Generational comparisons (F1 versus F3) of vinclozolin induced epigenetic transgenerational inheritance of sperm differential DNA methylation regions (epimutations) using MeDIP-Seq

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Abstract

Environmentally induced epigenetic transgenerational inheritance of disease and phenotypic variation has been shown to involve DNA methylation alterations in the germline (e.g. sperm). These differential DNA methylation regions (DMRs) are termed epimutations and in part transmit the transgenerational phenotypes. The agricultural fungicide vinclozolin exposure of a gestating female rat has previously been shown to promote transgenerational disease and epimutations in F3 generation (great-grand-offspring) animals. The current study was designed to investigate the actions of direct fetal exposure on the F1 generation rat sperm DMRs compared to the F3 transgenerational sperm DMRs. A protocol involving methylated DNA immunoprecipitation (MeDIP) followed by next-generation sequencing (Seq) was used in the current study. Bioinformatics analysis of the MeDIP-Seq data was developed and several different variations in the bioinformatic analysis were evaluated. Observations indicate needs to be considered. Interestingly, the F1 generation DMRs were found to be fewer in number and for the most part distinct from the F3 generation epimutations. Observations suggest the direct exposure induced F1 generation sperm DMRs appear to promote in subsequent generations alterations in the germ cell developmental programming that leads to the distinct epimutations in the F3 generation. This may help explain the differences in disease and phenotypes between the direct exposure F1 generation and transgenerational F3 generation. Observations demonstrate a distinction between the direct exposure versus transgenerational epigenetic programming induced by environmental exposures and provide insights into the molecular mechanisms involved in the epigenetic transgenerational inheritance phenomenon.

Key words: environmental epigenetics; transgenerational; MeDIP-Seq; DMR; bioinformatics; DNA methylation

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Introduction

One of the first observations of environmentally induced epigenetic transgenerational inheritance of disease involved the fungicide vinclozolin [1]. Vinclozolin is an antiandrogenic compound that is one of the most commonly used fungicides in the fruit and vegetable industry [2]. Vinclozolin has been shown to promote transgenerational phenotypes in mice and rats by a number of different laboratories [1, 3–7]. The current study was not designed to examine vinclozolin risk assessment, but to further investigate epigenetic transgenerational actions of vinclozolin and assess the different analysis methods. The molecular mechanism involved in the transgenerational inheritance of disease requires an epigenetic modification of the germline (sperm or egg) [1, 8, 9]. Initially, an alteration in DNA methylation was observed [1, 10, 11], but more recently alterations in non-coding RNA expression [12, 13] and histone retention [14, 15] in the germ cells have also been observed. Therefore, the integrated actions of DNA methylation, ncRNA, and histone retention may be involved in the epigenetic inheritance process [16, 17]. Over the past ten years a large number of laboratories have demonstrated environmentally induced epigenetic transgenerational inheritance phenotypes in a variety of organisms [8, 18, 19]. In the rat studies, similar actions have been observed with vinclozolin, plastic compounds bisphenol A (BPA) and phthalates, hydrocarbon mixture (jet fuel), dioxin, insect repellent DEET and pesticides permethrin, methoxychlor and DDT, and tributyltin [1, 20–29]. The phenomenon has now been observed in plants, insects, worms, fish, birds, pigs, rodents, and humans, so appears highly conserved [8, 30–34].

In considering the phenomenon of environmentally induced epigenetic transgenerational inheritance a distinction needs to be made for direct exposure impacts versus the transgenerational impacts in the absence of continued exposure [8, 35]. When a gestating female is exposed at the critical period of gonadal sex determination (days 8–14 rat or weeks 6–18 humans), the F0 generation female, the F1 generation fetus, and the germ-line that will generate the F2 generation are directly exposed, such that the F3 generation is the first transgenerational generation not having direct exposure [35]. When an adult male or non-pregnant female is exposed the F0 generation adult and the germ-line that will generate the F1 generation offspring are directly exposed, such that the F2 generation is the first transgenerational generation not having direct exposure [35]. Previous studies have demonstrated differences and similarities between the F1 and F3 generation disease and phenotypes for a number of different exposures [7, 25, 27, 28]. The focus of the current epigenetic analysis has been on the F3 generation sperm to address the transgenerational mechanism [10, 26]. An important question not previously investigated in mammals is how the epigenetics of the direct exposure F1 generation sperm compares with the transgenerational F3 generation sperm. One possibility is that the same epimutations are present in both the F1 and F3 generation sperm. Alternatively, the germ-line epigenetic developmental programming may be different between the generations leading to distinct epimutations. The current study was designed to investigate this comparison using more advanced molecular technologies.

The original analysis of the environmentally induced epigenetic transgenerational inheritance of sperm epimutations involved methylated DNA immunoprecipitation (MeDIP) followed by tiling array chips (Chip) of genome wide promoter regions of the genome [10, 11, 26]. This MeDIP-Chip analysis identified differential DNA methylation regions (DMRs) or epimutations in F3 generation sperm for a variety of toxicant induced transgenerational lineages [26]. Interestingly, the epimutation sites for the different exposures were found to be primarily exposure specific [26]. The MeDIP-Chip technology is useful but is limited to the oligonucleotides on the array, so does not consider the entire genome easily. A more advanced technology involves next-generation sequencing (NGS) for an MeDIP-Seq analysis that is genome-wide and not limited to selected oligonucleotides [36]. An MeDIP-Seq protocol was developed and is presented in the current study, along with the associated bioinformatics used to analyze the data. This study also investigates optimization of different bioinformatics approaches, pointing out the advantages and disadvantages of each.

The current study was designed to investigate and compare the genome-wide DMRs between the direct exposure F1 generation and transgenerational F3 generation sperm in control versus vinclozolin lineage rats. This is one of the first comparison of the F1 and F3 generation sperm epigenetic alterations. An advanced MeDIP-Seq protocol with associated bioinformatics was optimized and investigated. Interestingly, the DMRs of the F1 versus F3 generation sperm were found to be largely distinct. The mechanism and insights provided into environmentally induced epigenetic transgenerational inheritance phenomena are discussed.

Results

MeDIP-Seq Protocol and Bioinformatics

An MeDIP-Seq protocol was developed which used more advanced technology in the MeDIP procedure with the use of magnetic beads for the immunoprecipitation as outlined in the Methods. This increased the speed and reduced the non-specific binding associated with agarose bead protocols. The MeDIP DNA was used to generate NGS libraries with modifications to the manufacturer’s protocol to address the single stranded status of the MeDIP DNA, as described in the Methods. The libraries were then used for NGS to complete the MeDIP-Seq procedure. An outline of the major steps of the protocol is presented in Supplementary Fig. S1 and details presented in the Methods.

NGS was performed on an Illumina platform (2500) generating approximately 35 million reads per sample. The sequencing quality control and processing procedure is outlined in the Methods. A bioinformatics pipeline was developed for each step in the analysis as summarized in Supplementary Fig. S2. The details of the bioinformatics approach are presented in the Methods and include mapping, filtering, normalization, differential methylation analysis, genomic feature characterization, including size (kb) and CpG density, and gene association identification. Optimization of the pipeline involved varying the filtering parameters and normalization strategy to determine how these steps impacted the resulting DMR identification. The filtering was done using three different read depth thresholds. Similarly, three different normalization strategies were also evaluated. The specific analysis details are described in the Methods. Initially, the results using largely default parameters to the MEDIPS R package analysis are presented for all the DMR data. Subsequently, the results of analyses with deviations from these defaults are shown in the ‘Bioinformatics Optimization’ section.

Differential DMR Analysis

For the main analysis, parameters close to the defaults provided by the MEDIPS R package were used. A detailed summary of
these can be found in the Methods. The F1 generation control versus vinclozolin lineage sperm DMR analysis identified 290 total DMRs with a $P < 10^{-4}$ as shown in Fig. 1. The DMR numbers for various $P$ values are listed for all DMRs and those with multiple window (e.g. adjacent) DMR. Further analysis shows the majority of DMR include a single 100 bp window with an edgeR $P$-value $< 10^{-4}$ (Fig. 1B). The ratio of total reads associated with the DMR (control/total) for comparison identified DMRs with an increase (~70%) or decrease (~30%) in DNA methylation in the vinclozolin lineage sperm (data not shown). The F3 generation control versus vinclozolin lineage sperm DMR analysis identified 916 total DMR with an edgeR $P$-value $< 10^{-4}$ as shown in Fig. 2A. The $P < 10^{-4}$ was selected to allow a more balanced data analysis and stringent DMR selection. The DMR numbers for various $P$ values are listed for all DMRs and those with multiple 100 bp windows with an edgeR $P$-value $< 10^{-4}$. The majority of the DMR have a single 100 bp window, while a small number of DMR have ≥2 windows and some with greater than 5 windows (Fig. 2B). Similar to the F1 generation DMR, the F3 generation DMRs also had a (~70/30%) mixture of both an increase and decrease in DNA methylation (data not shown). Observations demonstrate a three-fold higher number of DMRs in the F3 generation sperm than the F1 generation sperm.

The genomic locations of the DMRs identified are presented using a chromosomal plot previously described [10]. The F1 generation DMRs was identified on all chromosomes except the Y chromosome and mitochondrial DNA (Fig. 3). When a statistically over-represented cluster of DMR is present, a black box under the chromosome bar is shown. Red arrow heads identify individual DMR. The genome locations of the F3 generation DMRs are presented in Fig. 4 and show all chromosomes contain DMRs except the Y mitochondrial chromosome. A list of the DMRs for F1 generation sperm is presented in Supplementary Table S1 and for F3 generation sperm in Supplementary Table S2. A list of the DMR clusters is presented in Supplementary Table S3 for both the F1 and F3 generation DMR clusters.

A comparison of the F1 and F3 generation sperm DMRs was made to see the degree of overlap versus generation specific DMR (Fig. 5A). None of the 290 DMR in the F1 generation ($P < 10^{-4}$) overlapped with any of the 916 DMR in the F3 generation ($P < 10^{-4}$). An extension of this analysis that included 10 kb flanking regions did not alter the lack of overlap observed. Therefore, the DMR were distinct between the F1 and F3 generations. Similarly, an overlap of the F1 and F3 generation DMR clusters demonstrated one overlap with the majority being distinct between the generations (Fig. 5B). Observations demonstrate the sperm DMRs are for the most part distinct between the direct exposure F1 generation sperm versus the transgenerational F3 generation sperm.

The genomic features of the DMR for both the F1 and F3 generation sperm were investigated. The DMR length was approximately a kilobase in size with few over 5 kb in size for either the F1 or F3 generation sperm DMR (Fig. 6A and B). The CpG density was found to be predominantly 1 CpG/100 bp with few above 4 CpG per 100 bp for both the F1 and F3 generation sperm DMR (Fig. 6C and D). Therefore, the environmentally modified DMR exist in CpG deserts as previously described [37]. Similar genomic features are identified between the F1 and F3 generation sperm, independent of their distinct chromosomal locations.

The DMR associated genes were identified to determine potential overlap with specific genes or associated pathways. The gene associations were identified with DMR when they were within 10 kb of a specific gene. Those genes associated with specific DMR are identified in Supplementary Tables S1 and S2.
The gene classification category for the DMR associated genes is presented in Fig. 7. The predominant gene classification categories for both the F1 and F3 generation sperm DMR were signaling, receptor and transcription. The relative ratios for each are shown in Fig. 7. Therefore, there is similarity in gene classification categories between the F1 and F3 generation sperm DMRs. Cellular and signal transduction pathways were analyzed using KEGG pathways as described in the Methods. A list of the predominant pathways and number of associated DMR genes is presented in Fig. 8. The metabolism, cAMP-signaling pathway (Supplementary Fig. S3), endocytosis (Supplementary Fig. S4), olfactory transduction (Supplementary Fig. S5), oxytocin pathway (Supplementary Fig. S6), and axon guidance (Fig. 9; random representative selected), pathways were in common between the F1 and F3 generation sperm DMR. Therefore, the DMR associated genes were distinct between the F1 and F3 generation sperm DMR, but some common pathways were identified.

Bioinformatics Optimization

The data presented used the default parameters in the MEDIPS R analysis [38]. Negligible information exists on how differences in parameter combinations affect the overall MEDIPS R analysis results. In order to help determine an appropriate parameter set, two aspects of the analysis, genomic window filtering and type of normalization, were varied over a limited range of values. The analysis was performed with three different levels of genomic window filtering. Genomic windows with fewer than a chosen number of reads summed across all samples were removed from the analysis prior to the differential coverage calculations. This affects the parameterization of the edgeR model. Three different minimum filtration read thresholds were used, min = 1, min = 10 (default), and min = 40. The min = 10 is the default threshold used in the edge R model analysis. All other analysis options were held constant, using the default normalization method TMM (Trimmed Mean of M-values) [39]. Observations indicate substantial differences in the DMR identified between min = 10 with the min = 1 versus min = 40 for the F1 and F3 generation filtration. This also involves shifts in the percentage increases versus decreases in DNA methylation in the DMR and patterns of changes observed (data not shown). The min = 40 is suggested for future analysis due to a decrease in potential false positive DMR.

Normalization is used to remove unwanted noise from the dataset. Ideally, normalization minimizes all technical variation between samples, for example, correcting for library size (total read numbers) variation. There are several normalization approaches that can be used for MeDIP-Seq data. We compare the default TMM normalization (control for library size) with two variations of Remove Unwanted Variation (RUV) normalization [40]. RUV normalization takes advantage of regions of the genome that are known to be constant across treatment groups. A key step in the RUV process is to identify these regions. While spike-in controls would be ideal, here we test two different techniques for identifying the control regions. First, since methylation occurs primarily at CpG sites, regions of the genome without any CpG sites should have constant (zero) methylation between samples. The first RUV normalization variation uses regions of the genome with no CpG within 1 kb as the control sites (min = 10 – RUV CpG). The second variation uses genomic windows that had an edgeR P-value ≥ 0.8 (no significant DMR) in a preliminary analysis with minimal genomic window filtering and only TMM normalization (min 10 RUVpv). Observations indicate the RUV CpG for both F1 and F3 generation also show shifts in the DMR increase or decrease in methylation (data not shown). The normalization for library size (total read numbers) is suggested for future analysis.

The DMR number is similar regardless of the filtering level used. The number of DMR at a given edgeR P-value threshold is similar in both F1 and F3 generation datasets. The DMR also have a similar CpG density of one or two CpG/100 bp and a length of 1 kb (data not shown). However, these DMR are not completely identical. Supplementary Figure S7 shows the
overlap between these DMR. The majority of DMR is unique to a single analysis or is shared by two of the three analyses (Supplementary Fig. S7A and B).

The different normalization approaches result in dramatically different numbers of DMR (Supplementary Fig. S7). Using an edgeR P-value threshold of $10^{-4}$, the analysis using RUV-CpG (CpG negative group) normalization results in mostly unique DMR, while the TMM normalization analysis DMR are largely a subset of the RUV-pv normalization analysis DMR (Supplementary Fig. S7C). This pattern is the same in both F1 and F3 generations, although the overlap between DMR for the RUV-pv and RUV-CpG analyses is higher in the F1 results (Supplementary Fig. S7). These patterns are generally maintained when the P values are adjusted with false discovery rates. Observations indicate the normalization dramatically varies the edgeR analysis and DMR data sets, which is further discussed in the ‘Discussion’ section.

A further analysis compared the negative-binomial approach of the edgeR based MEDIPS analysis with the non-parametric approach of SAM (significance analysis of microarrays) implemented in the SAMseq R function [41]. The SAMseq analysis provided similar DMR numbers as the edgeR analysis (data not shown). Therefore, these results do not show an obvious advantage for either the parametric approach of edgeR or the non-parametric approach.

A final analysis investigated the variation in the DMR data analysis using a permutation analysis and pairwise comparison analysis with individual pool comparisons. The permutation analysis (sub-sampled data set comparisons) does not address the validity of the individual DMR, but tests for an increase in DMR. The permutation analysis did not identify an increased number of DMR. The use of only three pools for comparison appears to be a limitation with a permutation analysis [42, 43]. Therefore, the future optimal experimental design suggested is to analyze individual animal sperm samples with independent sequencing libraries to obtain more significance in the DMR identified and reduce the variation in the analysis. The second analysis involved a pairwise comparison using individual pool comparisons to determine if variation in the data may impact DMR detection. The F3 generation had minimal random DMR identified that were in common between the comparisons showing low variation, however, in the F1 generation we did identify variation that resulted in DMR being identified. Therefore, the F1 generation does have increased epigenetic variation that can impact the DMR detected in comparison to the decreased variation observed in the F3 generation DMR analysis. This analysis also suggests that individual animal analysis will be more efficient at DMR identification than a limited number of pooled analyses.

Discussion

Previously, the identification of vinclozolin induced transgenerational sperm DMRs used a promoter MeDIP-Chip analysis and identified approximately 50 DMRs in the F3 generation sperm [10]. Subsequently, a genome wide MeDIP-Chip analysis was performed and approximately 200 DMRs were identified [11]. In the current study, the vinclozolin induced transgenerational sperm DMRs used an MeDIP-Seq analysis and compared the F3 generation DMRs with the F1 generation DMRs. Therefore, the current study used a more advanced technology with optimized bioinformatics. Interestingly, the number of DMR in the F1 generation vinclozolin lineage was less than the F3 generation. A total of 290 DMRs were identified in the F1
generation sperm and 981 identified in the F3 generation sperm. The overlap between these DMR sets identified no DMR common to both the F1 and F3 generation sperm. In considering larger chromosomal regions involving statistically over-represented clusters of DMR, there was only one overlapping cluster between the F1 and F3 generation DMR sets (Fig. 3). Therefore, the F1 generation and F3 generation sperm DMR sets are distinct.

The fetal exposure of the germline of the F1 generation involved a direct exposure toxicity to vinclozolin. The exposure period involved primordial germ cell (PGC) development and early onset of testis development. This period corresponds to the demethylation of the PGCs and initiation of DNA methylation in the prospermatogonia stage of the male germ-line [44–46]. Therefore, the direct exposure altered the normal epigenetic programming and generated different DNA methylation regions and ncRNA distinct from the control population [47, 48]. In contrast, the F3 generation vinclozolin lineage did not have any direct exposure and involved a transgenerationally altered DNA methylation programming in the fetal male germline. The presumption is that it is this epigenetic programming modification that will generate a similar set of DMR as seen in the F3 generation to subsequent generations, but this remains to be established. Therefore, considering this difference in direct exposure versus transgenerational epigenetic programming it is not surprising that the sperm epimutations in the F1 and F3 generation sperm are distinct. The simplistic concept that a DMR is directly induced, such as an imprinted-like gene site, that is then maintained generationally appears not to be the case. Instead the altered transgenerational developmental programming mechanisms appear to promote the transgenerational DMR.

The majority of the DMRs in either the F1 or F3 generation sperm were found to not be associated with promoters but instead be intergenic and distal to known genes. Previous studies have demonstrated the presence of statistically significant over-represented clusters of genes around DMR [49]. The existence of ‘epigenetic control regions’ or ECR was previously reported associated with transgenerational DMRs [49, 50]. These ECR are generally 2–5 Mb in size and have multiple genes that

Figure 7: DMR associated gene categories. (A) The F1 generation DMR number versus specific gene classifications (categories). (B) The F3 generation DMR number versus specific gene categories.
The advanced MeDIP-Seq procedure developed is described in the Methods and Supplementary Fig. S1 with the advantage of magnetic beads and modified sequencing library construction. The bioinformatics optimization is described in the Methods and Supplementary Fig. S2. Previously, the MeDIP-Chip analysis was used [10, 11, 26] and due to differential hybridization signal on the chip an intersection analysis was useful. Therefore, the DMR identified on each chip were compared previously utilized.

The bioinformatics analysis suggested the negative-binominal approach of edgeR may not be optional and a non-parametric approach like SAMseq needs to be considered in the future [52]. A preliminary analysis with SAMseq demonstrated similar results to the edgeR analysis. Therefore, SAMseq is a potentially useful alternative to consider [41]. Simulated MeDIP-Seq datasets may also be helpful for identifying optimal analysis parameters. While an exhaustive exploration of more analysis variations would be useful, it is beyond the scope of this paper.

Conclusion

Observations from the current study indicate that the direct fe-
tal exposure induced F1 generation sperm DMRs are distinct from the transgenerational F3 generation sperm DMRs identified. Although the current study demonstrated a larger number of DMRs in the F3 generation sperm, other types of toxicants or exposures may vary in the number of DMRs induced following direct exposure in comparison to transgenerational DMRs. Recently we have found methyl mercury induced epigenetic transgenerational inheritance in zebrafish have higher numbers of DMR in the F1 generation sperm than F3 generation sperm [53]. The more advanced technology of the MeDIP-Seq and associated bioinformatics provides a genome wide analysis. This is a significant advantage from the MeDIP-Chip analysis previously utilized.

The limitations of the MeDIP-Seq are more associated with read depth of the DMR sites. A higher read depth generally leads to more accurate DMR identification. A more stringent read depth filtration is anticipated to improve DMR identification. The minimum read depth of 40 reads per sequence was found to perform well in the current study. Genomic window normalization also has the potential to increase the accuracy of the DMR identification. Two RUV normalizations did not improve the analysis and dramatically altered the DMR obtained. Therefore, currently the normalization for library size appears the minimum necessary analysis step. The permutation analysis demonstrated pooled analysis as performed in the current study may not be optimal to assess or reduce variation in the data. Future analysis with alternate methods to confirm the DMR, such as bisulfite sequencing of the specific DMR, would be useful to further optimize the bioinformatics analysis. Preliminary studies with this approach suggest the alternate confirmation analysis may reduce the need to further optimize the bioinformatics. The variation in normalization provided preliminary distinct DMR sets and without follow up confirmation it is not possible to select a more accurate normalization strategy than correcting for library size (total read depth). Due to the dramatic change with the different normalization methods, the accuracy of the edgeR analysis computational tool needs to be investigated in the future. The sensitivity of the results to the normalization approach may indicate deviations of the data from the negative binomial distribution used by edgeR. In addition, the permutation analysis supports this limitation with the bioinformatics analysis. There is some evidence that parametric model approaches, such as edgeR, are not robust to bad model fit [52]. A preliminary analysis with SAMseq demonstrated similar results to the edgeR analysis. Therefore, SAMseq is a potentially useful alternative to consider [41]. Simulated MeDIP-Seq datasets may also be helpful for identifying optimal analysis parameters. While an exhaustive exploration of more analysis variations would be useful, it is beyond the scope of this paper.
is not an optimal experimental design and increased sample size needs to be considered in the future. In addition, the permutation analysis should be used in the future to help validate the bioinformatics analysis.

The transgenerational DMR identified are not simply induced from direct exposure to the toxicant, but instead appear derived from altered transgenerational epigenetic programming during germline development. Future studies need to examine the F2 generation and subsequent generations from the F3 generation to more fully investigate this epigenetic transgenerational phenomenon.

**Methods**

**Animal Studies and Breeding**

Female and male rats of an outbred strain Hsd:Sprague Dawley SD® (Harlan) at about 70 and 100 days 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 [54], the females were administered
daily intraperitoneal injections of vinclozolin (100 mg/kg BW/day) or dimethyl sulfoxide (DMSO) in oil (vehicle). The vinclozolin was obtained from Chem Service Inc. (West Chester, PA) and was injected in a 20 μl DMSO/oil vehicle as previously described [26]. 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 days 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. Individuals 70–90 days from F1 generation of control or vinclozolin lineages were the F1 generation. Non-littermate females and males aged the F0 generation. The offspring of the F0 generation rats 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 days 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. Individuals were maintained for 12 months and euthanized for sperm collection. The F1–F3 generation offspring were not themselves treated directly with vinclozolin. The control and vinclozolin lineages were housed in the same room and racks with lighting, food and water as previously described [26, 55, 56]. All experimental protocols for the procedures with rats were pre-approved by the Washington State University Animal Care and Use Committee (IACUC approval #02568-39).

Epididymal Sperm Collection and DNA Isolation

The epididymis was dissected free of connective tissue, a small cut made to the cauda and tissue 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 the pellet stored at −20 °C until processed further. The pellet was resuspended in NIM buffer and then 100 μl of sperm suspension was combined with 820 μl DNA extraction buffer and 80 μl 0.1M DTT. The sample was incubated at 65 °C for 15 min.

Following this incubation, 80 μl proteinase K (20 mg/ml) was added and the sample incubated at 55 °C for at least 2 h under constant rotation. Then 300 μl of protein precipitation solution (Promega Genomic DNA Purification Kit, A795A) was added, the sample mixed thoroughly and incubated for 15 min on ice. The sample was centrifuged at 13 500 rpm for 20 min at 4 °C. One milliliter of the supernatant was transferred to a 2 ml tube and 2 μl of glycobule and 1 ml of cold 100% isopropanol were added. The sample was mixed well by inverting the tube several times then left in −20 °C freezer for at least one hour. After precipitation the sample was centrifuged at 13 500 rpm for 20 min at 4 °C. The supernatant was taken off and discarded without disturbing the (blue) pellet. The pellet was washed with 70% cold ethanol by adding 500 μl of 70% ethanol to the pellet and returning the tube to the freezer for 20 min. After the incubation, the tube was centrifuged for 10 min at 4 °C at 13 500 rpm and the supernatant discarded.

The tube was spun again briefly to collect residual ethanol to bottom of tube and then as much liquid as possible was removed with gel loading tip. Pellet was air-dried at RT until it looked dry (about 5 min). Pellet was then resuspended in 100 μl of nuclease free water. Equal amounts of DNA from three individual sperm samples were used to produce three DNA pools per lineage and employed for methylated DNA immunoprecipitation (MeDIP).

Methylated DNA Immunoprecipitation MeDIP

Methylated DNA Immunoprecipitation (MeDIP) with genomic DNA was performed as follows: rat sperm DNA pools were generated using 2 μg of genomic DNA from each individual for three pools each of control and vinclozolin lineage animals. Each pool contained three individuals for a total of n = 9 rats per exposure group. Genomic DNA was sonicated using the Covaris M220 the following way: 6 μg of pooled genomic DNA was diluted to 130 μl with TE into the appropriate Covaris tube. Covaris was set to 300 bp program and the program was run for each tube in the experiment. About 10 μl of each sonicated DNA was run on 1.5% agarose gel to verify fragment size. The sonicated DNA was transferred from the Covaris tube to a 1.7 ml microfuge tube and the volume measured. The sonicated DNA was then diluted with TE buffer (10 mM Tris HCl, pH7.5; 1 mM EDTA) to 400 μl, heat-denatured for 10 min at 95 °C, then immediately cooled on ice for 10 min. Then 100 μl of 5× IP buffer and 5 μg of antibody (monoclonal mouse anti 5-methyl cytidine; Diagenode #C15200006) were added to the denatured sonicated DNA. The DNA-antibody mixture was incubated overnight on a rotator at 4 °C.

The following day magnetic beads (Dynabeads M-280 Sheep anti-Mouse IgG, 11201 D) were pre-washed as follows: The beads were resuspended in the vial, then the appropriate volume (50 μl per sample) was transferred to a microfuge tube. The same volume of Washing Buffer (at least 1 ml) was added and the bead sample was resuspended. Tube was then placed into a magnetic rack for 1–2 min and the supernatant discarded. The tube was removed from the magnetic rack and the beads washed once. The washed beads were resuspended in the same volume of 1× IP buffer as the initial volume of beads. About 50 μl of beads were added to the 500 μl of DNA-antibody mixture from the overnight incubation, then incubated for 2 h on a rotator at 4 °C.

After the incubation the bead–antibody–DNA complex was washed three times with 1× IP buffer as follows: The tube was placed into magnetic rack for 1–2 min and the supernatant discarded, then washed with 1× IP buffer three times. The washed bead-DNA solution is then resuspended in 250 μl digestion buffer with 3.5 μl Proteinase K (20 mg/ml). The sample was incubated for 2–3 h on a rotator at 55 °C and then 250 μl of buffered Phenol–Chloroform-Tsoamylalcohol solution was added to the supernatant and the tube vortexed for 30 s then centrifuged at 14 000 rpm for 5 min at room temperature. The aqueous supernatant was carefully removed and transferred to a fresh microfuge tube. Then 250 μl of chloroform were added to the supernatant from the previous step, vortexed for 30 s and centrifuged at 14 000 rpm for 5 min at room temperature. The aqueous supernatant was removed and transferred to a fresh microfuge tube. To the supernatant, 2 μl of glycobule (20 mg/ml), 20 μl of 5 M NaCl, and 500 μl ethanol were added and mixed well, then precipitated in −20 °C freezer for 1 h to overnight.

The precipitate was centrifuged at 14 000 rpm for 20 min at 4 °C and the supernatant removed, while not disturbing the pellet. The pellet was washed with 500 μl cold 70% ethanol in −20 °C freezer for 15 min then centrifuged again at 14 000 rpm for 5 min at 4 °C and the supernatant discarded. The tube was spun again briefly to collect residual ethanol to bottom of tube and as much liquid as possible was removed with gel loading tip. Pellet was air-dried at RT until it looked dry (about 5 min) then resuspended in 20 μl H2O or TE. DNA concentration was measured in Qubit (Life Technologies) with ssDNA kit (Molecular Probes Q10212).

MeDIP-Seq Analysis

The MeDIP pools were used to create libraries for NGS using the NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, San Diego, CA) starting at step 1.4 of the manufacturer’s protocol to generate double stranded DNA. After this step the manufacturer’s protocol was followed. Each pool received a separate
index primer. NGS was performed at WSU Spokane Genomics Core using the Illumina HiSeq 2500 with a PE50 application, with a read size of approximately 50 bp and approximately 35 million reads per pool. Six libraries were run in one lane.

**Statistics and Bioinformatics**

The basic read quality was verified using summaries produced by the FastQC program http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ (8 February 2017, date last accessed). The reads for each MeDIP sample were mapped to the Rnor 6.0 rat genome using Bowtie2 [57] with default parameter options. The mapped read files were then converted to sorted BAM files using SAMtools [58]. To identify DMRs, the reference genome was broken into 100 bp windows. The RUVseq R package was used for RUV normalization [40]. The MEDIPS R package [38] was used to calculate differential coverage between control and exposure sample groups. The edgeR P-value [59] was used to determine the relative difference between the two groups for each genomic window. Windows with an edgeR P-value < 10^{-4} were considered DMRs. For the SAM analysis, DMR were identified using the SAMseq function in the samr R package [52]. P values for individual genomic windows were extracted using the samr P values from perma function. The DMR edges were extended until no genomic window with a P-value < 0.1 remained within 1000 bp of the DMR. CpG density and other information were then calculated for the DMR based on the reference genome.

DMRs were annotated using the biomaRt R package [60] to access the Ensembl database [61]. The genes that overlapped with DMR were then input into the KEGG pathway search [62, 63] to identify associated pathways. The DMR associated genes were manually then sorted into functional groups by consulting information provided by the DAVID [64], Panther [65], and Uniprot databases incorporated into an internal curated database (www.skinner.wsu.edu under genomic data, 8 February 2017, date last accessed). All molecular data has been deposited into the public database at NCBI (GEO # GSE96850). The specific information provided by the DAVID [64], Panther [65], and Uniprot databases incorporated into an internal curated database (www.skinner.wsu.edu under genomic data, 8 February 2017, date last accessed).

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**Supplementary Data**

Supplementary data are available at EnuEpig online.

**Conflict of interest statement.** None declared.

**References**


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