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Environmentally induced epigenetic transgenerational inheritance of sperm epimutations promote genetic mutations

Michael K Skinner*, Carlos Guerrero-Bosagna, and M Muksitul Haque

Center for Reproductive Biology; School of Biological Sciences; Washington State University; Pullman, WA USA

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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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*Correspondence to: Michael K Skinner; Email: skinner@wsu.edu
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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.

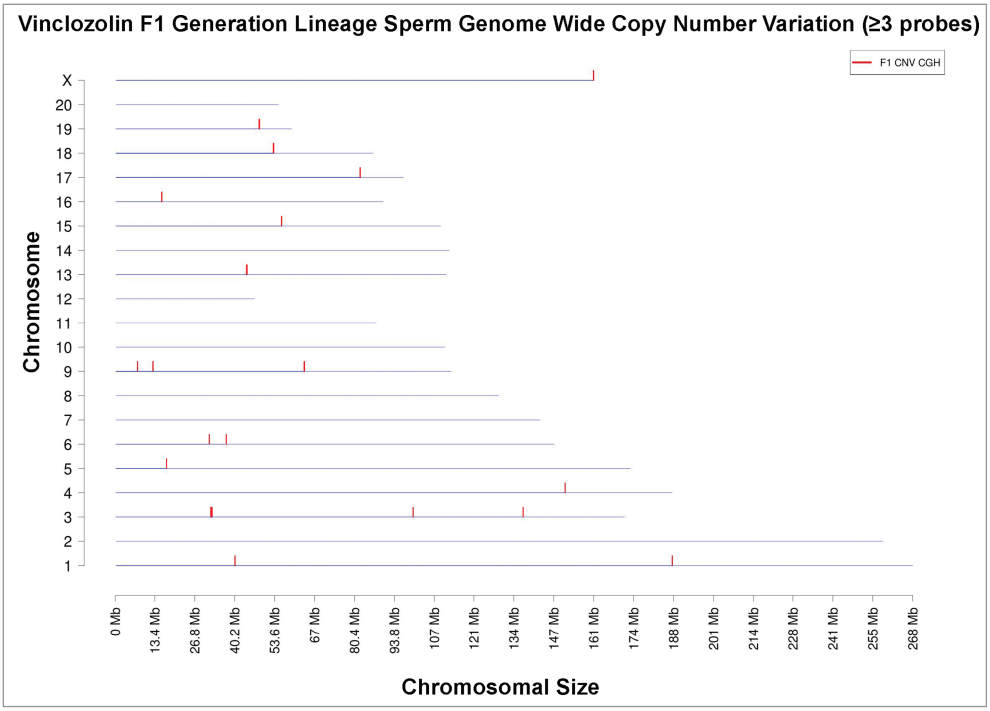

Figure 1. Transgenerational Vinclozolin F1 Generation Sperm Genome-wide Genetic CNV Mutation Analysis. F1 generation sperm genome-wide CNV (≥3 probe, 39 CNV) chromosomal locations and box indicates a statistically significant over-represented cluster of CNV.

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

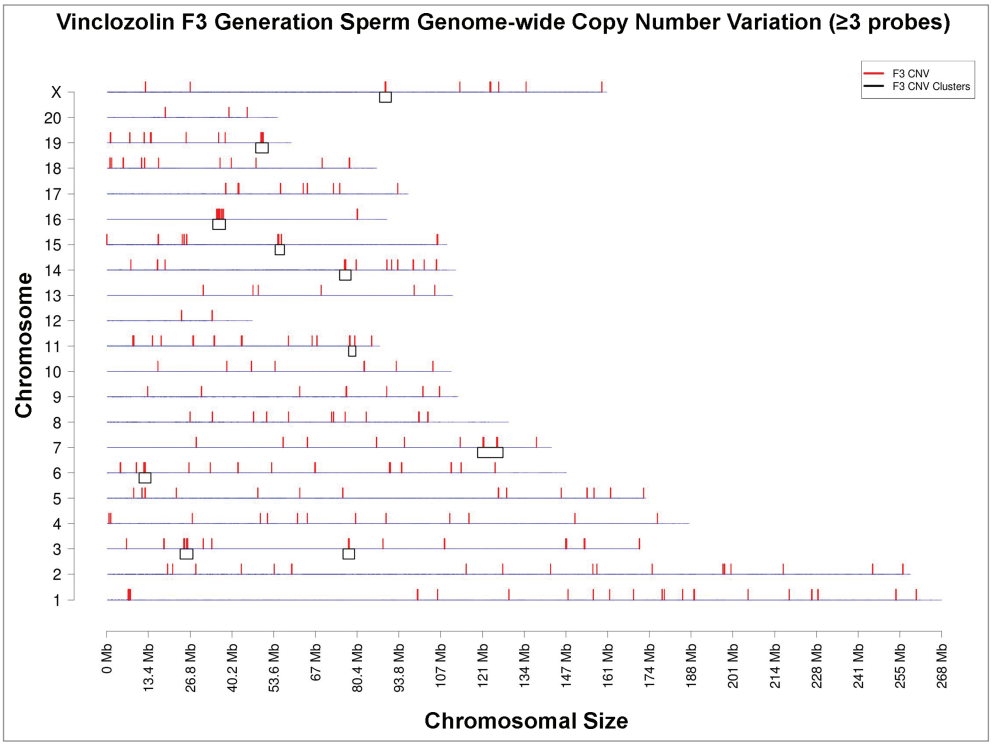

Figure 2. Transgenerational Vinclozolin F3 Generation Sperm Genome-wide Genetic CNV Mutation Analysis. F3 generation sperm genome-wide CNV (≥3 probe, 502 CNV) chromosomal locations; box indicates a statistically significant over-represented cluster of CNV.

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

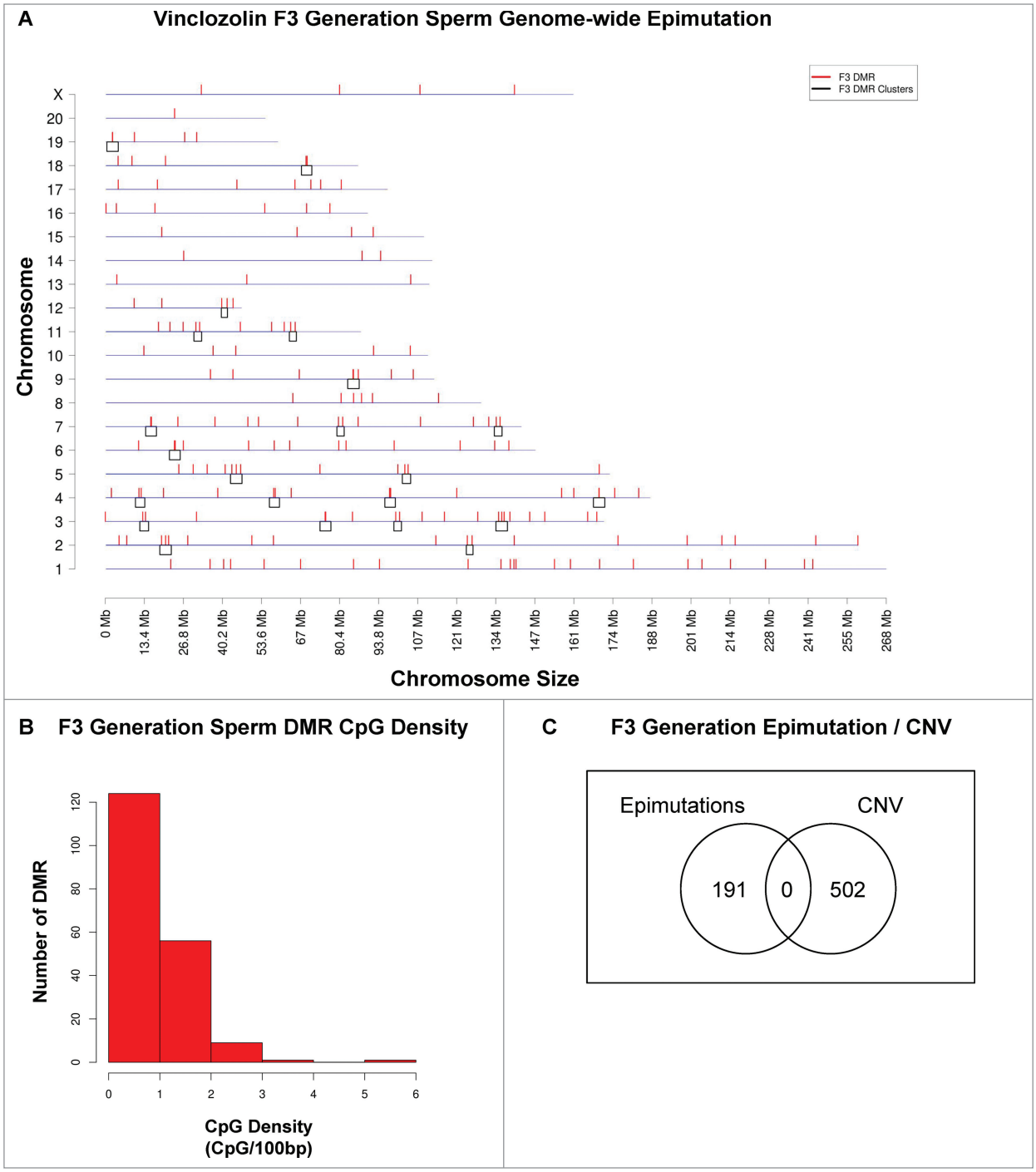

Figure 3. Transgenerational Vinclozolin F3 Generation Sperm Genome-wide Epimutation Analysis. (**A**) Vinclozolin lineage F3 generation sperm epimutations (191) chromosomal locations; box indicates a statistically significant over-represented cluster of epimutations. (**B**) F3 generation sperm DMR/epimutation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

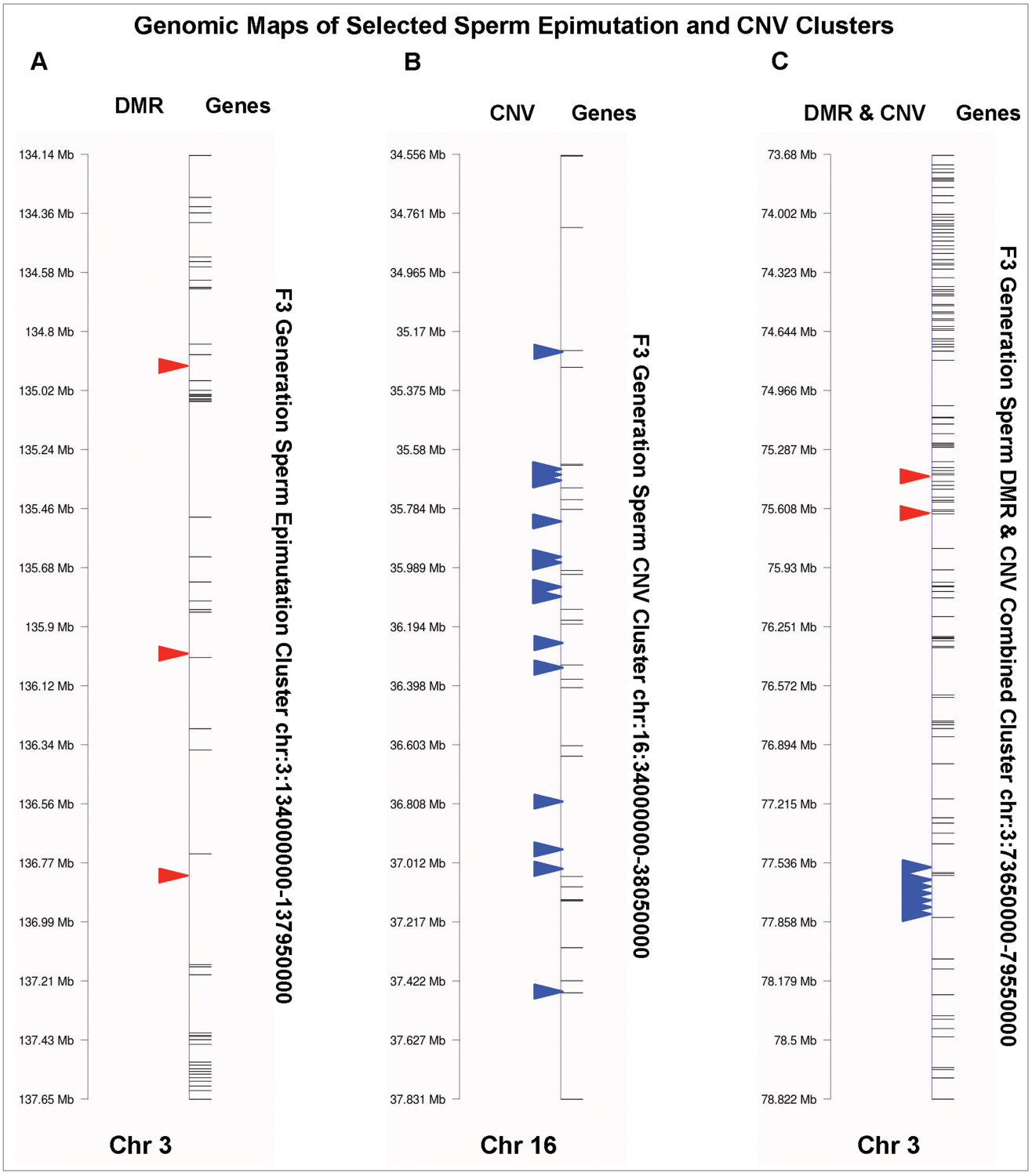

Figure 4. Genomic Maps of Selected Correlated Sperm Epimutations and CNV Cluster. (**A**) F3 generation epimutation cluster with genes (ticks) on right and epimutations (red arrow) on left aligned to chromosomal location. (**B**) F3 generation CNV cluster with genes (ticks) and CNV (blue arrow) aligned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