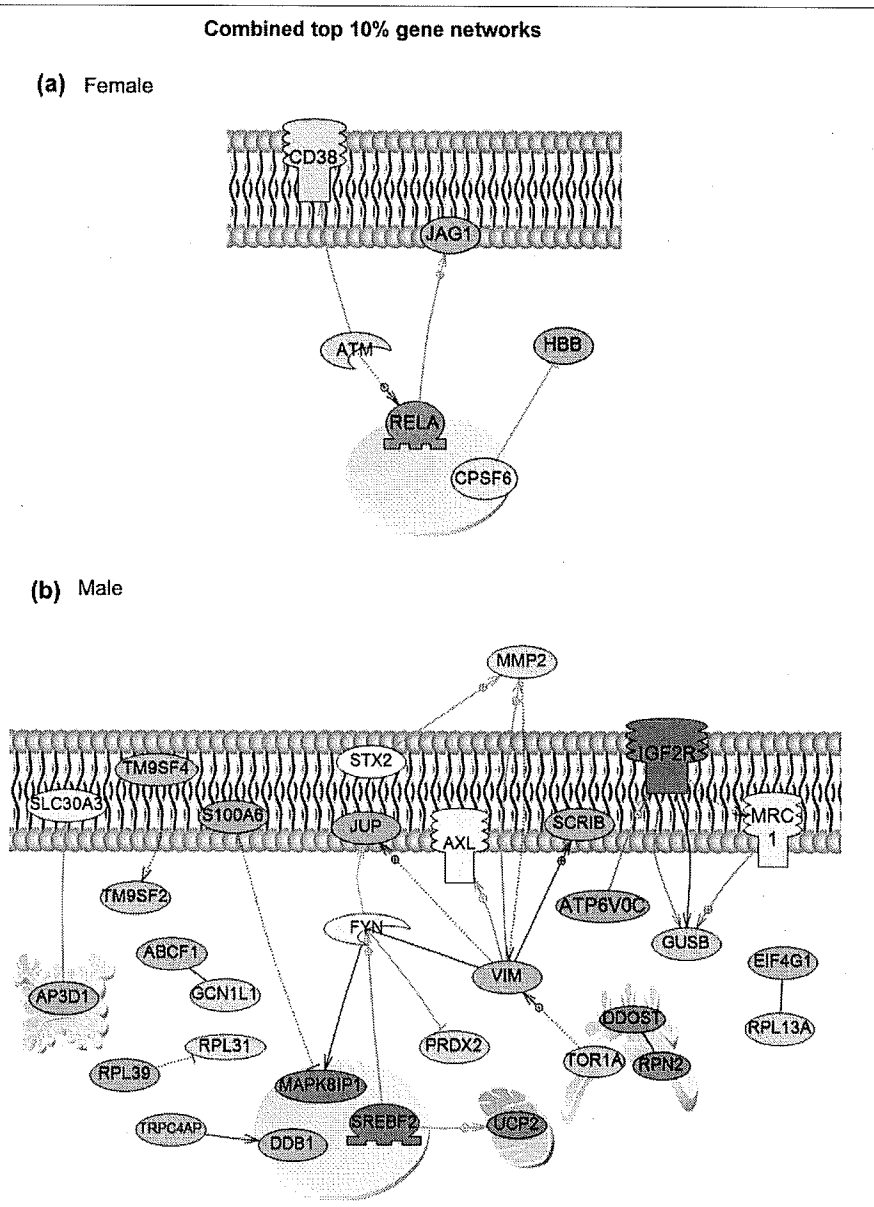

Figure 5 Direct connection gene sub-networks for the top 10% interconnected genes from each module of the separate networks for female and male obtained by global literature analysis. **(a)** Female; **(b)** male. Directly connected genes only are shown according to their location in the cell (on the membrane, in the Golgi apparatus, nucleus, or cytoplasm or outside the cell). Node shapes: 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. Color code: red, up-regulated genes; blue, down-regulated genes. Arrows with a plus sign indicate positive regulation/activation; arrows with a minus sign indicate negative regulation/inhibition; grey arrows represent regulation; lilac arrows represent expression; purple arrows represent binding; green arrows represent promoter binding; yellow arrows represent protein modification.

designed to investigate how an altered germline epigenome promotes transgenerational adult-onset disease in a variety of different tissues.

Upon fertilization, the germline (egg or sperm) forms the zygote and the developing embryo undergoes a de-methylation of DNA to create the totipotent embryonic stem cell. As the early blastula embryo develops, DNA re-methylation is initiated, promoting tissue- and cell-specific differentiation [14,15]. A set of imprinted gene DNA methylation regions are protected from this

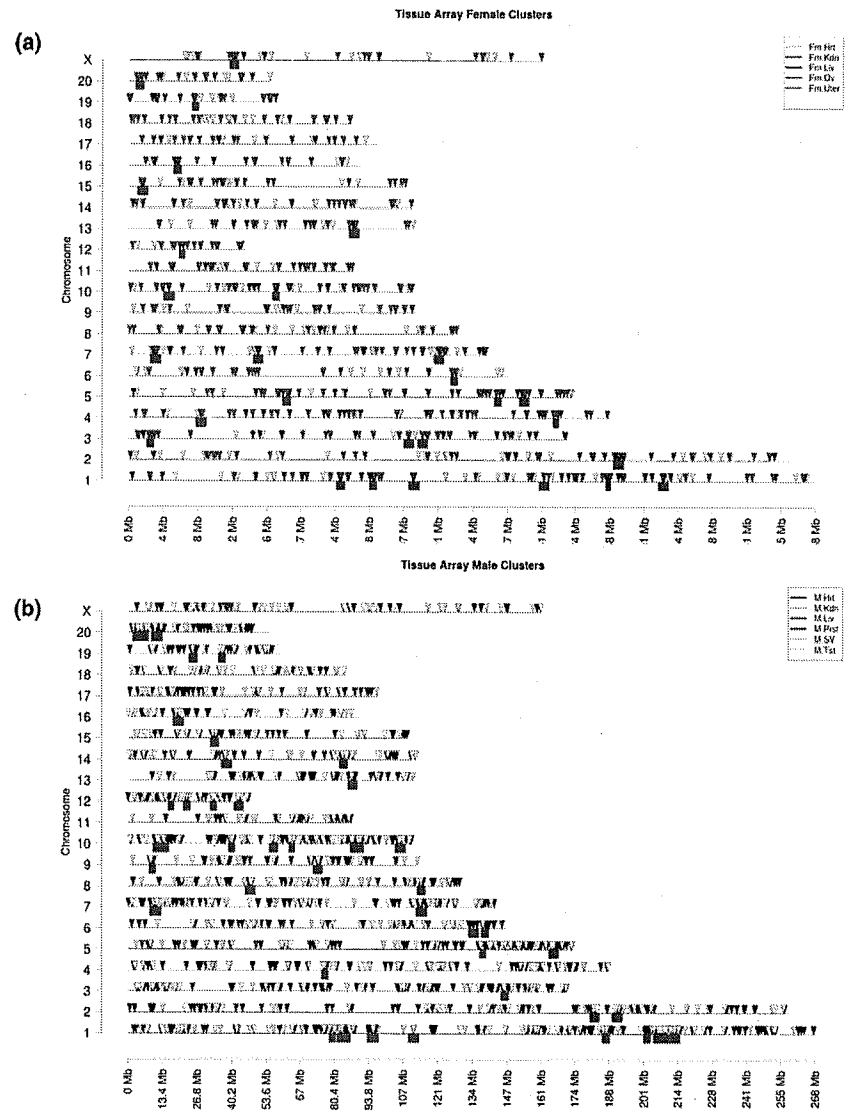

Figure 6 Chromosomal locations of differentially expressed genes. (a) Chromosomal plot of differential gene expression (arrow head) and ECRs (box) for five female tissue types (heart, kidney, liver, ovaries and uterus). **(b)** Chromosomal plot of ECRs for six male tissue types (heart, kidney, liver, prostate, seminal vesicle and testis). **(c)** Chromosomal plot showing clustering of male tissues and female tissues. Insets show tissue identification color code. F, female; M, male; SV, seminal vesicle.

de-methylation event to allow the specific DNA methylation pattern/programming to be transmitted between generations [17,41]. The identified vinclozolin-induced transgenerational alterations in the sperm epigenome (epimutations) [4] appear to be imprinted and transmit the altered DNA methylation regions between generations [2]. The mechanisms that allow a differential DNA methylation region to be protected from DNA de-methylation in the early embryo are not known, but are speculated to involve specific protein associations and/or other epigenetic factors. In addition, during early fetal gonadal development, the primordial germ cell DNA is de-methylated, which also involves imprinted genes. The imprinted sites are then re-methylated to maintain their original DNA methylation pattern/programming through unknown mechanisms. Therefore, how both imprinted sites and the transgenerational epimutations escape and/or reprogram to their original

Table 3 Gene clusters and epigenetic control regions

Cluster name	Chr.	Size (Mbp)	Start	Stop	*P*-value range		Number of genes regulated	Overlap opposite sex cluster
					Minimum	Maximum		
Female								
Chr1-109.35	1	4	109350000	113350000	1.1E-30	8.2E-03	6	No
Chr1-159.65	1	3.9	159650000	163550000	2.1E-05	5.6E-04	4	No
Chr1-185.85	1	2.15	185850000	1.88E+08	8.2E-03	8.2E-03	5	Yes
Chr1-206.4	1	3.95	206400000	210350000	4.4E-09	8.2E-03	10	Yes
Chr1-81.05	1	3.05	81050000	84100000	8.2E-03	8.2E-03	5	Yes
Chr1-93.9	1	2.8	93900000	96700000	5.6E-04	8.2E-03	6	Yes
Chr2-188.8	2	4.1	188800000	192900000	2.1E-05	8.2E-03	10	Yes
Chr3-107.4	3	3.8	107400000	111200000	2.1E-05	8.2E-03	5	No
Chr3-112.8	3	3.7	112800000	116500000	5.6E-04	8.2E-03	6	No
Chr3-7.2	3	2.6	7200000	9800000	8.2E-03	8.2E-03	5	No
Chr4-165.3	4	2	165300000	167300000	8.2E-03	8.2E-03	5	No
Chr4-26.2	4	3.8	26200000	3.00E+07	8.2E-03	8.2E-03	4	No
Chr5-142.1	5	2.6	142100000	144700000	5.6E-04	8.2E-03	6	No
Chr5-151.75	5	3.75	151750000	155500000	2.1E-05	8.2E-03	8	No
Chr5-59.9	5	3.25	59900000	63150000	8.2E-03	8.2E-03	6	No
Chr6-125.35	6	2.7	125350000	128050000	5.6E-04	8.2E-03	6	No
Chr7-118.8	7	3.85	118800000	122650000	8.2E-03	8.2E-03	7	No
Chr7-48.35	7	3.55	48350000	51900000	2.1E-05	8.2E-03	6	Yes
Chr7-8.5	7	3.9	8500000	12400000	2.1E-05	8.2E-03	8	No
Chr10-55.7	10	2.8	55700000	58500000	8.2E-03	8.2E-03	5	Yes
Chr12-19.65	12	2	19650000	21650000	8.2E-03	8.2E-03	5	No
Chr13-85.75	13	3.85	85750000	89600000	2.1E-05	8.2E-03	9	Yes
Chr15-3.45	15	3.85	3450000	7300000	4.2E-07	8.2E-03	6	Yes
Chr16-17.3	16	3	17300000	20300000	5.6E-04	8.2E-03	6	No
Chr19-24.55	19	2.4	24550000	26950000	8.2E-03	8.2E-03	5	Yes
Chr20-2.75	20	3.1	2750000	5850000	5.6E-04	8.2E-03	7	Yes
Chrx-39	X	3.25	3.90E+07	42250000	2.1E-05	8.2E-03	7	No
Male								
Chr1-78.4	1	2.6	78400000	8.10E+07	4.9E-02	1.1E-02	17	Yes
Chr1-81.6	1	4.9	81600000	86500000	4.9E-02	7.1E-04	21	Yes
Chr1-93.35	1	4.1	93350000	97450000	4.9E-02	2.2E-05	22	Yes
Chr1-109.4	1	3.95	109400000	113350000	7.4E-11	1.2E-19	16	No
Chr1-184.85	1	2.8	184850000	187650000	4.9E-02	1.1E-02	12	Yes
Chr1-200.8	1	2.65	200800000	203450000	4.9E-02	2.4E-02	11	No
Chr1-204.75	1	10.4	204750000	215150000	4.9E-02	7.6E-08	70	Yes
Chr2-180.15	2	3.45	180150000	183600000	4.9E-02	1.9E-03	18	No
Chr2-188.65	2	3.9	188650000	192550000	4.9E-02	2.2E-05	18	Yes
Chr3-144.75	3	3	144750000	147750000	4.9E-02	4.9E-02	11	No
Chr4-75.4	4	2.45	75400000	77850000	2.4E-02	1.1E-02	12	No
Chr5-136.75	5	2.2	136750000	138950000	4.9E-02	4.9E-02	11	No
Chr5-163.75	5	3.7	163750000	167450000	4.9E-02	4.8E-03	18	No
Chr6-132.3	6	4	132300000	136300000	7.4E-11	5.4E-65	19	No
Chr6-137.4	6	2.45	137400000	139850000	4.9E-02	1.1E-02	13	No
Chr7-8.5	7	4.4	8500000	12900000	2.1E-05	8.2E-03	25	Yes
Chr7-112.3	7	4.1	112300000	116400000	4.9E-02	7.7E-05	22	No
Chr8-45.4	8	3.9	45400000	49300000	4.9E-02	7.1E-04	15	No
Chr8-112.8	8	3.05	112800000	115850000	4.9E-02	4.8E-03	14	No
Chr9-72.25	9	3.3	72250000	75550000	4.9E-02	1.1E-02	12	No
Chr9-8.35	9	2.15	8350000	10500000	4.9E-02	4.9E-02	10	No
Chr10-9.85	10	2.5	9850000	12350000	4.9E-02	4.9E-02	10	Yes

Table 3 Gene clusters and epigenetic control regions *(Continued)*

Chr10-12.15	10	3.5	12150000	15650000	2.4E-02	1.1E-02	16	Yes
Chr10-38.9	10	2.3	38900000	41200000	4.9E-02	4.9E-02	10	No
Chr10-54.75	10	3.4	54750000	58150000	4.9E-02	4.8E-03	14	Yes
Chr10-62.45	10	2.35	62450000	64800000	4.9E-02	4.9E-02	12	No
Chr10-86.55	10	5.05	86550000	91600000	4.9E-02	7.7E-05	28	No
Chr10-104.15	10	3.85	104150000	1.08E+08	4.9E-02	7.1E-04	18	No
Chr12-15.65	12	2.1	15650000	17750000	4.9E-02	4.9E-02	10	No
Chr12-21.3	12	2.75	21300000	24050000	4.9E-02	4.9E-02	11	Yes
Chr12-31.75	12	2.55	31750000	34300000	4.9E-02	2.4E-02	11	No
Chr12-41	12	3.7	4.10E+07	44700000	4.9E-02	7.7E-05	10	No
Chr13-85.65	13	3.4	85650000	89050000	4.9E-02	4.8E-03	14	Yes
Chr14-36.1	14	3.95	36100000	40050000	1.9E-03	6.1E-06	15	No
Chr14-82.2	14	3.2	82200000	85400000	4.9E-02	2.4E-02	13	No
Chr15-31.9	15	2.95	31900000	34850000	2.4E-02	4.8E-03	15	Yes
Chr16-17.45	16	4.05	17450000	21500000	4.9E-02	6.1E-06	20	Yes
Chr19-23.6	19	3.2	23600000	26800000	4.9E-02	4.8E-03	14	Yes
Chr19-34.95	19	2.7	34950000	37650000	4.9E-02	4.9E-02	11	No
Chr20-2	20	6.05	2.00E+06	8050000	4.9E-02	2.4E-04	34	Yes
Chr20-9.25	20	3.9	9250000	13150000	2.4E-02	4.8E-03	16	Yes

state remains to be elucidated and is a critical mechanism to be investigated in future studies. The epigenetic transgenerational inheritance of the altered sperm epigenome results in a modified baseline epigenome in the early embryo that will subsequently affect the epigenetic programming of all somatic cells and tissues [16,19]. The epigenome directly influences genome activity such that an altered baseline epigenome will promote altered transcriptomes in all somatic cells and tissues [16]. The current study was designed to test this hypothesis and examine the transcriptomes of a variety of tissues.

The previously observed epigenetic transgenerational inheritance of adult-onset disease involved disease in a variety of different tissues (prostate, kidney, testis,

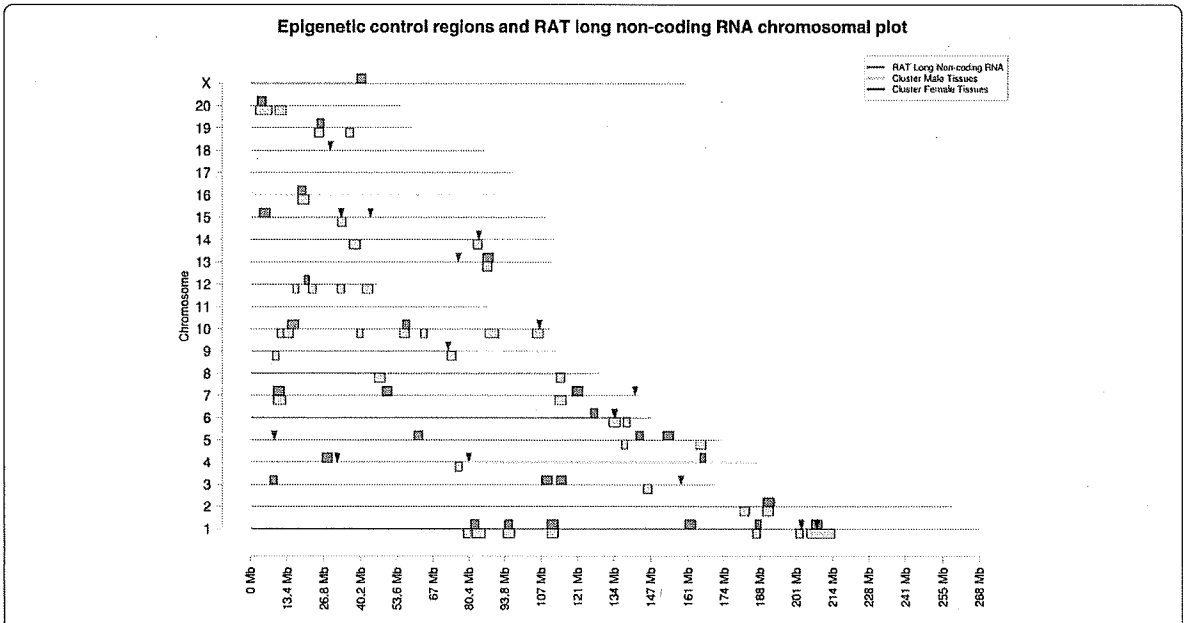

Figure 7 Chromosomal plot showing gene clustering in epigenetic control regions of male tissues and female tissues overlapped with rat long non-coding RNA (arrowheads). Inset provides color code.

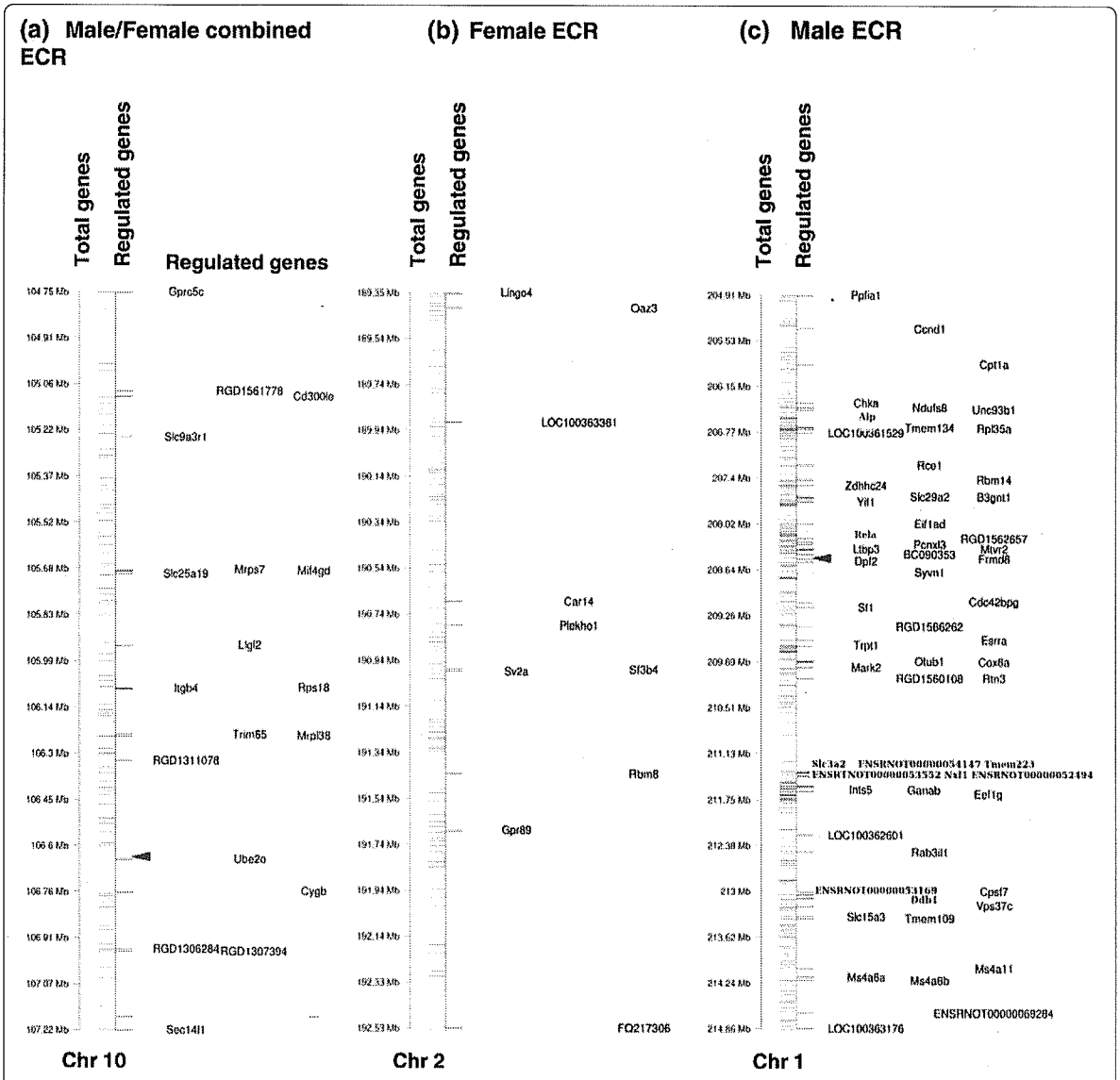

Figure 8 Representative epigenetic control regions (ECRs) identifying regulated genes in single ECRs. **(a)** ECR selected from male and female tissues combined (overlapped). **(b)** ECR selected from female only tissues. **(c)** ECR selected from male only tissues. The location of all genes (total genes) on chromosomes 1, 2 and 10 are shown in megabases and regulated genes are named. The arrowhead identifies the location of a known rat long non-coding RNA.

ovary), but no apparent disease in other tissues (liver, heart) [5]. Previous clinical observations have demonstrated that some tissues are more highly susceptible to develop disease than others. An alteration in the baseline epigenome and transcriptome of a tissue in certain tissues may increase susceptibility or promote disease, while others can tolerate the alterations and maintain normal function. The environmentally induced epigenetic transgenerational inheritance of adult-onset disease may be due to a baseline alteration in epigenomes and transcriptomes in somatic cells of tissues susceptible to these changes and disease.

The experimental design involved the isolation of six different tissues from males and five tissues from females. These tissues were obtained from young adult rats prior to any disease onset. The F3 generation control and vinclozolin lineage animals from different litters were used and tissues obtained from six different animals for each sex, tissue and lineage. A microarray analysis was used to assess transgenerational alterations in the tissue-specific

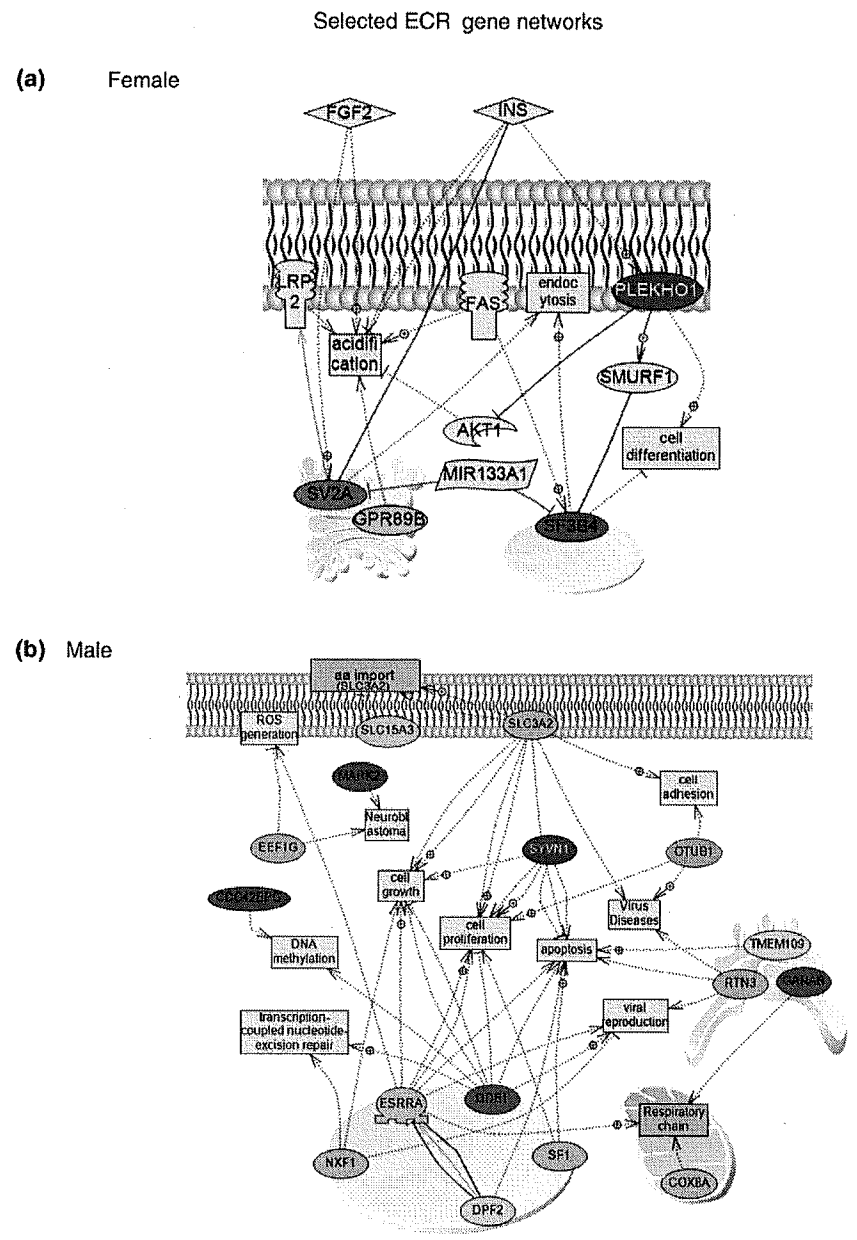

Figure 9 Shortest cell processes connection gene sub-networks for genes of selected female ECR chr2-188.8 and male ECR chr1-204.75. **(a)** ECR chr2-188.8. **(b)** Male ECR chr1-204.75. Node shapes: oval and circle, protein; diamond, ligand; circle/oval on tripod platform, transcription factor; ice cream cone, receptor; crescent, kinase or protein kinase; irregular polygon, phosphatase. Color code: red, up-regulated genes; blue, down-regulated genes. Arrows with a plus sign indicate positive regulation/activation; arrows with a minus sign indicate negative regulation/inhibition; grey arrows represent regulation; lilac arrows represent expression; purple arrows represent binding; green arrows represent promoter binding; yellow arrows represent protein modification. AA, amino acid; FAS; FGF, fibroblast growth factor; INS, insulin; LRP2, low density lipoprotein receptor-related protein 2; ROS, reactive oxygen species.

transcriptomes between control versus vinclozolin lineage animals. The differentially expressed genes for a specific tissue are referred to as a signature list. Analysis of the various tissue signature lists demonstrated negligible overlap among tissues or between sexes. Therefore, the transgenerational transcriptomes were observed in all tissues, but each tissue had a sexually dimorphic tissue-specific transgenerational transcriptome. The hypothesis that an altered

transgenerational germline epigenome would promote transgenerational alterations in all somatic transcriptomes is supported by the observations of the current study. The initial bioinformatics analysis involved examination of the various tissue signature lists to correlate the involvement of cellular signaling pathways or processes among the various signature lists. The majority of pathways included genes from each signature list, but none were predominant among the signature lists. Gene functional categories that were generally predominant in the cell, such as signaling or metabolism, were also the most predominant among the signature lists. Therefore, a common pathway or process was not present among the observed transgenerational transcriptomes.

A more extensive analysis of the differentially expressed genes in all the tissues involved a previously described gene bionetwork analysis [31,42]. The coordinated gene expression and connectivity between the regulated genes was considered in a cluster analysis (Figure 4). Gene modules of interconnected genes with coordinated gene expression were identified in both a combined male and female signature list analysis, and separate male and female analyses. Although defined modularity was identified in the combined analysis, the sexually dimorphic transgenerational transcriptomes and distinct tissue physiology suggested the separate male and female analyses would be more informative. The sex-specific modules were used to determine if any over-represented gene sets were present in specific tissues. Generally, each tissue had a specific module of differentially regulated genes (Table 2). For example, prostate was predominant in the male turquoise module and female heart in the female turquoise module. In contrast, in the analysis of cellular signaling pathways or processes, the gene modules did not have over-represented pathways (Additional file 7). The tissue-specific modules did not generally reflect a specific pathway or process. Therefore, the gene bionetwork analysis identified gene modules associated with specific tissues, but the modules did not generally contain predominant cellular pathways or processes.

The transgenerational transcriptome data analysis was extended with a literature-based gene network analysis. Direct connection networks (DCNs), involving genes with direct functional and/or binding links, were identified for a number of the male and female gene modules, but the majority did not have specific gene networks. Each DCN corresponds to a previously identified co-expressed gene module. Specifically, the nodes of a DCN were the members of the corresponding co-expressed gene module but the links in the DCN were based on the literature and known databases. The modules with an identified gene network suggest that those specific tissues and abnormal physiology are potentially regulated by the network (Table 2; Additional file 3). The female turquoise module associated with the heart, male yellow module associated with testis, male brown module associated with kidney, liver and seminal vesicle, and male turquoise module associated with prostate. Each of these gene networks is unique and provides a potential regulated gene set associated with abnormal tissue pathology. Future studies will need to consider these gene networks with regard to the pathophysiology of the specific tissues. An alternative gene network analysis involved the different tissue signature lists and tissue-specific direct connection gene network analysis (Additional file 4). Tissue-specific gene networks were identified for female heart, kidney, ovary and uterus, and for male heart, kidney and liver. Similar to the observed lack of overlap between the tissue-specific signature lists (Figure 2), negligible overlap was found between the tissue-specific gene networks (Additional file 4). These tissue-specific direct connection gene networks also provide regulated subnetworks of genes associated with the previously identified abnormal transgenerational tissue pathologies [5]. Interestingly, the gene network associated with the female turquoise module was similar to the female heart tissue-specific gene network. This regulated female heart network provides an interconnected gene set that could be investigated in future studies on heart pathophysiology. The final direct connection gene network analysis involved the combined male tissue and combined female tissue regulated gene sets. The combined female tissue network involved a small network of six genes, suggesting a gene network was not common among the different female tissues. The combined male tissue network involved a larger gene set of over 30 genes (Figure 5), which had elements similar to the male kidney network (Additional file 4). The similarities suggest this gene network may be associated with the observed kidney pathophysiology and needs to be investigated in future studies [5]. Although this combined male tissue direct connection gene network suggests a potential common regulatory gene set among the tissues, the tissue-specific transgenerational transcriptomes have negligible overlap (Figure 2) and distinct tissue-specific gene networks (Additional file 4). Observations suggest the transgenerational somatic transcriptomes are primarily tissue-specific without common gene networks or specific pathways associated with the adult-onset disease that developed in the specific tissues.

To understand how a limited number of sperm epimutations can lead to such a diverse gene expression profile between tissues, an epigenetic mechanism needs to be considered. As discussed, somatic cells and tissues will have a shift in the baseline epigenome derived from sperm that promotes distinct cellular and tissue differentiation [16,19]. Therefore, it is not surprising each cell type has a distinct epigenome and transcriptome to promote cell-specific differentiated functions. The classic dogma that a

gene's promoter is the central regulatory site involved in regulating its expression is not sufficient to explain the over 4,000 genes differentially regulated between the different tissues examined (Figure 1). A potential alternative epigenetic mechanism involves an ECR that can regulate gene expression within a greater than 2 Mb region together with, for example, lncRNAs and chromatin structure. An example of such a mechanism has been previously described as an ICR, where an imprinted DNA methylation site (for example, *H19* and *IGF2*) influences a lncRNA to regulate gene expression for over a megabase in either direction [17,22,23,27]. The imprinted *H19* and *IGF2* loci together with a lncRNA have been shown to distally regulate the expression of multiple different genes [17,25,26,28]. These ICRs are likely a small subset of a larger set of ECRs, most not involving imprinted gene sites. Another example has been shown in plants where lncRNAs regulate distal gene expression associated with specific plant physiological phenotypes [29,30]. The current study used the various tissue transgenerational transcriptomes to identify the potential presence of ECRs.

The ECRs were defined as having a statistically significant (Z test) over-representation of gene expression within an approximately 2 Mb region. The male and female sets of differentially expressed genes were used separately to identify regions with statistically significant (Z test) over-representation ($P < 0.05$). The differentially expressed genes were mapped to the chromosomes and then a 2 Mb sliding window was used to identify potential ECRs (Figures 6 and 7). For the male, over 40 ECRs were identified, and for the female, approximately 30 ECRs were identified. Approximately half the ECRs were found to be in common between male and female (Figure 7). The ECRs identified ranged from 2 to 5 Mb in size and the numbers of genes regulated ranged from 5 to 50 (Table 3). Interestingly, different genes in different tissues were found to be expressed within these ECRs (Additional file 8). The majority of the expression sites of currently known rat lncRNAs correlated with the identified ECRs (Figure 7; Additional file 9). Therefore, it is proposed that a single ECR could regulate tissue-specific gene expression that has been programmed during differentiation to express a specific set of genes within the ECR. This could explain how a limited number of epimutations could have a much broader effect on genome activity and clarify how tissue-specific transgenerational transcriptomes develop. The current study outlines the association of gene expression with the potential ECRs, but does not provide a functional link between epigenetic differential DNA methylation regions or lncRNAs and gene expression regulation within them. Therefore, future studies are now critical to assess the functional role of these ECRs and underlying epigenetic mechanisms.

Conclusions

A systems biology approach was taken to elucidate the molecular mechanism(s) involved in environmentally induced epigenetic transgenerational inheritance of adult-onset disease. The current study identifies tissue-specific transgenerational transcriptomes with tissue-specific gene networks. A combination of epigenetic and genetic mechanisms is required to reach these differentiated tissue states that can not be explained through genetic or epigenetic mechanisms alone. The identification of potential epigenetic control regions that regulate regions of the genome in a coordinated manner may help explain in part the mechanism behind the process of emergence [43]. In a revolutionary systems biology consideration the emergence of a phenotype or process involves the coordinated and tissue-specific development of unique networks (modules) of gene expression [44]. Since the initial identification of epigenetics [45], its role in system development at the molecular level has been appreciated. The current study suggests a more genome-wide consideration involving ECRs and tissue-specific transcriptomes may contribute, in part, to our understanding of how environmental factors can influence biology and promote disease states.

Combined observations demonstrate that environmentally induced epigenetic transgenerational inheritance of adult-onset disease [2] involves germline (sperm) transmission of an altered epigenome [4] and these epimutations shift the base line epigenomes in all somatic tissues and cells derived from this germline [16]. This generates tissue-specific transgenerational transcriptomes that do not involve common gene networks or pathways, which associate with the adult-onset disease in the tissues. All tissues develop a transgenerational transcriptome, which helps explain the phenotypic variation observed. Some tissues are sensitive to shifts in their transcriptomes and develop disease, while others are resistant to disease development. The observation that all tissues develop a specific transgenerational transcriptome can help explain the mechanism behind complex disease syndromes. Those tissues sensitive to developing disease will be linked into a complex disease association due to these transgenerational transcriptome modifications. This epigenetic mechanism involves ECRs that can have dramatic effects on genome activity and promote tissue-specific phenomena. Although the functional roles of these ECRs remain to be investigated, their potential impact on expanding our concepts of gene regulation, the elucidation of emergent properties of unique gene networks, and providing links to various tissue functions and diseases are anticipated to be significant. The observations provided help elucidate the molecular mechanisms involved in environmentally

induced epigenetic transgenerational inheritance of adult-onset disease and the phenotypic variation identified.

Materials and methods

Animal procedures

All experimental protocols involving rats were pre-approved by the Washington State University Animal Care and Use Committee. Hsd:Sprague Dawley®™SD®™ female and male rats of an outbred strain (Harlan, Indianapolis, IN, USA) were maintained in ventilated (up to 50 air exchanges per hour) isolator cages 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. In the event sperm were detected (day 0) the rats were tentatively considered pregnant. Vaginal smears were continued for monitoring diestrus status until day 7. Pregnant rats were then given daily intraperitoneal injections of vinclozolin (100 mg/kg/day) with an equal volume of sesame oil (Sigma, St. Louis, MO, USA) on days E8 through E14 of gestation [6]. Treatment groups were Control (DMSO vehicle) and Vinclozolin. The pregnant female rats treated with DMSO or vinclozolin were designated as the F0 generation.

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 DMSO or vinclozolin.

Six female and six male rats of the F3 generation Control and Vinclozolin lineages at 120 days of age were euthanized by CO_2 inhalation and cervical dislocation. Tissues, including testis, prostate, seminal vesicle, kidney, liver, heart, ovary and uterus, were dissected from rats and were processed and stored in TRIZOL (Invitrogen, Grand Island, NY, USA) at -80°C until RNA extraction. High quality RNA samples were assessed with gel electrophoresis and required a minimum OD260/280 ratio of 1.8. Three samples each of control and treated ovaries were applied to microarrays. For each of three Vinclozolin or Control microarray samples, RNA from two rats were pooled. The same pair of rats was used for each tissue type.

Microarray analysis

The microarray hybridization and scanning was performed by 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, cRNA was transcribed, and single-stranded sense DNA was synthesized, which was fragmented and labeled with biotin. Biotin-labeled single-stranded DNA was then hybridized to the Rat Gene 1.0 ST microarrays containing more than 30,000 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 software (Partek Incorporated, St Louis, MO, USA) using an RMA (Robust Multiarray Average), GC-content adjusted algorithm. Raw data pre-processing was performed in 11 groups, one for each male or female tissue. Comparison of array sample histogram graphs for each group showed that data for all chips were similar and appropriate for further analysis (Additional file 1).

The microarray quantitative data involve signals from an average 28 different oligonucleotides (probes) arrayed for each transcript and many genes are represented on the chip by several transcripts. The hybridization to each probe must be consistent to allow a statistically significant quantitative measure of the resulting gene expression signal. In contrast, a quantitative PCR procedure uses only two oligonucleotides and primer bias is a major factor in this type of analysis. Therefore, we did not attempt to use PCR-based approaches as we feel the microarray analysis is more accurate and reproducible without primer bias.

All microarray CEL files from this study have been deposited with the NCBI gene expression and hybridization array data repository Gene Expression Omnibus (GEO series accession number [GSE35839]) and can also be accessed through the Skinner Laboratory website [46]. For gene annotation, Affymetrix annotation file RaGene1_0stv1.na32.rn4.transcript.csv was used.

Network analysis

The network analysis was restricted to genes differentially expressed between the control and the treatment groups based on previously established criteria of fold change of group means ≥1.2, a mean difference >10, and P-value ≤ 0.05. A change in gene expression of 20% for many genes, particularly transcriptome factors, has been shown to have important cellular and biological effects. Therefore, the 1.2-fold cutoff was selected to maintain all expression information and not a more stringent one to simply reduce the gene list size. To eliminate baseline signal gene expression changes, a mean difference >10 was used. All genes required a statistical difference $P < 0.05$ to be selected. The union of the differentially expressed genes

from the tissues resulted in 5,266 genes for males and 1,909 for females being identified and used for constructing a weighted gene co-expression network [47,48]. Unlike traditional un-weighted gene co-expression networks in which two genes (nodes) are either connected or disconnected, the weighted gene co-expression network analysis assigns a connection weight to each gene pair using soft-thresholding and thus is robust to parameter selection. The weighted network analysis begins with a matrix of the Pearson correlations between all gene pairs, then converts the correlation matrix into an adjacency matrix using a power function: $f(x) = x^{\beta}$. The parameter β of the power function is determined in such a way that the resulting adjacency matrix (that is, the weighted co-expression network) is approximately scale-free. To measure how well a network satisfies a scale-free topology, we use the fitting index proposed by Zhang and Horvath [47] (that is, the model fitting index R^2 of the linear model that regresses $log(p(k))$ on $log(k)$ where k is connectivity and $p(k)$ is the frequency distribution of connectivity). The fitting index of a perfect scale-free network is 1.

To explore the modular structures of the co-expression network, the adjacency matrix is further transformed into a topological overlap matrix [49]. As the topological overlap between two genes reflects not only their direct interaction but also their indirect interactions through all the other genes in the network. Previous studies [47,49] have shown that topological overlap leads to more cohesive and biologically meaningful modules. To identify modules of highly co-regulated genes, we used average linkage hierarchical clustering to group genes based on the topological overlap of their connectivity, followed by a dynamic cut-tree algorithm to dynamically cut clustering dendrogram branches into gene modules [50]. Such networks were generated from combined 6 male or 5 female differentially expressed gene sets (2 networks) or from combined male and female 11-tissue signature lists. From 9 to 20 modules were identified in either of 3 networks and the module size range was from 7 to 1,040 genes.

To distinguish between modules, each module was assigned a unique color identifier, with the remaining, poorly connected genes colored grey. The hierarchical clustering over the topological overlap matrix (TOM) and the identified modules is shown (Figure 4). In this type of map, the rows and the columns represent genes in a symmetric fashion, and the color intensity represents the interaction strength between genes. This connectivity map highlights that genes in the transcriptional network fall into distinct network modules, where genes within a given module are more interconnected with each other (blocks along the diagonal of the matrix) than with genes in other modules. There are a couple of network connectivity measures, but one particularly important one is the within module connectivity (k.in). The k.in of a gene was determined by taking the sum of its connection strengths (co-expression similarity) with all other genes in the module to which the gene belonged.

Gene co-expression cluster analysis clarification

Gene networks provide a convenient framework for exploring the context within which single genes operate. Networks are simply graphical models composed of nodes and edges. For gene co-expression clustering, an edge between two genes may indicate that the corresponding expression traits are correlated in a given population of interest. Depending on whether the interaction strength of two genes is considered, there are two different approaches for analyzing gene co-expression networks: 1) an unweighted network analysis that involves setting hard thresholds on the significance of the interactions; and 2) a weighted approach that avoids hard thresholds. Weighted gene co-expression networks preserve the continuous nature of gene-gene interactions at the transcriptional level and are robust to parameter selection. An important end product from the gene co-expression network analysis is a set of gene modules in which member genes are more highly correlated with each other than with genes outside a module. Most gene co-expression modules are enriched for GO functional annotations and are informative for identifying the functional components of the network that are associated with disease [51].

This gene co-expression clustering/network analysis (GCENA) has been increasingly used to identify gene subnetworks for prioritizing gene targets associated with a variety of common human diseases such as cancer and obesity [52-56]. One important end product of GCENA is the construction of gene modules composed of highly interconnected genes. A number of studies have demonstrated that co-expression network modules are generally enriched for known biological pathways, for genes that are linked to common genetic loci and for genes associated with disease [42,47,51-55,57,58]. In this way, one can identify key groups of genes that are perturbed by genetic loci that lead to disease, and that define at the molecular level disease states. Furthermore, these studies have also shown the importance of the hub genes in the modules associated with various phenotypes. For example, GCENA identified *ASPM*, a hub gene in the cell cycle module, as a molecular target of glioblastoma [55] and *MGC4504*, a hub gene in the unfolded protein response module, as a target potentially involved in susceptibility to atherosclerosis [53].

Pathway and functional category analysis

Resulting lists of differentially expressed genes for each male or female tissue were analyzed for gene functional categories with GO categories from the Affymetrix annotation site. Each module generated in male or female network analysis were analyzed for KEGG (Kyoto

Encyclopedia for Genes and Genome, Kyoto University, Japan) pathway enrichment using the KEGG website 'Search Pathway' tool. Global literature analysis of various gene lists was performed using Pathway Studio 8.0 software (Ariadne Genomics, Inc., Rockville, MD, USA) and used to generate the direct and indirect gene connection networks.

Chromosomal location of ECRs

An R-code was developed to find chromosomal locations of ECRs. A 2 Mb sliding window with 50,000 base intervals was used to find the associated genes in each window. 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 termed ECRs. Typical ECR regions range from 2 to 5 Mb, with the largest being 10 Mb.

Additional material

Additional file 1: Figure S1 - microarray histogram quality control. **(a-l)** Sample histograms and box plots for microarray raw (a) and pre-processed signal values, using a RMA (Robust Multiarray Average), GC-content-adjusted algorithm for 11 male and female tissues (b-l).

Additional file 2: Figure S2 - cluster coefficient and connections. **(a,b)** Cluster coefficient versus number of connections for male (a) and female (b) network modules.

Additional file 3: Figure S3 - gene networks from gene modules. **(a-d)** Direct connection sub-networks for female and male modules: (a) female turquoise; (b) male yellow; (c) male brown; (d) male turquoise. Shape and color codes are the same as for Figure 5.

Additional file 4: Figure S4 - gene networks from signature lists. **(a-d)** Direct connection sub-networks for female and male tissue signature lists: (a) female heart; (b) female kidney; (c) male ovary; (d) uterus; (e) male heart; (f) male kidney; (g) male liver. Shape and color codes are the same as for Figure 5.

Additional file 5: Table S1 - differentially expressed genes in tissues.

Additional file 6: Table S2 - pathway enrichment in signature lists.

Additional file 7: Table S3 - pathway enrichment in tissue modules.

Additional file 8: Table S4 - epigenetic control regions and gene expression.

Additional file 9: Table S5 - lncRNA and epigenetic control regions.

Abbreviations

DCN: direct connection network; DMSO: dimethylsulfoxide; E: embryonic day; ECR: epigenetic control region; GCENA: gene co-expression clustering/networks analysis; GO: Gene Ontology; ICR: imprinting control region; k.in: connectivity index; lncRNA: long non-coding RNA.

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Authors' contributions

MKS designed the study; MM, MiS, MH and BZ performed the experiments and analysis; all authors reviewed the data; MKS wrote the manuscript; all authors edited the manuscript; all authors have read and approved the manuscript for publication.

Competing interests

The authors declare that they have no competing interests.

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RESEARCH PAPER

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Environmentally induced epigenetic transgenerational inheritance of sperm epimutations promote genetic mutations

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Keywords: copy number variation, CNV, disease etiology, epigenetic, evolution, genome instability, inheritance, review, transgenerational

A variety of environmental factors have been shown to induce the epigenetic transgenerational inheritance of disease and phenotypic variation. This involves the germline transmission of epigenetic information between generations. Exposure specific transgenerational sperm epimutations have been previously observed. The current study was designed to investigate the potential role genetic mutations have in the process, using copy number variations (CNV). In the first (F1) generation following exposure, negligible CNV were identified; however, in the transgenerational F3 generation, a significant increase in CNV was observed in the sperm. The genome-wide locations of differential DNA methylation regions (epimutations) and genetic mutations (CNV) were investigated. Observations suggest the environmental induction of the epigenetic transgenerational inheritance of sperm epimutations promote genome instability, such that genetic CNV mutations are acquired in later generations. A combination of epigenetics and genetics is suggested to be involved in the transgenerational phenotypes. The ability of environmental factors to promote epigenetic inheritance that subsequently promotes genetic mutations is a significant advance in our understanding of how the environment impacts disease and evolution.

Introduction

Environmental factors such as toxicants, nutrition, and stress all have been shown to promote the epigenetic transgenerational inheritance of disease and phenotypic variation.[1] One of the first observations in mammals involved the actions of the agricultural fungicide vinclozolin on a gestating F0 generation female promoting transgenerational disease in the F3 and F4 generation progeny.[2] This was found to be mediated in part through differential DNA methylation regions (termed epimutations) in the sperm that are transmitted between generations and correlate with transgenerational disease phenotypes.[3] Transgenerational disease was found in the testis, ovary, kidney, prostate, and mammary gland.[1] Subsequently, a large number of toxicants (plastics, pesticides, hydrocarbons),[4] nutritional abnormalities (high fat and caloric restriction),[1] and stress (social and aversion)[5] have been shown to promote the transgenerational phenomenon.[1] Epigenetic transgenerational inheritance has now been observed in plants, flies, worms, fish, mice, rats, pigs, and humans.[1,6] The phenomenon of environmentally induced epigenetic transgenerational inheritance appears highly conserved and is a form of non-Mendelian genetic inheritance.

Epigenetic transgenerational inheritance requires the germline transmission of epigenetic information in the absence of any direct exposure or genetic manipulation.[7] In contrast, direct exposure of an individual at a specific developmental stage (e.g., fetal) does not involve a generational process. The initial germline epimutations identified involved differential DNA methylation regions (DMRs). These sperm epimutations have been identified following a variety of different toxicant ancestral exposures, and the transgenerational sets of DMRs were found to be exposure specific.[4] Therefore, the specific epimutation signature provides potential biomarkers for ancestral exposures. Analysis of the epimutation genomic features demonstrated all DMR identified occurred in genomic regions having less than 10 CpG/100 bp density, such that the epimutations were in deserts of CpG.[8] Although these initial studies focused on DNA methylation,[3] more recent studies with different species have suggested histone modifications and non-coding RNA are also involved in the epigenetic transgenerational inheritance phenomenon.[1,9,10]

Epigenetic mechanisms have previously been shown to influence and promote the occurrence of a number of DNA sequence mutations. For example, the highest frequency point mutation known is a C to T transition, nearly ten-fold higher than other

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single nucleotide polymorphisms (SNPs). The methylation of CpGs increases CG to TG transition to an over twelve-fold higher frequency than all other SNPs.[11] The DNA methylation status of the cytosine has been shown to directly influence the development of this genetic mutation.[12] Translocation and chromosomal breakpoint events in the genome have also been shown to be influenced by epigenetic alterations at the genomic regions involved.[13,14] Transposable elements, such as retrotransposons, are suppressed by DNA hypermethylation of the elements.[15] Previous studies in cancer biology have demonstrated that altered epigenetic mechanisms can promote genome instability and influence the transformation event and cancer progression.[16] Although genetic manipulations can influence epigenetic changes,[17] much of the molecular information available suggests epigenetics can also influence the development of genetic mutations by promoting genome instability.[18] Therefore, this previous literature suggests alterations in epigenetics can promote genetic mutations.

The current study focused on another important genetic mutation: the copy number variation (CNV). CNV involve the amplification (duplication) or deletion of repeat elements and sequences.[19,20] CNVs are the most frequent genetic mutation and are stable in the genome once established. The DNA methylation state of these repeat elements directly correlates with the emergence of CNV.[14,21] CNVs have previously been shown to be a useful genetic mutation to monitor in both disease etiology and evolution.[22]

Since epigenetics can influence the development of genetic mutations and promote genome instability, the current study was designed to investigate the influence of the epigenetic transgenerational inheritance of germline epimutations on the occurrence of genetic mutations using CNV. The hypothesis tested is that developmental exposure to environmental factors (e.g., toxicant vinclozolin) can promote the epigenetic transgenerational inheritance of germline epimutations that influences genome instability and genetic mutations (e.g., CNV). Therefore, the transgenerational phenomenon is initially induced through epigenetic inheritance but, in later generations, the transgenerational phenotype may involve a combination of the effects of epimutations and derived genetic mutations.

Results

The experimental design involved the exposure of gestating female F0 generation Sprague-Dawley (outbred) rats transiently to a vehicle control (dimethylsulfoxide, DMSO) or vinclozolin during fetal gonadal sex determination (embryonic day 8–14, E8-E14). Generally, sister littermates were used as the control and vinclozolin lineage F0 generation females to maintain genetic similarity between the lineages. The F1 generation offspring was bred to generate the F2 generation, followed by breeding to the F3 generation, as described previously [2,4] and in the Methods. No sibling or cousin breeding was used to avoid any inbreeding artifacts. Two different experiments were performed, one for CNV analysis, in which the F1 generation (n = 9 per lineage) and F3 generation (n = 9 per lineage) were studied, and one for DNA methylation F3 generation (n = 6 per lineage). Each experiment involved different control and vinclozolin lineages with DNA samples from 3 different individual animals from different litters being pooled. The epigenetic and genetic analysis used different experiments involving distinct F0 generation female lineages. The direct developmentally exposed F1 generation males and transgenerational F3 generation males were aged to 120 d and epididymal sperm collected and DNA obtained as previously described.[2,4] The genomic DNA samples were analyzed using chromosomal genomic hybridization (CGH) for CNV analysis.[3,4,22]

A genome-wide CNV analysis of control vs. vinclozolin lineage F1 or F3 generation sperm is summarized in **Table 1** and Supplemental **Tables S1 and S2**. The F1 generation vinclozolin CNV analysis identified 540 single-probe sites and 39 three-adjacent-probe minimum CNV sites with a statistical significance of $P < 0.05$. The three-adjacent-probe minimum represents 3 or

Table 1. (A) Vinclozolin F3 Generation Sperm Genome-wide CNV and Epimutations

Parameters	F1 Generation Sperm CNV	F3 Generation Sperm CNV	F3 Generation Epimutation Sperm
Number (Single Probe)	540(294 Gain / 246 Loss)	4912(4648 Gain / 264 Loss)	9932
Number (≥3 Probe)	39(21 Gain / 18 Loss)	506(461 Gain / 45 Loss)	191
Mean Size (base)	11,633	12,637	2,131
Mean CpG Density (CpG/100 bp)	1.1	1.0	0.9

(B) Gene Correlation with F3 Generation Sperm CNV and Epimutations

Epimutation F3 (191)	CNV F3 (506)
Prap1	LOC366431
Olr1442	
Hdgfl1	
Osap	
Slc39a13	
RaD1560481	
Wdp43	
Rab13	

more adjacent probes on the tiling array with statistically significant differences for each consecutive probe, such that a larger region is altered. The three-adjacent-probe minimum was found to be more reflective of the CNV and reduced the potential false positive calls with the single-probe analysis. The average size of the CNV was found to be 11.6 kb (**Table 1**). The 39 F1 generation vinclozolin sperm CNV were found to be within the probability of random animal variation and general individual CNV variation (**Fig. 1** and **Table S1**).[20,23] Therefore, the vinclozolin lineage F1 generation sperm did not appear to have a significant increase in genetic CNV mutations. In contrast, the vinclozolin F3 generation sperm had 4,912 single-probe CNV sites and 502 three-adjacent-probe minimum CNV sites (**Table 1** and **Table S2**). This statistically significant ($P < 0.05$) transgenerational increase in CNV indicates the vinclozolin F3 generation sperm have an altered level of genetic CNV mutations, which was not present in the F1 generation sperm. Single- vs. three-probe minimum numbers for average size and amplification vs. deletion in CNV compared to control are summarized in **Table 1**. The genetic similarity of the control vs. vinclozolin lineages (i.e., F0 female sister littermates and similar set of F0 males for each lineage) suggests the CNV variation in the F3 generation is not due to genetic divergence in the animal populations.

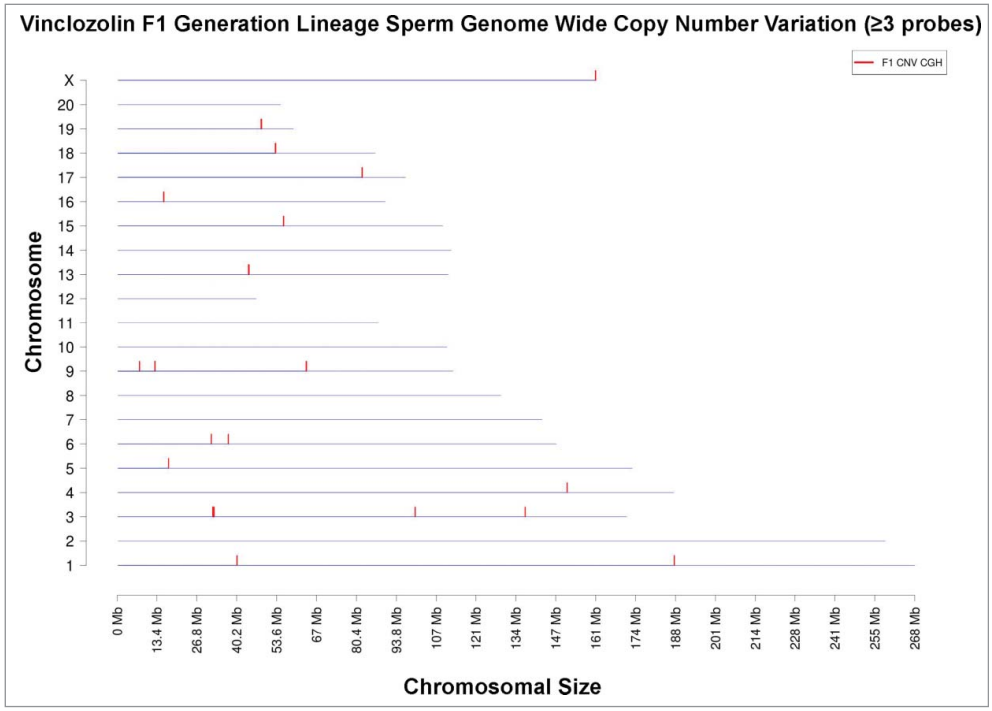

Figure 1. Transgenerational Vinclozolin F1 Generation Sperm Genome-wide Genetic CNV Mutation Analysis. F1 generation sperm genome-wide CNV (≥3 probe, 39 CNV) chromosomal locations and box indicates a statistically significant over-represented cluster of CNV.

The genome-wide chromosomal locations of the vinclozolin F3 generation sperm CNV are shown in **Figure 2**. All chromosomes had CNV and some high-density regions of CNV are observed. Therefore, a cluster analysis of the CNV was performed as previously described [22] and 10 clusters with statistically significant over-representation of CNV in regions of the genome are shown in **Figure 2**. The mean size of these clusters is 4.03 Mb, containing 7 to 17 CNVs (**Table 1** and **Table 2**). Interestingly, both gains and losses appeared to be clustered together in these locations. These regions may represent genomic sites that are more sensitive to an epigenetic influence promoting genetic CNV mutation formation.

In addition to CNV analysis, using a different set of experiments (i.e., different F0 generation female lineage animals) the DNA was fragmented and used in a methylated DNA immunoprecipitation (MeDIP) with methyl-cytosine antibody to isolate methylated DNA. The MeDIP samples were then analyzed on a genome-wide tiling array (MeDIP-Chip) for differential DNA methylation region (DMR) analysis. [3] Although a previous study identified the genome-wide promoter sites for F3 generation vinclozolin epimutations,[3,4] a genome-wide analysis, not restricted to promoters, was needed to allow a comparison with the CNV analysis. Therefore, a separate genome-wide analysis of the F3 generation vinclozolin epimutations was performed using MeDIP-Chip on control vs. vinclozolin lineage sperm (**Fig. 3**). A total of 9,932 DMRs were identified using a single oligonucleotide resolution and 191 DMR using a ≥3 (adjacent) oligonucleotide selection criterion, with a $P < 1 \times 10^{-7}$ statistical significance (**Table 1**). All subsequent analysis used the more stringent ≥3 adjacent probe selection data. The average size of the vinclozolin epimutation was found to be 2.1 kb; therefore, the three-adjacent-probe (50–60 bp each probe, with 200 bp spacing between probes) provides the most statistically significant and reliable epimutation estimate (**Table 1** and Supplemental **Table S3**). A chromosomal genome-wide map of the 191 epimutations is shown in **Figure 3A**. As previously described,[1] epimutations often cluster in similar regions of the genome, so a cluster analysis identifying statistically significant over-represented clusters of epimutations is also shown in **Figure 3A**. A comparison of this genome-wide analysis with the 52 DMR previously identified in promoters was investigated.[3] However, the probe distribution on the 2-chip set genome-wide custom tiling array used was found to be different with negligible correlation with the promoter tiling array previously used.[3] Therefore, the 52 DMRs could not be accurately compared with the newer genome-wide tiling arrays. An overlap was found with 15 of the 52 previously associated genes,[3] but not at the specific previously identified DMR sites. Future analyses will need to utilize less biased procedures, such as next generation sequencing. A genomic

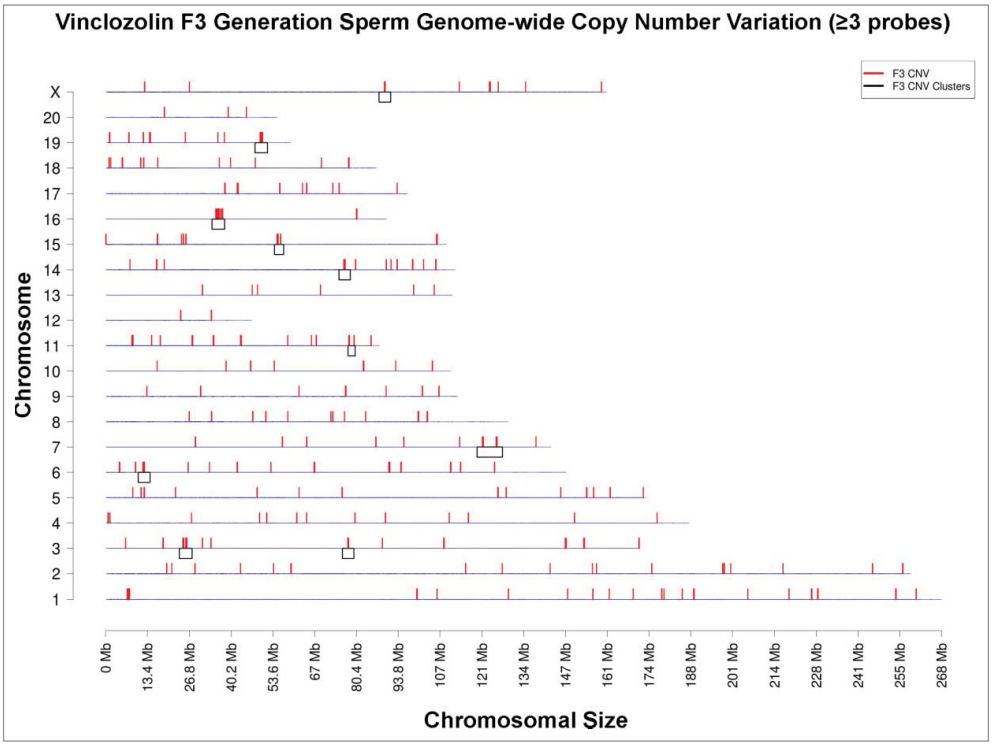

Figure 2. Transgenerational Vinclozolin F3 Generation Sperm Genome-wide Genetic CNV Mutation Analysis. F3 generation sperm genome-wide CNV (≥3 probe, 502 CNV) chromosomal locations; box indicates a statistically significant over-represented cluster of CNV.

feature found in all previously identified promoter epimutations was a low density CpG content.[8] In the current study, the genome-wide DMRs identified had a CpG density of less than 2 CpG/100 bp (**Fig. 3B**). The mean CpG content for all 191 genome-wide epimutations was 0.9 CpG/100 bp (**Table 1**). Therefore, the epimutations appear in CpG deserts with small clusters of CpG being the DMR, as previously described.[3,8] This genome-wide vinclozolin F3 generation sperm epimutation signature is correlated below to the CNV analysis. Future studies will need to compare the F1 generation and F2 generation sperm epimutations with the F3 epimutations, which were not compared in the current study due to lack of Nimblegen arrays and financial constraints.

Correlation of the epimutations and genetic CNV mutations demonstrated no overlap in a Venn diagram, shown in **Figure 3C**. Therefore, none of the epimutations and CNV had common genomic locations in the vinclozolin F3 generation sperm. Only three CNVs were found to be within 100 kb of an epimutation. The CNV clusters and epimutation clusters also had only one overlap on chromosome 3 at 75–77 Mb (**Table 2**). A technical limitation is that the F3 generation sperm samples used for the CNV and DMR were distinct DNA preparations and experiments (different F0 generation female lineages), which may influence the direct comparison. Although similar chromosomal regions may have epimutations and genetic CNV mutations, no direct overlap was found. The speculation is that the original epimutation may be lost upon development of the genetic mutation. Therefore, development of the CNV at a specific genomic location when compared to the control lineage sperm DNA may eliminate the ability to detect the differential DNA methylation site in the F3 generation. Examples of several representative epimutations and CNV clusters are shown in **Figure 4**. Therefore, future studies are needed to clearly establish the relationship of the epimutations and CNV.

The final analysis determined the gene associations with CNV and epimutations (**Table 1B**). The CNVs were found to associate with one potential gene in the rat genome. The epimutations were found to correlate with 8 annotated genes (**Table 1B**). Analysis of the single-probe correlated genes for the 4,912 CNVs had 149 correlated genes, while the epimutations with 9,771 had 538 correlated genes. Although higher level of false positives is anticipated with the single-probe sites, less than 5% of the single-probe sites correlated with the genes. Therefore, the majority of the epimutations and CNV identified in the F3 generation vinclozolin sperm are not directly associated or proximal to genes, but are intergenic. Previous studies have demonstrated transgenerational epimutations can exist in Epigenetic Control Regions (ECR) of approximately 4–5 Mb, such that distal effects on gene expression for multiple genes within the ECR can be observed.[24]

Discussion

Environmentally induced epigenetic transgenerational inheritance of disease or phenotypic variation requires the germline transmission of epimutations between generations.[1-4] There are 2 developmental periods when DNA methylation is dramatically reprogrammed (or reset): the primordial germ cell (PGC), prior to and during fetal gonadal sex determination,[25,26] and the early embryo, following fertilization.[27] Interestingly, imprinted genes also undergo erasure and reprogramming during gonadal sex determination and re-methylate correctly through unknown mechanisms and are therefore protected from DNA de-methylation after fertilization.[28] Epigenetic transgenerational inheritance of germ cell epimutations are thought to act as imprinted-like sites and have the same reprogramming events,[29] but this remains to be experimentally established. In the event the germline (sperm) has an altered epigenome that escapes reprogramming following fertilization, the embryonic stem cells derived will have an altered epigenome. All cell types and tissues derived from the

Table 2. Transgenerational CNV and Epimutation Genomic Clusters

(A) F3 Generation CNV Clusters

CNV Cluster Name	Chromosome	Cluster Start	Cluster End	Size (Mb)
CNVcChr3-23.65	3	23650000	27600000	3.95
CNVcChr3-75.85	3	75850000	79550000	3.7
CNVcChr6-10.45	6	10450000	14250000	3.8
CNVcChr7-118.95	7	118950000	127100000	8.15
CNVcChr11-77.65	11	77650000	79950000	2.3
CNVcChr14-74.75	14	74750000	78400000	3.65
CNVcChr15-54.05	15	54050000	57000000	2.95
CNVcChr16-34	16	34000000	38050000	4.05
CNVcChr19-47.8	19	47800000	51800000	4
CNVcChrX-87.6	X	87600000	91350000	3.75

(B) F3 Generation Epimutation Clusters

Epimutation Cluster Name	Chromosome	Cluster Start	Cluster End	Size (Mb)
DMRcChr2:18.7	2	18700000	22650000	3.95
DMRcChr2:123.85	2	123850000	126150000	2.3
DMRcChr3:11.85	3	11850000	14850000	3
DMRcChr3:73.65	3	73650000	77400000	3.75
DMRcChr3:99	3	99000000	101600000	2.6
DMRcChr3:134	3	134000000	137950000	3.95
DMRcChr4:10.35	4	10350000	13550000	3.2
DMRcChr4:56.3	4	56300000	59750000	3.45
DMRcChr4:95.9	4	95900000	99500000	3.6
DMRcChr4:167.45	4	167450000	171350000	3.9
DMRcChr5:42.95	5	42950000	46900000	3.95
DMRcChr5:101.9	5	101900000	104750000	2.85
DMRcChr6:22	6	22000000	25700000	3.7
DMRcChr7:13.85	7	13850000	17550000	3.7
DMRcChr7:79.45	7	79450000	81950000	2.5
DMRcChr7:133.45	7	133450000	136100000	2.65
DMRcChr9:83.15	9	83150000	87100000	3.95
DMRcChr11:30.4	11	30400000	33050000	2.65
DMRcChr11:63.15	11	63150000	65550000	2.4
DMRcChr12:39.8	12	39800000	41900000	2.1
DMRcChr18:67.3	18	67300000	70850000	3.55
DMRcChr19:0.55	19	550000	4450000	3.9

stem cells will have alterations in their epigenomes and gene expression.[7,24] This is how an altered germline epigenome can promote the etiology of disease in various tissues and cell types.[30] Transgenerational transmission requires the absence of direct exposure or genetic manipulation. For example, if a gestating female is exposed to an environmental factor, the F0 generation female, F1 generation fetus, and germline in the fetus, which will generate the F2 generation, are directly exposed.[1] The effects on the F0, F1, and F2 generation can be due to direct multigenerational exposure and not necessarily involve transgenerational transmission. The first unambiguous transgenerational phenotype involving no direct exposure would be observed in the F3 generation.[1,31] In contrast, when an adult male or female (not pregnant) F0 generation is exposed, the F0 generation and germline that will generate the F1 generation are directly exposed. Therefore, the F2 generation is the first that is considered transgenerational.[31] Direct exposure germline epigenetic modifications need to become permanently programmed and transmit the epigenetic information to subsequent generations. The environmentally induced epigenetic transgenerational inheritance phenomenon has been observed in a variety of species from plants to humans.[1,6] Therefore, the phenomenon is initially induced through an epigenetic alteration in the germline. The question addressed in the current study was the potential role of transgenerational epimutations in altering genetic mutations in later generations.

Previous studies have demonstrated that alterations in the epigenome can promote genome instability.[11-16,18-21] One of the best examples is the role of epigenetics in influencing genetic events resulting in cell transformation and cancer.[18,32] The role of DNA methylation in the formation of SNPs and CNVs has been described.[12,14,21] Additional epigenetic processes, such as non-coding RNA and histone modifications, have also been shown to promote genetic mutations.[33,34] Therefore, increasing evidence suggests epigenetics has an important role in promoting genetic mutations. [18,32,35] In contrast, recent studies have also demonstrated that genetic manipulations can promote alterations in the epigenome.[36-38] For example, incompatible cross-pollination promotes the transgenerational mobilization of transposons that promote epigenetic instability.[39] This has led to the proposal that genetics drives epigenetics.[40] Although a genetic focus is

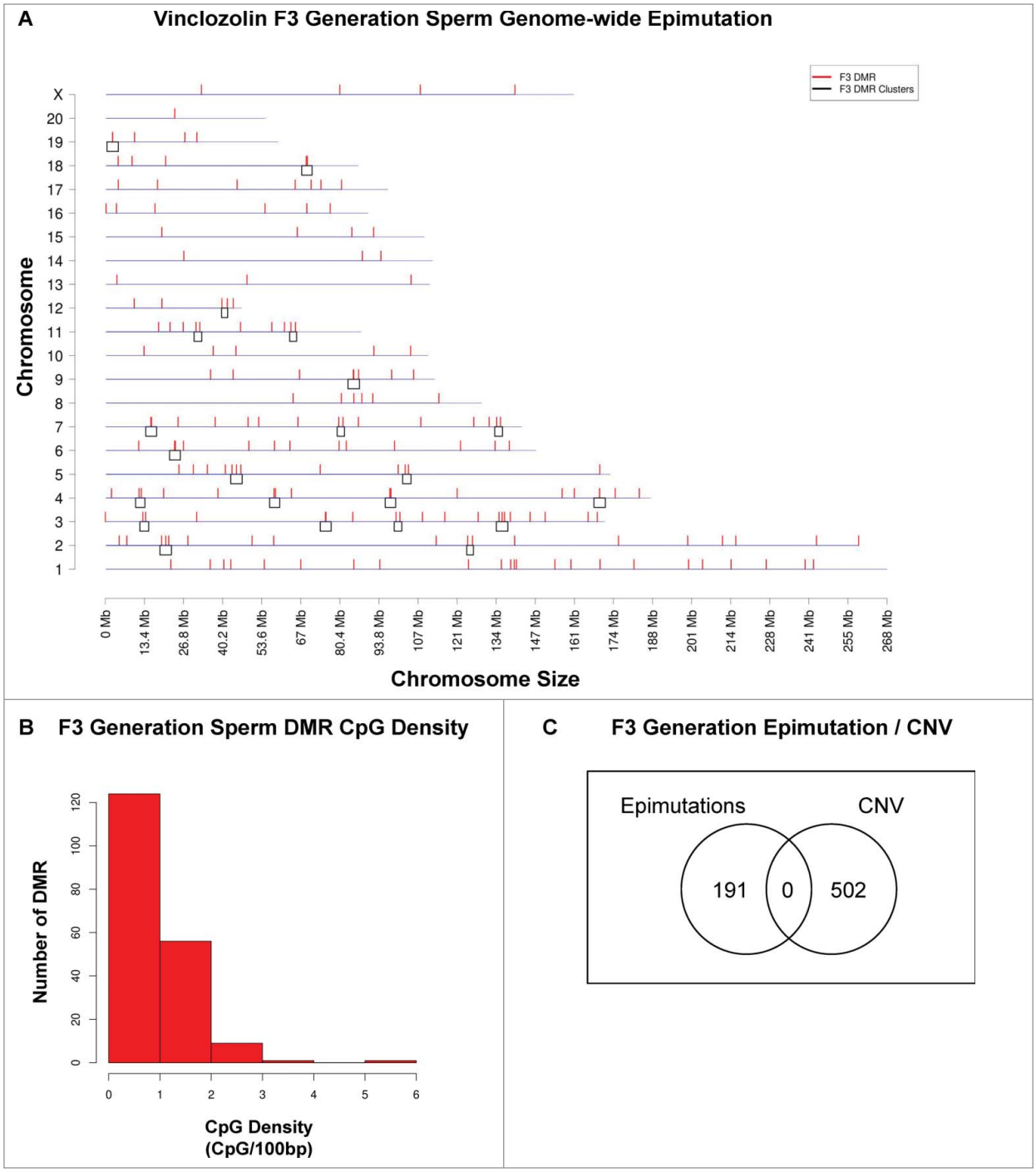

Figure 3. Transgenerational Vinclozolin F3 Generation Sperm Genome-wide Epimutation Analysis. (**A**) Vinclozolin lineage F3 generation sperm epimutations (191) chromosomal locations; box indicates a statistically significant over-represented cluster of epimutations. (**B**) F3 generation sperm DMR/epimutation CpG density. (**C**) F3 generation epimutation and CNV overlap Venn diagram.

predominant in the current concepts of biology, it will be an integration of epigenetics and genetics, with neither being dominant, that likely will provide a more accurate perspective on the molecular control of biological processes.[1,7,18] Therefore, some phenomena will involve "epigenetics driving genetics." The influence of the environment on epigenetic transgenerational inheritance appears to be a good example of this type of phenomena.

Previously, we have demonstrated the ability of vinclozolin to promote the epigenetic transgenerational inheritance of sperm epimutations using a genome-wide promoter analysis.[3] Subsequently, we found that a variety of environmental toxicants promoted transgenerational sperm epimutations in an exposure-specific manner.[4] In the original analysis, one of the differentially methylated regions detected was found to be a CNV in the

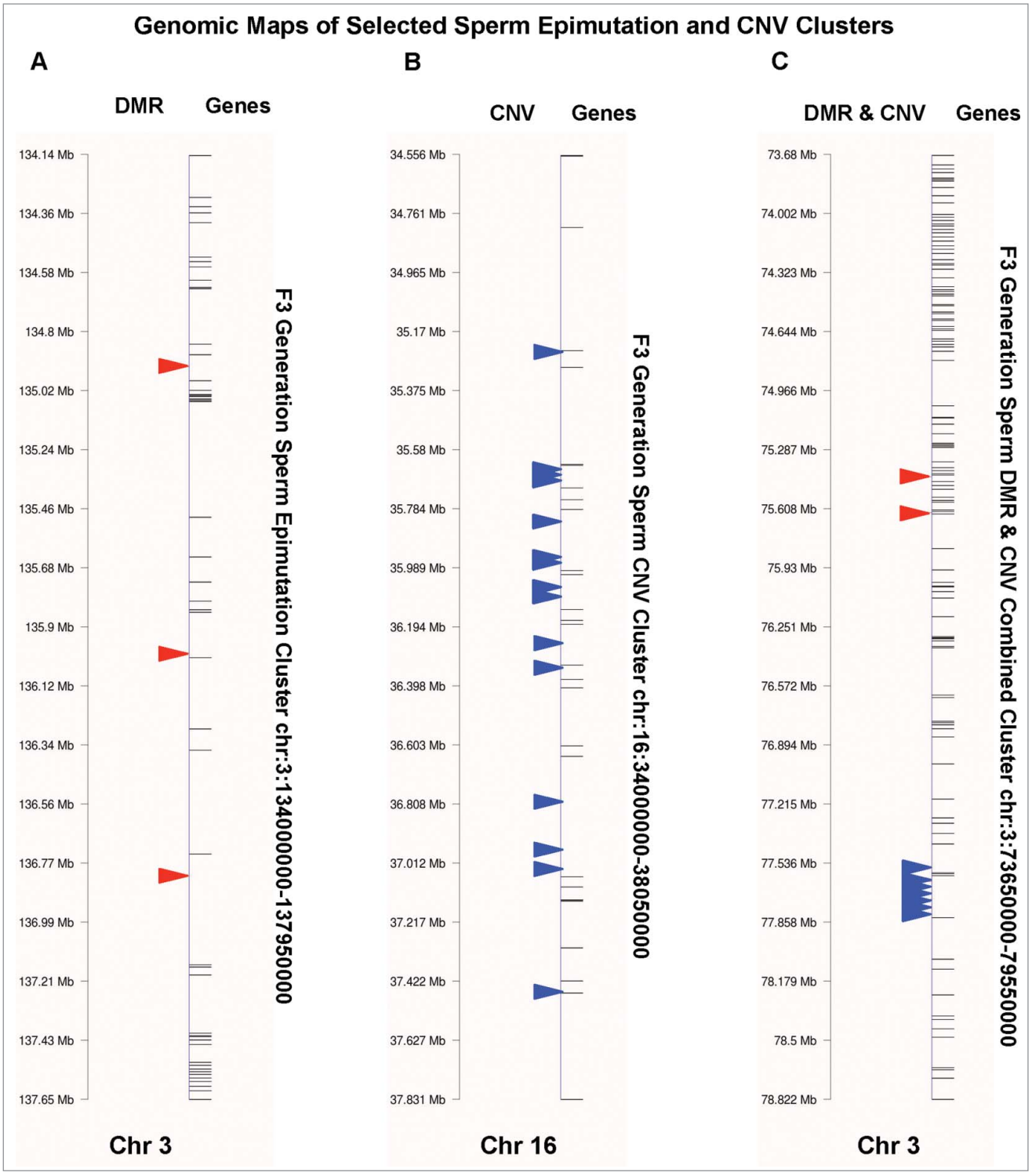

Figure 4. Genomic Maps of Selected Correlated Sperm Epimutations and CNV Cluster. (**A**) F3 generation epimutation cluster with genes (ticks) on right and epimutations (red arrow) on left aligned to chromosomal location. (**B**) F3 generation CNV cluster with genes (ticks) and CNV (blue arrow) aligned to chromosomal location. (**C**) F3 generation overlapped CNV and epimutation clusters with genes (ticks) and epimutation (red arrow) and CNV (blue arrow) aligned to chromosomal location.

Fam111a promoter.[3] This led to the hypothesis that transgenerational epigenetic alterations may influence genetic mutation frequency.[3] The current study used a genome-wide analysis to identify vinclozolin induced transgenerational epimutations (i.e., DMRs). Most of the 191 epimutations were found not to be associated with gene promoters but rather are in regions of the

genome with no associated genes. These genome-wide epimutations were also primarily present in CpG deserts, as previously described.[8] This analysis provides the first genome-wide view of the transgenerational epimutations. The current study used a CNV analysis as a reflection of the genetic alterations that may occur transgenerationally. The vinclozolin lineage F1 generation sperm was found not to have a significant increase in CNV, with the small change of 39 CNV being within the anticipated genetic variation within this outbred rat population of animals.[20,23] Interestingly, the vinclozolin lineage F3 generation sperm had a dramatic increase of 502 CNV. Therefore, the environmentally induced epigenetic transgenerational inheritance of sperm epimutations correlated with a significant increase in genetic CNV mutations in the transgenerational F3 generation. Although the specific genomic locations of the F3 generation epimutations and CNV did not overlap, there were some correlations in larger genomic regions. However, the epimutation and CNV experiments were distinct using different F0 generation female lineages, such that future experiments will be needed to better determine the relationship between the transgenerational epimutations and genetic mutations. Observations suggest the environmental toxicant vinclozolin promotes an epigenetic reprogramming of the germline in the first generation that induced increased genomic instability and genetic mutations transgenerationally.

Clearly, epigenetics and genetics are integrated, such that most phenomena require the interplay of both.[7,18] The current study demonstrates that an environmental factor can promote epigenetic transgenerational inheritance of germline epimutations and appearance of genetic mutations (i.e., CNVs) in later generations (i.e., F3). Likely, other types of genetic mutations are also involved, and a study is in progress to investigate epigenetically induced point mutations. Therefore, the transgenerational phenotype will likely involve an integration of epigenetics and genetics. Our observations provide an example of the ability of epigenetic mechanisms to drive genetic change. Environmental epigenetics may be the major molecular mechanism involved in environment-gene interactions and emergence of genetic variation. The predominant current view for the origin and evolution of disease considers genetic mutations as the primary molecular mechanism involved. Environmental impacts on the epigenome that have the ability to promote genetic mutations extend these previous views and help clarify how the environment may have direct impact on disease etiology and on the origins of phenotypic and genotypic variation in evolutionary processes.

Methods

Animal studies and breeding

Female and male rats of an outbred strain Hsd:Sprague Dawley (Harlan) at 70 to 100 d of age were fed *ad lib* with a standard rat diet and *ad lib* tap water for drinking. To obtain time-pregnant females, the female rats in proestrus were pair-mated with male rats. The sperm-positive (day 0) rats were monitored for diestrus and body weight. On days 8 through 14 of gestation,[41] the females were administered daily intraperitoneal injections of vinclozolin (100 mg/kg BW/day) or dimethyl sulfoxide (vehicle) as a control. Generally, sister littermates were selected for the control and vinclozolin lineages F0 generation females. In addition, similar groups of F0 generation males were used between the control and exposure lineages. Vinclozolin was obtained from Chem Service Inc., West Chester, PA, USA, and was injected in a 200-μl DMSO/sesame oil vehicle, as previously described.[4] Treatment lineages are designated "control" or "vinclozolin" lineages. The gestating female rats treated were designated as the F0 generation. The offspring of the F0 generation rats were the F1 generation. Non-littermate females and males aged 70–90 d from F1 generation of control or vinclozolin lineages were bred to obtain F2 generation offspring. The F2 generation rats were bred to obtain F3 generation offspring. Only the F0 generation gestating female was directly treated transiently with vinclozolin. Different F0 generation females were used for the different experiments and one male per litter was selected for the individual animals for a specific experiment. Two different experiments with different groups of animals were performed with an $n = 6$ for epigenetic analysis (F3 generation), and $n = 18$ for genetic analysis ($n = 9$ for F1 generation and $n = 9$ for F3 generation). Therefore, the epimutation and CNV studies were distinct, involving different F0 generation female lineages. The control and vinclozolin lineages were housed in the same room and lighting racks, food, and water as previously described.[4,42,43]

Epididymal sperm collection, DNA isolation, and methylated DNA immunoprecipitation

The epididymis was dissected free of connective tissue, and a small cut was made to the cauda, which was then placed in 5 ml of F12 culture medium containing 0.1% bovine serum albumin for 10 min at 37°C and then kept at 4°C to immobilize the sperm. The epididymal tissue was minced and the released sperm centrifuged at 13,000 × g and stored in fresh nucleus isolation medium (NIM) buffer at −20°C until processed further. Sperm heads were separated from tails through sonication, following previously described protocol (without protease inhibitors),[44] and then purified using a series of washes and centrifugations [45] from a total of 9 F1 generation and 15 F3 generation rats per lineage (control or vinclozolin) that were 120 d of age. DNA extraction on the purified sperm heads was performed as described.[3] Equal concentrations of DNA from different sets of 3 individual sperm samples were used to produce 2 different DNA pools per lineage. A total of 6 of the F3 generation pools were used for chromatin immunoprecipitation of methylated DNA fragments (MeDIP). MeDIP was performed as previously described.[3,4] The DNA samples and animals used for the MeDIP were distinct from those used for the CNV analysis.

MeDIP-chip analysis

The comparative MeDIP-Chip were performed with Roche Nimblegen's custom whole-genome array, which contains 2 different array sets, with 4,085,426 probes per sub-array. Probe sizes ranged from 50–75 bp in length with the median probe spacing of approximately 300 bp. Two different comparative (MeDIP vs. MeDIP) hybridization experiments were performed for the

F3 generation vinclozolin lineage vs. control, with each array set encompassing DNA samples from 6 animals (3 each from vinclozolin and control). MeDIP DNA samples from experimental lineages were labeled with Cy3 and MeDIP DNA samples from the control lineage were labeled with Cy5.[46,47]

Copy number variation (CNV) analysis

The array used for the copy number variation analysis was a chromosomal genomic hybridization (CGH) custom design by Roche Nimblegen that consisted of a whole-genome tiling array of the rat genome with 385,102 probes per array. Probe size ranged from 50–75mer in length with median probe spacing of 1,395 bp. Three different comparative (CNV vs. CNV) hybridization experiments were performed (2 array set) for each experiment having vinclozolin vs. control lineage, with each array including hybridizations from DNA pools from different experiments. For each array, genomic DNA samples from the vinclozolin lineage were labeled with Cy3 and genomic DNA samples from the control lineage were labeled with Cy5. Equal concentrations of DNA from sperm samples from groups of 3 individuals were used to produce 3 different DNA pools per lineage and employed for the CNV analysis. These sperm DNA samples were distinct from those used for the MeDIP-Chip analysis. The Nimblegen tiling arrays are no longer available due to its acquisition by Roche.

Bioinformatics and statistics

For the MeDIP-Chip and CNV experiments, raw data from 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]. Within-array and between-array normalizations were performed as previously described.[4] For the CGH, following normalization, the average value of each probe was calculated and 2 different copy number variation algorithms were used on each of these probes: CGHseg [48] and cghFlasso.[49] These 2 algorithms were used with the default parameters. Average values from the output of these algorithms were obtained. A threshold of 0.05 as a cut-off was used on the summary (average of the log-ratio from the 2 algorithms), where gains are probes above the positive threshold and losses are probes below the negative threshold. Consecutive probes ($\geq$3) of gains and losses were used to identify separate CNV regions. A cut-off of three-probe minimum was used and those regions were considered a valid CNV. Statistically significant copy number variation regions (CNVs) were identified.

The chromosomal location of CNV and DMR clusters used an R-code developed to find chromosomal locations of clusters.[24] A 2-Mb sliding window with 50,000-base intervals was used to find the associated CNVs and DMRs in each window. A Z-test statistical analysis with $P < 0.05$ was used on these windows to find the ones with over-represented; CNVs and DMRs were merged together to form clusters. A typical cluster region averaged approximately 3–4 megabases in size.

The DMR and CNV association with specific rat genes and genome locations used the Gene NCBI database for rat gene locations and correlated the epimutations associated (overlapped) with the genes. The three-adjacent-probes constituted approximately a 200 bp homology search. Statistically significant over-representation uses a Fisher's exact analysis. All DMR and CNV genomic data obtained in the current study have been deposited in the NCBI public GEO database (GEO #: GSE61480).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

Ethics Statement

All experimental protocols for the procedures with rats were pre-approved by the Washington State University Animal Care and Use Committee (IACUC approval # 02568-032).

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