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**Research Article**

# Epigenetic transgenerational inheritance of toxicant exposure-specific non-coding RNA in sperm

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## Abstract

Environmentally induced epigenetic transgenerational inheritance of phenotypic variation and disease susceptibility requires the germ cell (sperm or egg) transmission of integrated epigenetic mechanisms involving DNA methylation, histone modifications, and non-coding RNA (ncRNA) actions. Previous studies have demonstrated that transgenerational exposure and disease-specific differential DNA methylation regions (DMRs) in sperm are observed and that ncRNA-mediated DNA methylation occurs. The current study was designed to determine if transgenerational exposure-specific ncRNAs exist in sperm. Specifically, toxicants with distinct mechanisms of action including the fungicide vinclozolin (anti-androgenic), pesticide dichlorodiphenyltrichloroethane (estrogenic), herbicide atrazine (endocrine disruptor at cyclic adenosine monophosphate level), and hydrocarbon mixture jet fuel (JP8) (aryl hydrocarbon receptor disruptor) were used to promote transgenerational disease phenotypes in F3 generation outbred rats. New aliquots of sperm, previously collected and used for DNA methylation analyses, were used in the current study for ncRNA sequencing analyses of nuclear RNA. Significant changes in transgenerational sperm ncRNA were observed for each transgenerational exposure lineage. The majority of ncRNA was small noncoding RNAs including piwi-interacting RNA, tRNA-derived small RNAs, microRNAs, rRNA-derived small RNA, as well as long ncRNAs. Although there was some overlap among the different classes of ncRNA across the different exposures, the majority of differentially expressed ncRNAs were exposure-specific with no overlapping ncRNA between the four different exposure lineages in the transgenerational F3 generation sperm nuclear ncRNAs. The ncRNA chromosomal locations and gene associations were identified for a small number of differential expressed ncRNA. Interestingly, an overlap analysis between the transgenerational sperm DMRs and ncRNA chromosomal locations demonstrated small populations of overlapping ncRNA, but a large population of non-overlapping ncRNAs. Observations suggest that transgenerational sperm ncRNAs have both exposure-specific populations within the different classes of ncRNA, as well as some common populations of ncRNAs among the different exposures. The lack of co-localization of many of the ncRNAs with previously identified transgenerational DMRs suggests a distal integration of the different epigenetic mechanisms. The potential use of ncRNA analyses for transgenerational toxicant exposure assessment appears feasible.

**Keywords:** ncRNA; epigenetic; transgenerational; inheritance; sperm; exposure; toxicants; vinclozolin; DDT; jet fuel; atrazine

## Introduction

Epigenetic transgenerational inheritance is a non-genetic form of inheritance that has been demonstrated in all organisms examined from plants to humans [1–3]. Environmental factors ranging from nutrition to toxicants promote germline epigenetic alterations that are transmitted to subsequent generations and promote embryonic stem cell alterations that subsequently influence the development of all somatic cell types in the organism to promote phenotypic variation and disease susceptibility [4, 5]. This germline (i.e. sperm or egg)-mediated inheritance has been shown in all organisms examined, sometimes even for hundreds of generations following the initial F0 generation exposure [6–8]. Chemical toxicants are environmental factors commonly used to promote epigenetic transgenerational inheritance [9]. The toxicant-induced transgenerational inheritance has been shown to involve DNA methylation [1, 10], non-coding RNA (ncRNA) [11, 12], and chromatin structure change [13, 14]. Recently, the integration of these three epigenetic mechanisms has been shown to potentially mediate transgenerational phenotypic variation and disease susceptibility [15, 16].

The ncRNAs represent a critical epigenetic component that has been shown to be altered in epigenetic transgenerational inheritance [15]. Initially, small ncRNAs were found to be altered in toxicant-induced transgenerational sperm [17]. Subsequent

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examples of all categories of ncRNAs have been shown to be altered in sperm [18, 19] and several in eggs [20, 21]. As discussed, a recent study observed the combined alteration of DNA methylation, ncRNA expression, and histone retention at common chromosomal sites in transgenerational sperm [15]. Previous studies have suggested that ncRNA can interact at genomic sites to help recruit DNA methyltransferase and facilitate DNA methylation at the sites, termed ncRNA-directed DNA methylation [22, 23]. The ncRNA has been suggested to facilitate protein binding to DNA, such as chromatin remodeling proteins [23, 24], and histone modification enzymes to facilitate histone modifications [25, 26]. For this study, the small ncRNA types investigated include microRNAs (miRNAs), which possess complementary seed sequences that target the 3′UTRs of mRNAs, thereby regulating translational efficiency [20]. Another small ncRNA type examined is piwi-interacting RNAs (piRNAs), which have roles in silencing repetitive sequences and post-transcriptional regulation of gene expression during gametogenesis [27]. As tRNA-derived fragments, tRNA-derived small RNAs (tsRNAs) also affect post-transcriptional gene regulation and are being investigated [17, 28].

During spermatogenesis, altered ncRNAs may facilitate epigenetic programming and modifications of other epigenetic factors such as DNA methylation. All early-stage spermatogenic cells in the testes have normal nuclear gene expression, similar to somatic cells. However, in the haploid phase of spermatogenesis, transcription ceases when round spermatids start to differentiate into elongating and then elongated spermatids due to nuclear condensation mediated by protamine replacement of histones. When elongating spermatids differentiate into spermatozoa, gene transcription and translation is silenced and shut down due to the loss of cytoplasm and the translational machinery [29, 30]. The spermatozoa have no classic cytoplasm and thus lack most of the typical cellular organelles [31]. The sperm head contains an acrosome, a structure filled with various enzymes (e.g. hyaluronidases) known to facilitate fertilization [32]. The remaining part of the spermatozoa is the mid-piece that contains the mitochondria that will be involved with sperm tail motility after ejaculation into the female reproductive tract [33, 34]. Since the current study used sperm head nuclear-derived ncRNA, we assume negligible contribution of epididymal ncRNA to the observations presented [35–38]. The sperm were sonicated to remove the tail and mitochondria, as well as remove the acrosome structure and fluid, to leave only the head of the sperm that contains only the nucleus with compacted DNA. All somatic cell contamination is also destroyed through sonication of the sperm.

The toxicant exposures used in the current study involved an intraperitoneal injection of toxicant into F0 generation pregnant females during the transient period of gonadal sex determination when the primordial germ cells differentiate into male or female germline cells to facilitate the initial testis or ovary development [39]. Therefore, the embryonic germ cells are initiating differentiation, along with the gonadal somatic cell differentiation. Following this transient period, no further toxicant exposure is applied, and the later stages of testis development proceed to adult to produce mature sperm that transmit epigenetic alterations to the next generation and promote epigenetic transgenerational inheritance [2]. Although primordial germ cells could be a target for toxicant exposure, it is anticipated that exposure of the somatic cells in the fetal gonad and corresponding alterations in their epigenetic programming will be critical later during adult spermatogenesis and oogenesis to induce the epigenetic alterations in the germline to promote the germ cell-mediated epigenetic transgenerational inheritance.

The initial transgenerational experiments focused on the impacts of individual environmental toxicants to promote the epigenetic inheritance of disease susceptibility and DNA methylation alterations in the sperm [1]. A large number of different gestating female rat toxicant exposures, including vinclozolin [40], DDT [41], atrazine [42], jet fuel [43], glyphosate [44], plastics [45], and pesticides [46], were all found to promote the epigenetic transgenerational inheritance of disease susceptibility along with unique sperm DNA methylation alterations in the F3 transgenerational generation. Interestingly, the transgenerational sperm DNA methylation alterations were distinct for each exposure with negligible overlapping DNA methylation regions (DMRs) [47]. Although sperm number and motility were reduced in the F1 generation males, the F3 transgenerational sperm number and motility were not altered significantly. Previous studies with ncRNA have demonstrated ncRNA-directed DNA methylation [48, 49]. Therefore, the current study was designed to use the previously developed sperm samples from several transgenerational F3 generation toxicant exposure studies. New aliquots from these previously collected sperm samples were used in the current study to do ncRNA analyses to allow correlations between the two epigenetic processes. Therefore, no RNA data were used from the previous studies, only aliquots of sperm for ncRNA analysis in the current study. The questions were if the ncRNA alterations would have exposure specificity, and if correlations to DNA methylation sites previously identified occurred. Further investigation into the molecular mechanisms of environmentally induced epigenetic transgenerational inheritance can be used to investigate the exposure-specific ncRNA alterations and allow correlations to previously identified DNA methylation alterations. The potential role of exposure specificity in the transgenerational transmission of ncRNA is investigated.

## Results

As previously described and outlined in the Methods, F0 generation female Sprague Dawley outbred rats at 7 days of gestation were exposed through intraperitoneal injection of toxicants or control dimethylsulfoxide (DMSO) daily until 14 days of gestation. The control and exposure lineage adult rats were then bred to produce the F3 generation “transgenerational” rats for the collection of sperm and pathology analyses [5]. Transgenerational F3 generation sperm sample aliquots from previous studies with vinclozolin [40], jet fuel [50], DDT [51], and atrazine [52] exposures were used for ncRNA analyses. New aliquots from the same sperm samples that had been used for DNA methylation analyses previously were used in the current study. An aliquot of sperm was used to do sperm nuclear RNA isolation followed by next generation sequencing analyses. The control lineage samples were similar for all treatments except the jet fuel lineage controls that were distinct for that study. Control lineage sperm and the different exposure lineages’ sperm samples were used to identify ncRNA present and alterations due to the various individual toxicant exposures. The objective was to identify the epigenetic transgenerational inheritance of sperm ncRNA alterations in the different exposure lineages, and compare with the previously identified transgenerational DNA methylation alterations identified [47]. The cauda epididymal sperm were collected as previously described [40, 50–52], and then sonicated to remove/destroy contaminating somatic cells, remove the tail and mitochondria, and remove/destroy the acrosome, yielding sperm heads/nuclei for isolation of large and small ncRNAs, as described in the Methods [47].

The small and large ncRNA preparations were sequenced, and the control versus the exposure transgenerational (e.g. F3 generation) lineage samples were compared to identify differentially expressed ncRNA, as described in the Methods. The subcategories of ncRNA were identified based on molecular characteristics, size, and sequence. The sequencing data were labeled and categorized, as described in the Methods. The individual sample sequencing depths for lncRNA averaged 17 M reads per sample and sncRNA average of 19.3 M reads per sample with quality control parameters for unique reads 80%–90% for each sample, as described in the Methods. The differentially expressed ncRNAs for each of the transgenerational exposures in the F3 generation sperm are presented in Fig. 1. The data for vinclozolin (Fig. 1a), jet fuel (Fig. 1b), DDT (Fig. 1c), and atrazine (Fig. 1d) are presented for F3 generation sperm ncRNAs. The lncRNAs and sncRNAs are presented for each with the number of differential expressed ncRNA at different edgeR *P*-values, with the different ncRNA categories indicated for false discovery rate (FDR) <0.1 for each exposure (Fig. 1). Observations indicate the lncRNA are negligible in number compared to the sncRNA for all transgenerational differentially

a Vinclozolin

| p-value | lncRNA | miRNA | piRNA | rsRNA | tsRNA |
|---|---|---|---|---|---|
| 0.001 | 11 | 28 | 781 | 16 | 77 |
| 1e-04 | 4 | 20 | 513 | 10 | 72 |
| 1e-05 | 3 | 14 | 324 | 9 | 66 |

| FDR | lncRNA | miRNA | piRNA | rsRNA | tsRNA |
|---|---|---|---|---|---|
| 0.1 | 62 | 57 | 1386 | 37 | 132 |
| 0.05 | 7 | 37 | 1263 | 30 | 117 |
| 0.01 | 3 | 22 | 973 | 17 | 82 |

b Jet Fuel

| p-value | lncRNA | miRNA | piRNA | rsRNA | tsRNA |
|---|---|---|---|---|---|
| 0.001 | 459 | 231 | 1201 | 55 | 194 |
| 1e-04 | 358 | 202 | 1084 | 53 | 166 |
| 1e-05 | 277 | 175 | 1000 | 45 | 141 |

| FDR | lncRNA | miRNA | piRNA | rsRNA | tsRNA |
|---|---|---|---|---|---|
| 0.1 | 797 | 329 | 1511 | 64 | 262 |
| 0.05 | 684 | 304 | 1426 | 62 | 252 |
| 0.01 | 509 | 259 | 1304 | 61 | 222 |

c DDT

| p-value | lncRNA | miRNA | piRNA | rsRNA | tsRNA |
|---|---|---|---|---|---|
| 0.001 | 19 | 61 | 746 | 15 | 91 |
| 1e-04 | 11 | 25 | 450 | 11 | 72 |
| 1e-05 | 6 | 17 | 251 | 9 | 54 |

| FDR | lncRNA | miRNA | piRNA | rsRNA | tsRNA |
|---|---|---|---|---|---|
| 0.1 | 21 | 133 | 1417 | 41 | 146 |
| 0.05 | 15 | 99 | 1282 | 27 | 123 |
| 0.01 | 10 | 61 | 942 | 15 | 110 |

d Atrazine

| p-value | lncRNA | miRNA | piRNA | rsRNA | tsRNA |
|---|---|---|---|---|---|
| 0.001 | 36 | 386 | 1042 | 46 | 212 |
| 1e-04 | 17 | 348 | 949 | 40 | 196 |
| 1e-05 | 3 | 326 | 875 | 37 | 185 |

| FDR | lncRNA | miRNA | piRNA | rsRNA | tsRNA |
|---|---|---|---|---|---|
| 0.1 | 106 | 477 | 1317 | 67 | 248 |
| 0.05 | 47 | 450 | 1253 | 61 | 241 |
| 0.01 | 19 | 414 | 1145 | 51 | 222 |

**Figure 1.** Differential ncRNA analysis. Differential expressed ncRNA in sperm between control and exposure samples at various edgeR *P*-value threshold levels. (a) Vinclozolin; (b) jet fuel; (c) DDT; and (d) atrazine. The ncRNA categories at bottom column have FDR <0.1.

expressed ncRNAs. The predominant ncRNAs were piRNAs for all the transgenerational exposure ncRNAs. The number of differentially expressed miRNAs, tsRNAs, and rRNA-derived small RNA (rsRNAs) were generally comparable for each of the exposure lineages (Fig. 1). Therefore, comparable numbers of differentially expressed ncRNAs in the different subcategories were identified for each of the different transgenerational exposures. The number of piRNAs showed more variation.

The differentially expressed small ncRNAs for each of the exposures are presented with volcano plots, showing those up-

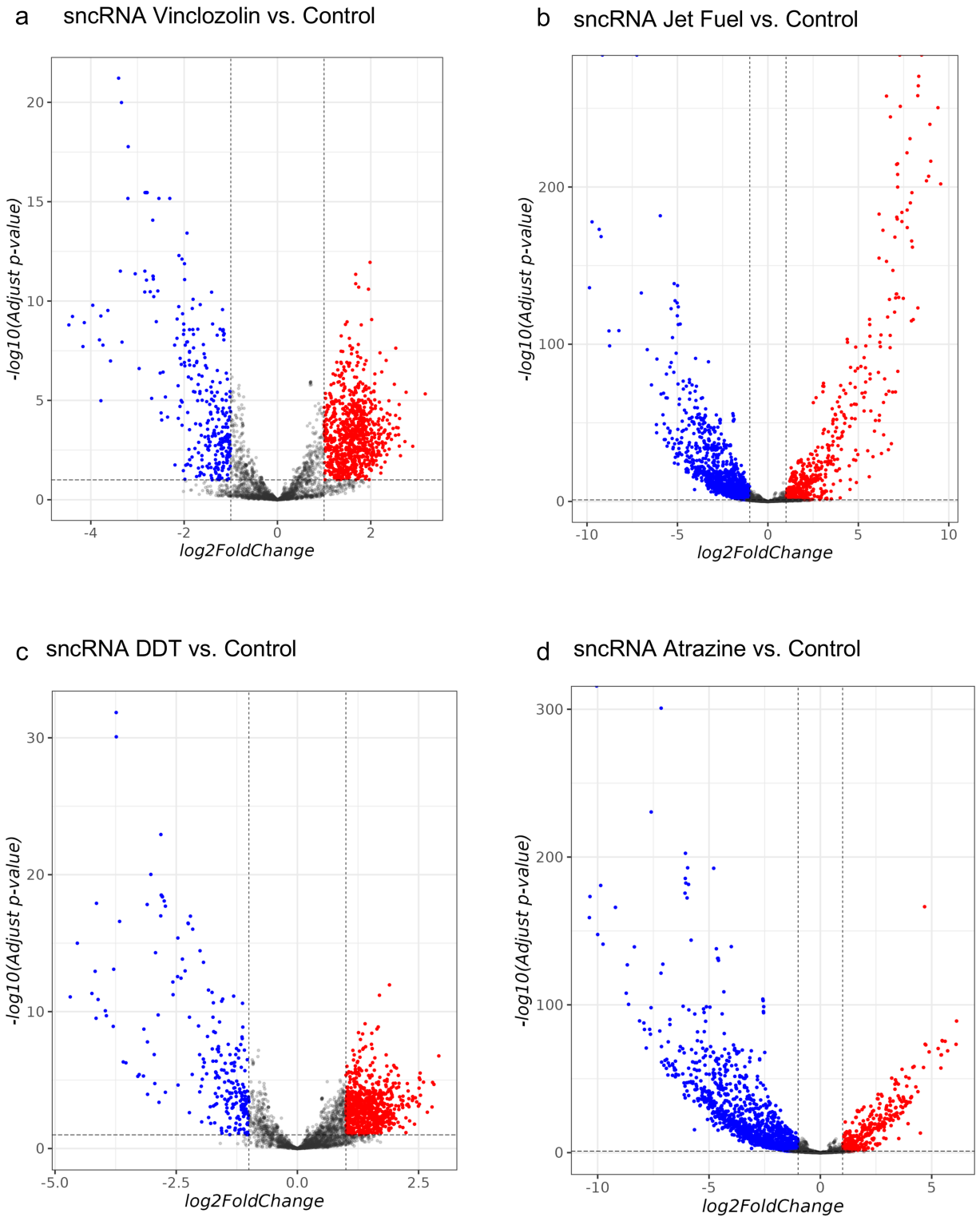


**Figure 2.** Volcano plots for differential expressed ncRNA**.** An increase (red) or decrease (blue) in expression. A volcano plot represents the log of the ncRNA for color, and < log2 fold change for black, as a function of the log ratio of differential expression. (a) Vinclozolin versus control—small ncRNA (b) jet fuel versus control—small ncRNA (c) DDT versus control—small ncRNA, and (d) atrazine versus control—small ncRNA.

or down-regulated sncRNAs in the different exposures (Fig. 2). The vinclozolin had predominantly decreased in small ncRNA expression compared to controls (Fig. 2a); the jet fuel showed equal increases or decreases in expression (Fig. 2b); the DDT predominantly increased in expression (Fig. 2c); and atrazine showed more decreases in expression (Fig. 2d). The negative log indicates a decrease in expression and positive log (red) an increase in expression (Fig. 2). The small ncRNAs had variable responses between transgenerational exposures regarding an increase, decrease, or mix of ratios. This analysis used a >log2 fold change and FDR <0.1 in color, and < log2 fold change and no FDR for black to allow all data to be represented. The volcano plots for each specific exposure in the F3 generation sperm lineage are presented in Supplementary Fig. S1a–p. Each exposure sncRNA group (biotype) is presented and demonstrates some variation between the exposure lineage and type of sncRNA (Supplementary Fig. S1).

The chromosomal locations for those differentially expressed ncRNAs that could be annotated and aligned to the genome are presented in a chromosomal plot for each transgenerational exposure (Fig. 3). These plots are limited to the top 1000 statistically significant differentially expressed ncRNAs. The small ncRNAs for all the exposure lineage sperm generally had genome-wide distribution of the locations of the differentially expressed ncRNAs. The red arrowheads represent differentially expressed ncRNAs and black boxes show clusters of differentially expressed ncRNAs. The chromosome size and number are presented (Fig. 3). The number of long lncRNAs is orders of magnitude lower, but chromosomal distributions are shown in Fig. 3b, d, f and h. The DDT and atrazine lncRNAs were higher in number and also had a genome-wide distribution (Fig. 3).

The next analysis was to determine the potential overlap or lack of overlap of the transgenerational ncRNAs between the different exposure lineages (Fig. 4). The top 1000 differentially expressed small ncRNAs were primarily exposure-specific for all transgenerational exposure lineages (Fig. 4a). The differentially expressed small ncRNAs were divided into piRNAs and non-piRNAs (composed of miRNAs, tsRNAs, and rsRNAs). Overlaps between various exposures also had large numbers of ncRNAs in common. Interestingly, there were overlapping ncRNAs that were common to all transgenerational exposure lineages (Fig. 4a). Therefore, exposure-specific and common exposure ncRNA exist within all the comparisons. The differentially expressed lncRNAs were much lower in number and were predominantly exposure-specific (Fig. 4b). The small non-pi RNA also predominantly had exposure-specific ncRNAs, but some ncRNAs were common between different exposures, and 39 non-piRNA were common to all transgenerational exposures (Fig. 4c). The piRNA category had high numbers of transgenerational exposure-specific piRNAs and 298 piRNAs common for all transgenerational exposures (Fig. 4d). All the various subclasses of piRNA were combined and were present for all exposure differential expressed piRNA. Therefore, this is in contrast to differential DMRs from the same sperm collection and treatment lineages where no common DMRs were observed, and the vast majority were exposure-specific [47]. The ncRNAs have a mix of unique and common ncRNA between exposure lineages (Fig. 4). A principal component analysis (PCA) was ran for each ncRNA group and demonstrated variation with the larger groups being isolated due to bias for the larger groups in the PCA (Supplementary Fig. S2).

The annotated ncRNAs identified for all the different transgenerational exposures for all categories are presented in Supplementary Tables S1–8. The tables contain the ncRNA identification, name, ncRNA type, chromosome location, start position, log2 fold change, *P*-value, adjusted *P*-value FDR, and gene annotation and category when available (Supplementary Tables S1–8). Some ncRNAs were identified that were within 10 kb of a gene, so potential promoter distal and proximal sites for regulatory associations are considered. The ncRNA annotation only considered ncRNA with chromosomal locations. The Supplemental Tables were generated to identify all the differentially expressed ncRNA with the various transgenerational exposures, and indicate those with relevant genome location. For those ncRNAs that had gene associations, the gene categories were combined and presented in Supplementary Tables S1–8 and Fig. 5 to illustrate the spectrum of gene categories that the differentially expressed ncRNAs may regulate and/or associate with. The main gene association categories for the sncRNA included transcription, transport, and metabolism for most ncRNA (Fig. 5a). The main gene association categories for the lncRNAs included signaling, metabolism, cytoskeleton, transport, and transcription (Fig. 5b). Therefore, similar categories were predominant for both the sncRNAs and lncRNAs (Fig. 5). In addition, the ncRNA-associated genes were also put into a KEGG pathway analysis to see if any general pathways were impacted. The differentially expressed ncRNA gene associated pathways are summarized for each exposure transgenerational lineage in Fig. 6. The number of different ncRNAs within each pathway (i.e. top five pathways) is presented in brackets for those exposures that had sufficient numbers of pathway associations. The lncRNAs for atrazine exposure identified the metabolic pathway; for jet fuel exposure, the metabolic pathway; and for sncRNAs, the cancer pathway (Fig. 6a and b). The vinclozolin, DDT, atrazine, and jet fuel transgenerational sncRNAs all had metabolism pathways and pathways in cancer (Fig. 6c–f). Some unique pathways were also present in the different exposures as well (Fig. 6). Observations suggest the transgenerational ncRNAs have gene associations that may impact and coordinate with similar observations with differential DMRs [47].

The final experiment was to use the transgenerational ncRNAs identified for each of the distinct exposure lineages and compare with the previous studies on the same sperm samples for DMR analyses [47]. The objective was to determine if the ncRNA had similar genomic locations to suggest potential interactions with the DMR sites. Within the same exposure F3 generation lineage transgenerational sperm sample, those ncRNAs that had the same chromosomal sites as previously identified DMRs are indicated (Supplementary Tables S1–8). The specific exposure for lncRNA, Supplementary Tables S1–4, and sncRNA, Supplementary Tables S5–8, demonstrates that a subset of the ncRNA has chromosomal locations and gene associations. The subset of DMR associations for this ncRNA subset demonstrated the only major exposure that had reasonable overlap was the jet fuel exposure for both lncRNA and sncRNA (Fig. 7). The vast majority of ncRNA did not have associated DMRs. Therefore, the DMR and ncRNA sites were primarily distinct with negligible overlap. This suggests distal regulation between the two epigenetic factors.

All the lncRNA were successfully localized to chromosomal sites, and most of the sites had gene associations (Supplementary Tables S1–4). However, only a subset of sncRNA had chromosomal sites and gene associations (Supplementary Tables S5–8). The vast majority of sncRNA did not have chromosomal locations identified, and so no gene associations. The sncRNA lists for each exposure and biotype of sncRNA are presented in Supplementary Tables S9–24. These tables provide the name, initial sequence, length, ncRNA type, base mean, log2 fold change, starts, *P*-value, and adjusted *P*-value FDR. The different exposure

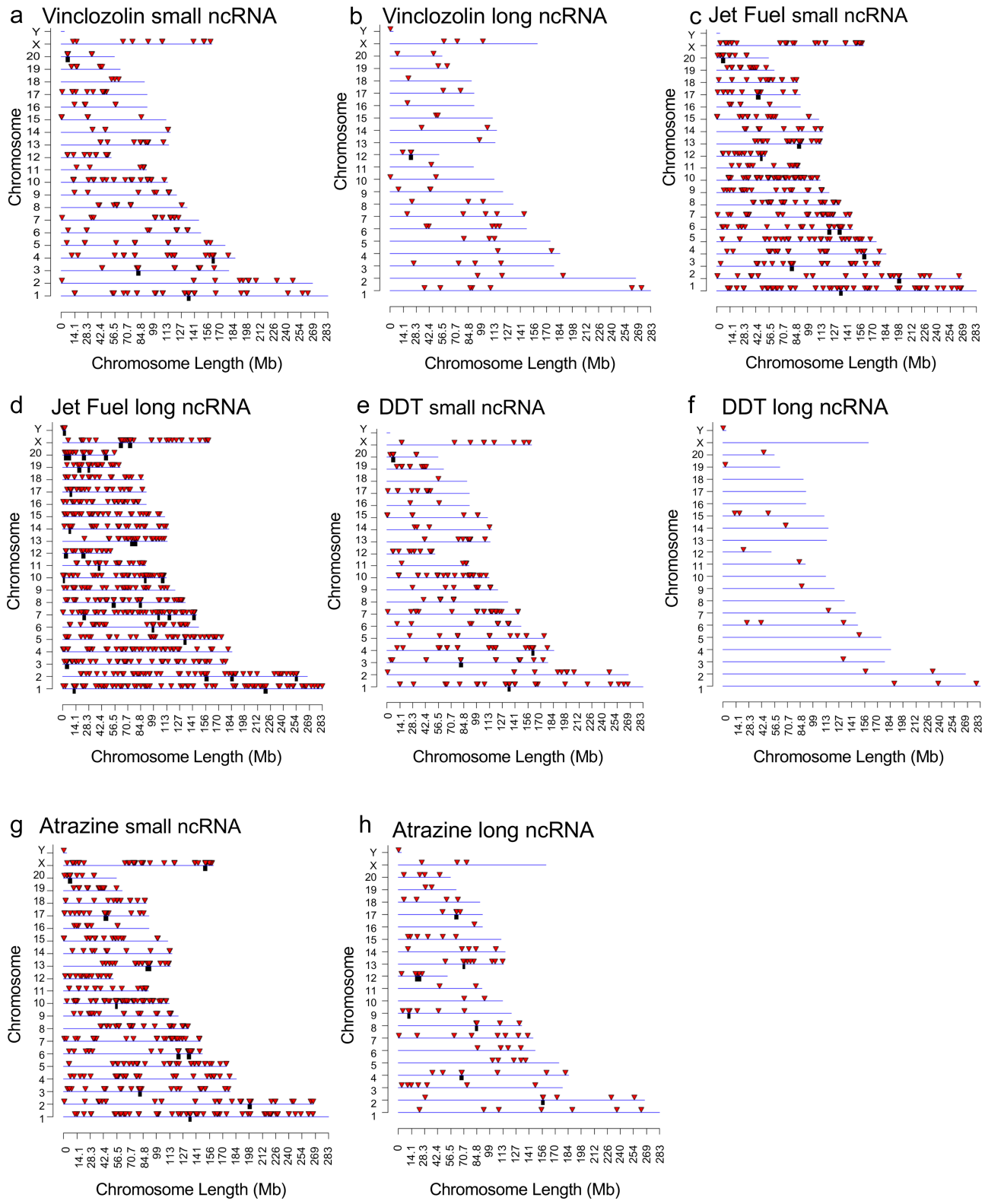


**Figure 3.** DMR chromosomal locations differential ncRNA. The (a) Vinclozolin small ncRNA; (b) vinclozolin long ncRNA; (c) jet fuel small ncRNA; (d) jet fuel long ncRNA; (e) DDT small ncRNA; (f) DDT long ncRNA; (g) Atrazine small ncRNA; and (h) Atrazine long ncRNA. The red arrowheads identify ncRNA locations and black box clusters of ncRNA.

miRNA (Supplementary Tables S9–12), piRNA (Supplementary Tables S13–16), rsRNA (Supplementary Tables S17–20), and tsRNA (Supplementary Tables S20–24) are presented. This presents all the transgenerational ncRNA that were differentially expressed following the specific ancestral environmental exposures (Supplementary Tables S9–23).

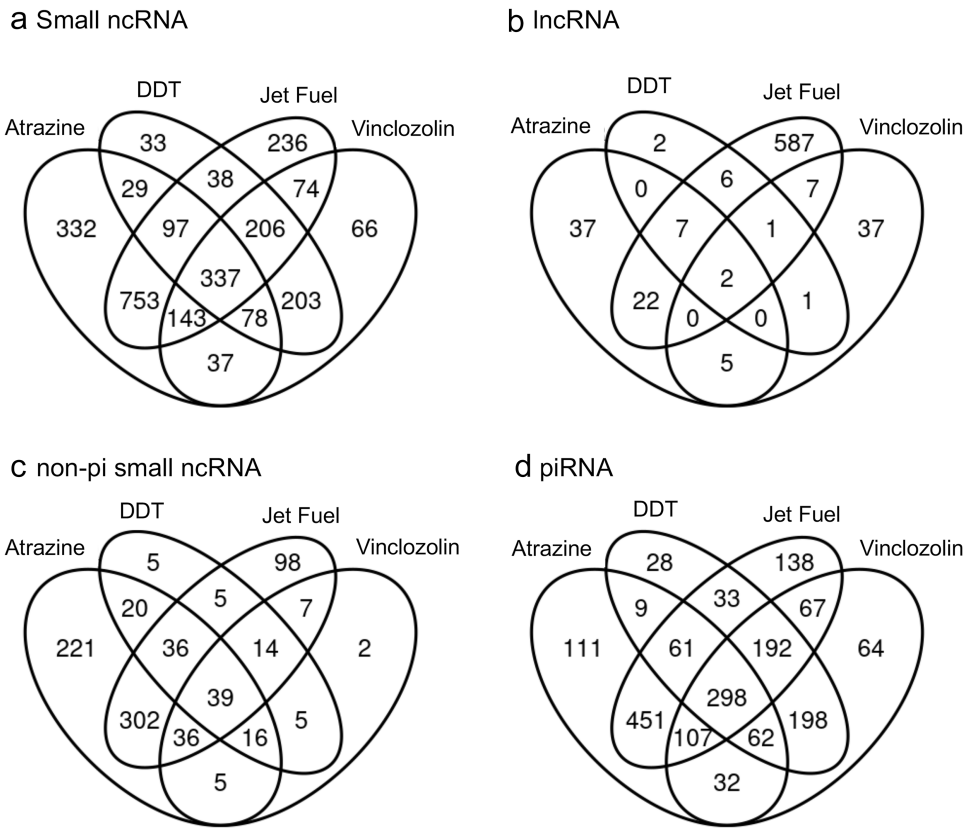


**Figure 4.** Venn diagram overlap differential expressed ncRNA. The (a) Small ncRNA. (b) Long lncRNA. (c) non-pi ncRNA. (d) pi ncRNA. The various exposure ncRNA are compared with top 1000 statistically significant ncRNA.

## Discussion

Epigenetics is defined as "molecular factors and processes around DNA that regulate genome activity independent of DNA sequence, and are mitotically stable" [53]. When epigenetic processes are altered in the germ cells (sperm or egg), then epigenetics at fertilization alters the zygote and subsequent embryonic pluripotent stem cell epigenetics [4] and corresponding gene expression, which alters the epigenetic cell specificity of all subsequently derived somatic cells to influence phenotypic variation and disease susceptibility [47]. As this individual develops, the germline development carries these epigenetic changes to subsequent generations [4]. Therefore, epigenetics can be inherited and this process is termed "epigenetic transgenerational inheritance" [2]. Although the initial observations used DNA methylation as the epigenetic process to identify this phenomenon [1], recently all epigenetic processes have been shown to be involved in epigenetic transgenerational inheritance [47]. The DNA methylation, non-coding RNA, chromatin structure, histone modifications, and RNA modifications all can regulate gene expression and genome activity independent of DNA sequence, and participate in epigenetic inheritance [15, 16]. The ncRNA-directed DNA methylation and DNA methylation-directed chromatin structure alterations are examples of the integration of epigenetic processes in epigenetic inheritance [54]. The ncRNAs have been shown to be crucial in epigenetic inheritance [17, 55].

A number of studies in a variety of different species have demonstrated a role for ncRNAs in epigenetic transgenerational inheritance [15, 19, 56]. When ncRNAs are assessed along with DNA methylation in epigenetic inheritance, both are altered [15, 16]. The analyses of ncRNA and DNA methylation in the same sperm sample in epigenetic transgenerational inheritance have shown that both epigenetic processes are coordinatingly impacted and can be co-localized in the genome [15]. This appears to be associated with ncRNA-directed DNA methylation to facilitate epigenetic inheritance [15, 57]. In the analysis of ncRNA associated with sperm, the unique structure of the spermatozoa and lack of normal cellular structure and organelles need to be considered. The sperm is not a normal cell with a plasma membrane, transport processes, or nucleus that is active or has gene expression. The sperm has a head with a membrane that is impermeable to the entry of molecules such as ncRNAs. An acrosome is present on the head that has a permeable membrane to allow substances to enter the acrosome, but this is a separate structure to hold proteins and hyaluronidases to facilitate fertilization, and is not connected or permeable to the remainder of the sperm head structure [32, 58]. The mid-piece holds mitochondria that facilitate motility of the sperm tail and motility of the sperm upon ejaculation. The head is independent of the acrosome or mid-piece and contains compacted DNA that is predominantly complexed with protamines to maintain the compacted DNA structure, and that is not able to express RNAs. No other normal cellular structures, such as endoplasmic reticulum, lysosomes, or any normal signaling systems, exist in the sperm. It is primarily an inactive transport vesicle for the compacted and inactive DNA. Although ncRNAs from the epididymis can associate with the mid-piece and enter the acrosome, they cannot enter the head membrane or the nucleus of the sperm due to the impermeability of these membranes [59]. The ncRNAs in the nuclear structure of the sperm

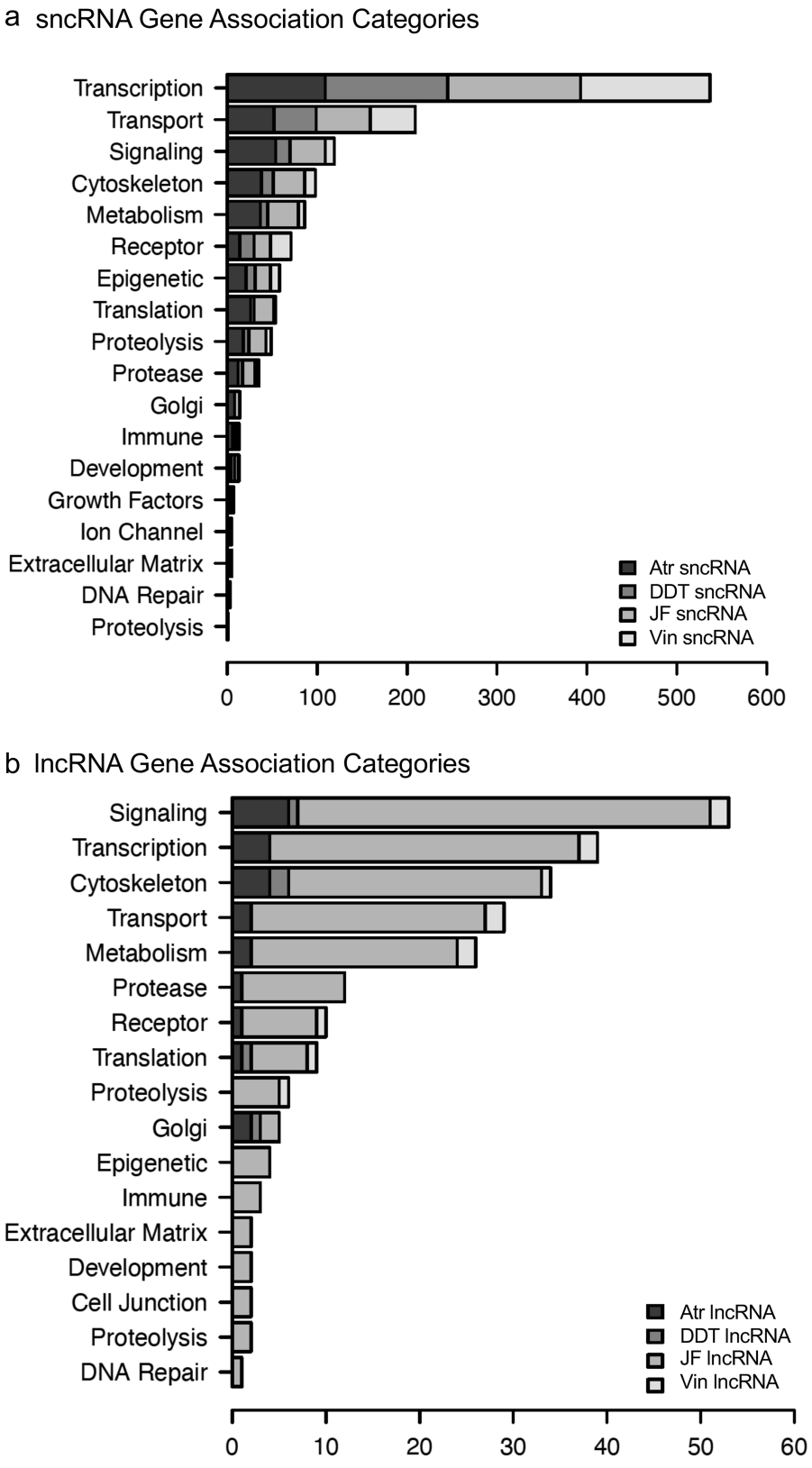


**Figure 5.** Differential expressed ncRNA gene association categories. The (a) Small ncRNA associated gene categories for each exposure. (b) Long ncRNA associated gene categories for each exposure. Number of genes on x-axis and gene categories on y-axis.

were derived during the spermatogenesis process in the seminiferous tubule of the testis, prior to spermiogenesis, when the haploid round spermatid DNA has histones replaced with protamines to compact the DNA and form the spermatozoa with the normal sperm structure. The ncRNA produced during the spermatogenic germ cell developmental period will be what is delivered to the

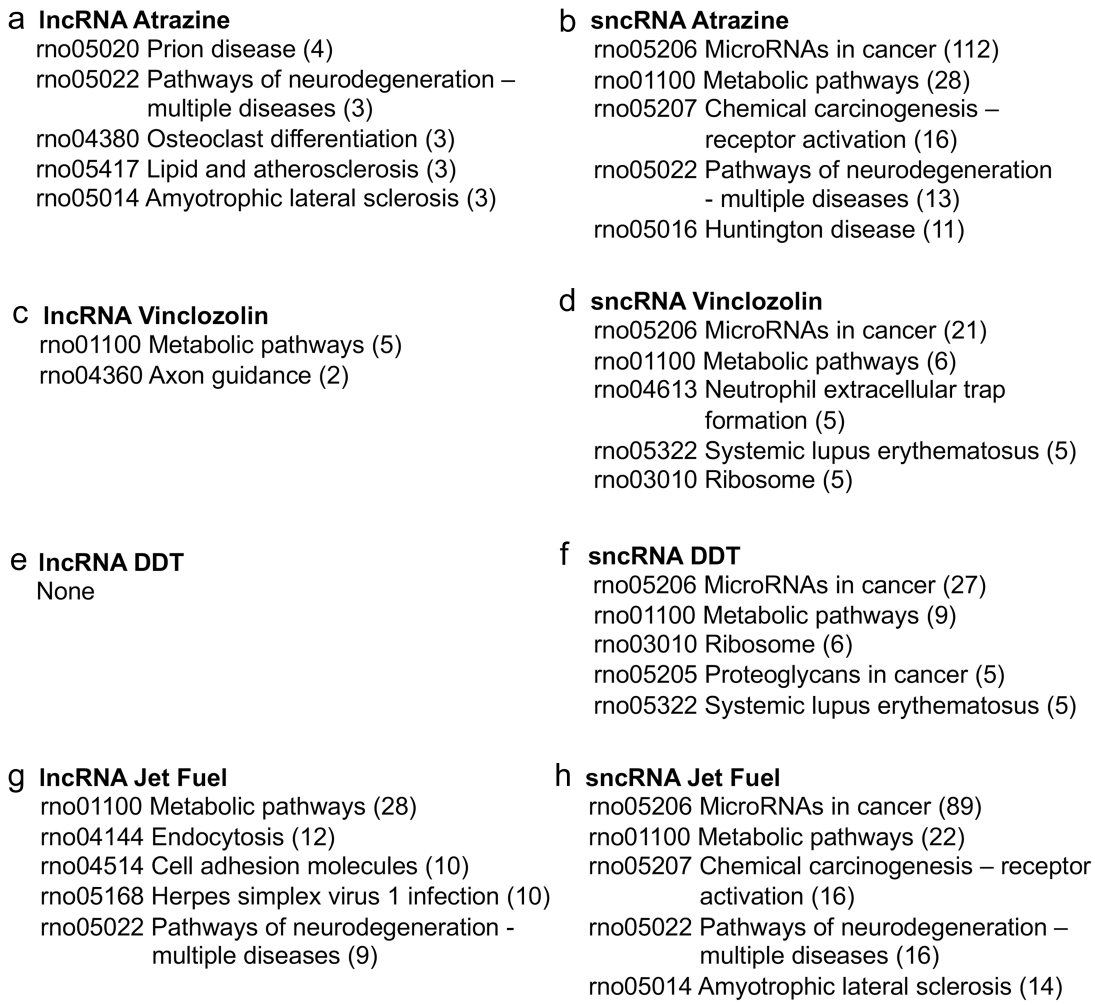


**Figure 6.** Differential expressed ncRNA gene association pathways. The (a) lncRNA atrazine, (b) sncRNA atrazine, (c) lncRNA vinclozolin, (d) sncRNA vinclozolin, (e) lncRNA DDT, (f) sncRNA DDT, (g) lncRNA jet fuel, and (h) sncRNA jet fuel. The number in brackets identifies the number of gene associations within the gene category.

| lncRNA | No | Yes |
|---|---|---|
| Vinclozolin | 62 | 0 |
| Jet Fuel | 620 | 177 |
| DDT | 20 | 1 |
| Atrazine | 104 | 2 |
| | | |
| **sncRNA** | **No** | **Yes** |
| Vinclozolin | 773 | 0 |
| Jet Fuel | 1080 | 61 |
| DDT | 699 | 0 |
| Atrazine | 1109 | 0 |

**Figure 7.** ncRNA overlap with DMR The lncRNA top and sncRNA bottom for lack overlap (no) and overlap (yes) with the specific ncRNA numbers listed.

egg during fertilization. Therefore, the sperm ncRNAs that will impact the embryo epigenetics are derived during spermatogonial development and reside in the nucleus of the sperm. The current study used sonication to remove the tail and mid-piece from the sperm head and destroy the acrosome and release its components to isolate the sperm nucleus that is the structure involved in fertilization and fusion with the egg structure within the zona pellucida structure. Therefore, the current study examined the sperm ncRNAs that are involved in epigenetic transgenerational inheritance that can impact the early zygote and subsequent embryo development. The ncRNAs derived from the mid-piece during epididymal transport are likely important for sperm motility following ejaculation, while those in the acrosome may facilitate entry of the sperm through the zona pellucida at the initial steps in sperm–egg interactions. However, these epididymal ncRNAs have not been shown to directly impact epigenetic inheritance nor have a mechanism to enter the sperm nucleus. Although the presence of epididymal ncRNAs and their association with sperm is interesting and may be critical in motility and the acrosome reaction, a role in the sperm nucleus and epigenetic inheritance remains to be established and requires further investigation. The resident ncR-

NAs acquired during spermatogenesis within the sperm nucleus clearly can have a role in epigenetic transgenerational inheritance, and was the focus of the current study.

Previous studies have demonstrated that various toxicant exposures promote unique transgenerational differential DMR profiles in sperm [47]. Interestingly, these unique toxicant DMR profiles also are associated with unique disease-specific epigenetic profiles [47]. Therefore, DNA methylation has been shown to have specific epigenetic alterations in the sperm. The current study was designed to assess potential exposure-specific ncRNA profiles in the sperm that are associated with epigenetic transgenerational inheritance. Sperm sample aliquots from previous studies that examined the unique DNA methylation profiles of DMRs from separate experiments for vinclozolin, DDT, jet fuel, and atrazine in lineage F3 generation rat sperm collection samples were used [40–43]. In the current study, an aliquot of these specific studies' samples were used to investigate toxicants that have different modes of action including vinclozolin (antiandrogenic), DDT (estrogenic), atrazine (endocrine disruptor at cyclic adenosine monophosphate), and a hydrocarbon mixture (jet fuel JP8), which disrupts the aryl hydrocarbon receptor signaling. These transgenerational sperm samples were used to isolate ncRNAs from the head of the sperm. This allowed the ncRNA data obtained to be compared with the previous DNA methylation data. The objective was to assess the potential exposure specificity of the ncRNA present in the sperm, and correlate this with the previously identified DNA methylation alterations in the sperm [47]. Observations support a role for ncRNA in the epigenetic transgenerational inheritance, as previously described [15, 16].

The ncRNAs were isolated from the cauda epididymal sperm nuclei for all of the different exposure groups. The ncRNAs were sequenced to identify the altered ncRNA between the control versus exposure F3 generation sperm. The ncRNAs was separated informatically into total small ncRNAs, long ncRNAs, non-piRNAs, and piRNAs (Fig. 5). The piRNAs were generally highest in number, but have the least functional information currently known about them. Therefore, the small ncRNAs and non-piRNAs were the focus of the subsequent analysis. The lncRNAs were separated from the mRNAs. Generally, the lncRNAs were less than 1% of the ncRNAs in abundance compared to the small sncRNAs (Fig. 1). The comparison of the differentially expressed ncRNAs demonstrates the majority of the ncRNAs were specific for each exposure (Fig. 4). However, overlap between the different exposures was also observed. Therefore, in contrast to previous observations on DNA methylation when few DMRs were in common between the exposures, the ncRNA had a mixture of unique ncRNAs and some that are common between the exposures. Observations indicate that exposure-specific ncRNAs exist and could be used to assess various environmental exposures, similar to DNA methylation DMRs that were previously identified.

All the various classes of ncRNAs were observed, including miRNAs, tsRNAs, rsRNA, piRNAs, and lncRNAs (Fig. 1). A mixture of both increased and decreased ncRNA expression was observed. The different exposures generally impacted the observations with vinclozolin primarily decreasing the ncRNA presence and DDT primarily increasing the presence of the ncRNAs (Fig. 2). The ncRNAs altered were generally found genome-wide and present on most chromosomes (Fig. 3). The gene association categories with the ncRNAs were analyzed and found common gene categories among the different exposures. The gene pathways impacted were also assessed and demonstrated common pathways including miRNAs in cancer, metabolic pathways, and pathways in cancer (Fig. 6). These observations suggest that alterations in ncRNA expression in the germline will impact the transgenerational inheritance phenotypes previously observed [47]. One limitation of the small RNA sequencing kit used is its reduced efficiency in capturing small RNAs that have extensive modifications, which many lead to an under representation of these molecules in the sequencing results.

An interesting analysis was done with the individual ncRNAs with respect to the different exposures and comparison with the previously identified DNA methylation DMRs for the same samples and exposures [47]. Observations indicated negligible overlap between the ncRNAs and DMRs. The only exposure that had some overlap was the jet fuel that had 109 sncRNA out of a total of 3621 differentially expressed sncRNAs, and 42 lncRNAs out of a total of 181 lncRNAs. Therefore, the ncRNAs appear to act distal to the DMR sites to integrate the epigenetic regulation. The ncRNA are known to act distally to regulate gene expression, so are anticipated to act similarly with other epigenetic factors such as DNA methylation. As observed with DMRs, the differentially expressed ncRNA can also act as an epigenetic biomarker for exposure specificity and potential disease susceptibility. Therefore, the current study provides one of the initial observations that ncRNA can act as a sperm biomarker for specific exposures received ancestrally and will be transgenerationally passed to future generations. The use of exposure-specific ncRNA biomarkers may help elucidate an individual's ancestral exposure and potential disease susceptibility.

## Methods

### Animal studies and breeding

As previously described [15, 16], 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 received 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 [60], the females received daily intraperitoneal injections of vinclozolin (100 mg/kg BW/day), DDT (25 mg/kg BW/day), atrazine (25 mg/kg BW/day), jet fuel (JP8) (500 mg/kg BW/day), or DMSO(vehicle). The exposures of gestating female are near the lowest observed effect level for each of the exposures, and no further exposure occurred for the subsequent F1, F2, or F3 generation animals. The vinclozolin, atrazine, and DDT were obtained from Chem Service Inc. (West Chester, PA) and were injected, dissolved in a DMSO vehicle, as previously described [40, 51, 52, 61]. The jet fuel (JP-8 hydrocarbon) was obtained from Lt Dean Wagner, Dayton, OH, and was injected with an equal volume of sesame oil [50]. Treatment lineages are designated "control," "vinclozolin," "DDT," "atrazine," or "jet fuel" 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 exposure lineages were bred to obtain F2 generation offspring within the lineage. The F2 generation rats were bred to obtain F3 generation offspring within the lineage. Individuals were aged to 1 year and euthanized for sperm collection. The F1–F3 generation offspring were not treated directly with the treatment compounds. The control and exposure lineages were housed in the same room and racks with the same lighting, food, and water, as previously described [61–63]. All experimental protocols for the procedures with rats were preapproved by the Washington State University Animal Care and Use Committee (IACUC approval # 02568-39).

### Epididymal sperm collection and DNA and RNA isolation

As previously described [15, 16], the epididymis was dissected free of connective tissue, a small cut made to the cauda and tissue placed in 5 ml of 1X phosphate buffer saline for 10 min at 37°C and then kept at 4°C to immobilize the sperm. The cauda epididymal tissue was minced, and the released sperm was centrifuged at 13 000 × *g*. The sample was resuspended and sonicated to destroy any contaminating somatic cells and acrosome. This removed any somatic cell contamination due to the sonication resistance of the sperm head nuclei [64]. The pellet was resuspended in Nucleus Isolation Medium buffer [65] and stored at −80°C until RNA isolation.

### RNA isolation

As previously described [15, 16], the F3 generation exposure and control lineage male epididymal sperm were collected, processed, and stored at −80°C until use [66]. The total RNA (messenger RNA; long, noncoding RNA; ribosomal RNA; transfer RNA; small, noncoding RNA) was isolated using the mirVana miRNA Isolation Kit (Life Technologies) following the manufacturer's instructions with modifications at the lysis stage. In brief, after addition of lysis buffer, the sperm pellets were manually homogenized, followed by a 5-min incubation at 65°C. Samples were then placed on ice, and the default protocol was resumed. For quality control, RNA integrity numbers (RIN) were obtained by RNA 6000 Pico chips run on an Agilent 2100 Bioanalyzer (Agilent). A RIN of 2–4 indicates good sperm RNA quality. Concentration was determined using the Qubit RNA HS Assay Kit (ThermoFisher). Equal amounts of RNA were used in the final analysis. The number of samples in each treatment group is presented in Supplementary Table S25 for both the sncRNA and lncRNA analyses, as well as sequencing quality.

### ncRNA sequencing analysis

As previously described [15, 16], total RNA was used to construct large RNA libraries utilizing a ribosomal RNA depletion approach to capture all non-ribosomal RNA species, including mRNA and long non-coding RNA (lncRNA). Libraries were constructed using the KAPA Stranded RNA-seq Library Preparation kit with RiboErase, according to the manufacturer's instructions, with some modifications. The adaptor and barcodes used were from NEBNext Multiplex Oligos for Illumina. Prior to PCR amplification, libraries were incubated at 37°C for 20 min with the USER enzyme (NEB). PCR cycle number was determined using qPCR with the KAPA RealTime Library Amplification kit before final amplification. Size selection (300–700 bp) was performed using Agencourt AMPure XP beads (Beckman Coulter). Quality control was performed using Agilent DNA High Sensitivity chips (Agilent) and Qubit dsDNA high-sensitivity assay (ThermoFisher). Libraries were pooled and loaded onto an Illumina NovaSeq S1 2 × 50 flowcell, and sequenced on an Illumina NovaSeq 6000 sequencer. Bioinformatics analysis was used to separate mRNA transcripts from ncRNA transcripts (see ncRNA bioinformatics section below).

Prior to small library preparation, total sperm RNA samples were enriched for small RNAs using the supplemental protocol for miRNA enrichment with SPRIselect by Beckman Coulter. Small RNA-enriched samples were used for small RNA library preparation, using the NEBNext Multiplex Small RNA Library Prep Set for Illumina, and barcoded with NEBNext Multiplex Oligos for Illumina. Size selection (135–170 bp) was performed using the Pippin Prep (Sage Science). Quality control was performed using Agilent DNA High Sensitivity chips (Agilent) and Qubit dsDNA high sensitivity assay (ThermoFisher). Libraries were pooled and loaded onto an Illumina HiSeq High Output 1 × 50 flowcell, and sequenced on an Illumina HiSeq 2500 sequencer. The sequencing read depth was on average 17–19 million reads per sample, and the sequencing characteristics were 80%–90% unique reads per sample, as previously described [15, 16].

### ncRNA bioinformatic analysis and statistics

The quality of sequencing data was assessed using FastQC [67] and MultiQC [68] tools. Adapters were trimmed, and reads shorter than 15 nt or with a Phred score lower than 20 were discarded using Cutadapt [69]. The samples with low <1 million unique reads, and not used in the analysis, are indicated in Supplementary Table S25.

For lncRNA-Seq analysis, the remaining reads were aligned to the *Rattus norvegicus* reference genome (Rnor_6.0, release 104) from the Ensembl database [70] using Hisat2 [71]. FeatureCounts [72] (using the parameters -M and -O) was employed to quantify the aligned reads. Prior to the Differential Expression Analysis (DEA), 3249 lncRNAs (including 59 antisense, 3090 lincRNAs, 81 processed transcripts, and 19 sense_intronic) were selected from count matrix based on GTF file information (ENSEMBL, Rnor_6.0, release 104). Subsequently, 29 of these lncRNAs with CPAT [73] coding probability score (CP) > 0.35 were filtered out. The remaining 3220 lncRNAs were included in the DEA.

For sncRNAseq analysis, the cleaned reads were aligned against known Rattus novergicus miRNAs (496 mature, 764 precursor sequences) from miRBase [74] (release 22.1), piRNAs (2578 gold standard sequences) from piRbase [73], tsRNAs (385 sequences) from GtRNAdb/rn6 [75], and rsRNAs (329 sequences) from Ensembl (ncRNAs subset, Rnor_6.0 release 104) using AASRA [76] with default parameters.

DEA was conducted using DESeq2 [77] methodology. lncRNAs with at least 10 counts and sncRNAs with at least 20 counts were selected for analysis. The expression levels of lncRNAs and sncRNAs from sample groups Atrazine, DDT, Jet Fuel, and Vinclozolin were compared against the Control sample group using an adjusted *P*-value threshold lower than 0.1 and a fold change threshold greater than 2. For sncRNAs, DEA was conducted separately for the subtypes of interest, including miRNA, piRNA, rRNA, and tsRNA. ncRNA were annotated by comparing genomic locations of the ncRNA with NCBI provided annotations. Gene targets were not considered in this analysis. The genes that overlapped with differential ncRNA (within 10 kb) were then input into the KEGG pathway search [78, 79] to identify associated pathways. The ncRNA associated genes were then sorted into functional groups by reducing Panther protein classifications into more general categories. All molecular data have been deposited into the public database at NCBI and can be accessed under the PRJNA1072708 and PRJNA1073216 BioProject accession numbers. The specific scripts used to perform the analysis can be accessed at https://github.com/WeiYanLab/ncRNAseq/.

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## Author contributions

Hayden McSwiggin (ncRNA Molecular Analysis, edited manuscript), Rubens Magalhães (ncRNA Molecular Analysis and

Bioinformatics, Data Analysis, edited manuscript), Eric E. Nilsson (Sample Processing, Data Analysis, edited manuscript), Wei Yan (Supervised ncRNA Analysis and ncRNA Informatics, edited manuscript), Michael K. Skinner (Conceived, Data Analysis, Funding Acquisition, Wrote and Edited manuscript).

## Supplementary data

Supplementary data is available at *EnvEpig* online.

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## Data availability

All molecular data have been deposited into the public NCBI SRA Database under the PRJNA1072708 and PRJNA1073216 BioProject accession numbers, and R code computational tools are available at https://github.com/WeiYanLab/ncRNAseq/.

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