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Hydrocarbons (Jet Fuel JP-8) Induce Epigenetic Transgenerational Inheritance of Obesity, Reproductive Disease and Sperm Epimutations

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Abstract

Environmental compounds have been shown to promote epigenetic transgenerational inheritance of disease. The current study was designed to determine if a hydrocarbon mixture involving jet fuel (JP-8) promotes epigenetic transgenerational inheritance of disease. Gestating F0 generation female rats were transiently exposed during the fetal gonadal development period. The direct exposure F1 generation had an increased incidence of kidney abnormalities in both females and males, prostate and pubertal abnormalities in males, and primordial follicle loss and polycystic ovarian disease in females. The first transgenerational generation is the F3 generation, and the jet fuel lineage had an increased incidence of primordial follicle loss and polycystic ovarian disease in females, and obesity in both females and males. Analysis of the jet fuel lineage F3 generation sperm epigenome identified 33 differential DNA methylation regions, termed epimutations. Observations demonstrate hydrocarbons can promote epigenetic transgenerational inheritance of disease and sperm epimutations, potential biomarkers for ancestral exposures.

Keywords

Environmental Epigenetics; Transgenerational; Obesity; Ovary; Puberty; DNA Methylation; Hydrocarbons; Systems Biology

Introduction

Environmentally induced epigenetic transgenerational inheritance involves the transmission of phenotypic changes to the offspring of unexposed subsequent generations. This occurs through heritable germline epigenetic alterations following exposure of gestating F0 generation females to environmental factors [1, 2]. Previous studies in both mice [3] and rats [1, 4], using exposures to the fungicide vinclozolin during the fetal gonadal sex determination period, have demonstrated that the male germline undergoes a modification in epigenetic reprogramming resulting in altered somatic cell gene expression and development of adult onset disease [5]. These germline epigenetic changes appear imprinted-like and allow transgenerational inheritance to subsequent generations that have not been directly

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exposed [6–8]. For example, epigenetic marks acquired in the male germline at the F2 generation persisted in somatic tissue of F5 mice, despite passage through the female germline in the F3 and F4 generations [9]. When the F0 generation gestating female is exposed, not only the F0 generation female but also the F1 generation embryo and its germ cells (that will become the F2 generation) are directly exposed. Therefore, the F3 generation is the first unexposed generation and is the first transgenerational progeny [10].

Epigenetic alterations can be induced by environmental factors such as caloric restriction during pregnancy [11] or exposure to various environmental compounds such as vinclozolin [1]. Endocrine disrupting compounds such as herbicides, fungicides, and pesticides have been released into the environment and expose both humans and animals [12]. Recent events highlight a major environmental contamination as hydrocarbons. In 2010 the Deepwater Horizon oil rig explosion created the largest environmental disaster in U.S. history and the second largest oil spill in human history, releasing over 800 million liters of oil (hydrocarbons) into the Gulf of Mexico over a period of 3 months [13]. In the United States alone there are on average more than 70 spills reported per day [14]. Hydrocarbons represent one of the largest chemical exposures to the US military as it is used for fueling military vehicles and dust control on road surfaces [15–17]. JP-8 (Jet Fuel) is a hydrocarbon mixture that is most commonly used by the military [16, 18, 19]. Over 2 million military and civilian personnel are exposed to an estimated 60 billion gallons of JP-8. The first estimate of premature mortalities attributable to aircraft emissions globally is 8000 people per year [15]. These reports indicate that there is extensive exposure of humans and animals to hydrocarbon mixtures such as jet fuel.

Individuals exposed to hydrocarbons have reported emotional dysfunction, decreased attention spans, fatigue, skin irritation, postural sway imbalances, and adverse effects on sensorimotor speed, liver function, and the respiratory system [17, 20–23]. JP-8 was found to be potentially toxic to the immune system, respiratory tract, and nervous system [24]. In animal models low toxicity of JP-8 has been reported to cause slight dermal irritation, weak dermal sensitization, respiratory tract sensory irritation, hearing loss, nephropathy, and immunosuppressive and neurobehavioral effects [24]. Previous studies have also shown adverse effects in lung ventilatory function [17], immune dysfunction [18, 25], increased tumor formation [26] and suppressed immune response to viral infections [27]. There are no reports regarding potential epigenetic transgenerational inheritance of disease from JP-8 exposed gestating females.

The current study was designed to examine the transgenerational actions of a hydrocarbon mixture (JP-8) after exposure of a gestating female rat. A dose of 25% of the oral LD50 was employed to minimize direct toxicity. Similar in vivo doses have been used in previous studies [28–33]. The exposure duration used in the current study was shorter than most previous studies. This study was not designed to evaluate risk assessment, but to assess potential transgenerational effects. These observations can now be used to design more efficient risk assessment studies in the future. The hypothesis tested was that the exposure to hydrocarbons, specifically JP-8 (jet fuel), during fetal gonadal development will promote permanent changes to the DNA methylation pattern of the male germline to induce epigenetic transgenerational inheritance of adult onset disease. Abnormalities and disease were analyzed in the F1 and F3 generation progeny to determine the direct exposure and transgenerational phenotypes, respectively. The F3 generation sperm from control (vehicle) and jet fuel lineage animals were previously compared to identify differential DNA methylation regions (DMR) induced transgenerationally in the germline [34]. These transgenerational epigenetic alterations in the F3 generation sperm are termed epimutations. A previous publication documented the comparative actions of jet fuel with a plastics mixture (bisphenol-A, DEHP and DBP), dioxin and a pesticide mixture (permethrin and

DEET) on postnatal day 120 rats and showed reproductive abnormalities in F3 generation animals [34]. Interestingly, exposure specific transgenerational sperm epimutations were identified [34]. The current study extends these observations to examine the jet fuel hydrocarbon actions on 1-year old F1 and F3 generation animals, since transgenerational adult onset disease primarily appears to develop between 6–12 months of age [8]. In addition, the current study more thoroughly examines the previously identified [34] jet fuel induced transgenerational sperm epimutations.

Materials and Methods

Animal Studies

Experimental protocols with rats were pre-approved by the Washington State University Institutional Animal Care and Use Committee. The Washington State University Department of Environmental Health and Safety approved the protocols for the use of environmental chemicals. Female and male rats of an outbred strain Sprague Dawley SD (Harlan) of about 70 and 100 days of age were maintained in ventilated isolator cages containing Aspen Sani-chips. Rats were fed ad libitum with a standard rat diet and ad libitum tap water for drinking. During the injection, vaginal smear collection, weaning and puberty checking procedures rats were held in an animal transfer station. To obtain time-pregnant females the female rats in proestrus were pair-mated with male rats. The sperm-positive (day 0) rats were considered pregnant and monitored for diestrus and body weight. On embryonic day 8 (E8) through E14 the gestating females were administered daily intraperitoneal injections [6] of JP-8 hydrocarbon (jet fuel obtained from Lt Dean Wagner, Dayton, OH), 500 mg/kg BW/day, or dimethyl sulfoxide (DMSO) (vehicle) with an equal volume of sesame oil (Sigma) to prevent irritation at the injection site. The gestating females rats treated with vehicle control or jet fuel were designated the F0 generation.

Breeding

The offspring of the F0 generation rats were the F1 generation. Non-littermate females and males aged 70-90 days from F1 generation control or jet fuel lineages were bred to obtain F2 generation offspring. The F2 generation rats were bred to obtain F3 generation offspring. Individual animals were selected to represent a litter for all mating studies. The number of animals per litter (litter representation) mean ± SEM used for a specific disease/abnormality for the F1 or F3 generation control versus jet fuel lineage was found not to be statistically different ($p>0.05$). Therefore, the litter representation was the same between control and jet fuel comparison for specific disease/abnormality comparisons and litter bias was not detected. No sibling or cousin breeding was used to avoid any inbreeding artifacts. Suckling rats were weaned from their mothers at 21 days of age. It is important to note that only the F0 generation gestating females were directly exposed to the control vehicle or jet fuel treatment, and the F1- F3 generations were not subjected to any treatment.

Tissue Harvest and Histology Processing

One-year old rats were euthanized by CO_2 inhalation for tissue harvest. Body and organ weights were measured at dissection time for all animals. Testis, epididymis, prostate, seminal vesicle, ovaries, uterus and kidney were collected and fixed in Bouin's solution (Sigma) and 70% ethanol, then processed for paraffin embedding by standard procedures for histopathology examination. Five-micrometer thick tissue sections were made and were either unstained or stained with hematoxylin and eosin stain and examined for histopathology. Blood samples were collected at the time of dissection, allowed to clot, centrifuged and serum samples stored at −20°C for steroid hormone radioimmunoassays (RIA), performed by the Washington State University Center for Reproductive Biology Assay Core Laboratory.

Histopathology Examination and Disease Classification

Three different observers independently examined each unmarked tissue slide and identical criteria were applied to identify diseased tissue. A marked central portion of each prostate, kidney, ovary and testis section was microscopically examined under 200x magnification. An additional peripheral portion of each testis section was also examined. Testis histopathology criteria included the presence of a vacuole, azoospermic atretic seminiferous tubule and other abnormalities including sloughed spermatogenic cells in the center of the tubule and a lack of a tubule lumen. Testis spermatogenic cell apoptosis was also assessed as described in the Supplemental Methods. Prostate (Ventral Lobe) histopathology criteria included the presence of vacuoles, atrophic epithelial layer of ducts and hyperplasia of prostatic duct epithelium. Kidney histopathology criteria included reduced size of glomerulus, thickened Bowman's capsule and the presence of proteinaceous fluid-filled cysts. A cut-off was established to declare a tissue 'diseased' or abnormal based on the mean number of histopathological abnormalities plus two standard deviations from the mean number of abnormalities in control lineage tissues as determined by each of the three individual observers. This number was used to classify rats into those with and without disease in testis, prostate or kidney in each lineage. A rat tissue section was declared 'diseased' only when at least two of the three observers marked the same tissue section as such. Necropsy and histopathology examinations on rats that died prior to 1 year of age and also pathology analysis of tissues found with unknown or suspected diseases were performed by the WSU Washington Disease Diagnostic Laboratory and these results were also included in the study. The proportion of rats with obesity or tumor development was obtained by tallying those that had these conditions out of all the animals evaluated. The primary tumors detected were mammary tumors, while some skin, kidney and uterine tumors were detected as well. Obesity was determined with the combined characteristics of increased body weight and the presence of significantly increased abdominal adiposity, as previously described [35]. The litter representation for males or females per litter used for a specific disease state or abnormality assessment was found to be statistically the same for control versus jet fuel comparisons.

Ovary sections were assessed for two diseases, primordial follicle loss associated with primary ovarian insufficiency (POI) and polycystic ovarian (PCO) disease. Every 30th section of ovaries was stained with hematoxylin and eosin and stained sections (150 μm apart), with a focus on the central portion of the ovary with the largest cross section were evaluated. Primordial follicle loss was determined by microscopically counting the number of primordial follicles per ovary section. Primordial follicle loss was considered present in an ovary when the primordial follicle number was less than the control mean minus two standard deviations. Polycystic ovaries were determined by microscopically counting the number of small cystic structures. The mean number of primordial follicles or small cysts per section was calculated from multiple sections per ovary. Polycystic ovarian disease was considered present when the number of small cysts was more than the 'control mean plus two standard deviations'. Follicles had to be non-atretic and showing an oocyte nucleus in order to be counted. Primordial follicles had an oocyte surrounded by a single layer of either squamous or both squamous and cuboidal granulosa cells [36, 37]. Cysts were defined as fluid-filled structures of a specified size that were not filled with red blood cells, had no oocyte and negligible granulosa cells. A single layer of cells may line cysts. Small cysts were 5 to 25 μm in diameter measured from the inner cellular boundary across the longest axis. Percentages of females with primordial follicle loss or polycystic ovarian disease were computed.

Onset of puberty was assessed in females by daily examination for vaginal opening from 30 days of age and in males by balano-preputial separation from 35 days of age. For identifying a rat with a pubertal abnormality (either an early or delayed onset of puberty), first a mean

and standard deviation of the age of pubertal onset was calculated for control lineage rats. A range of normal pubertal onset was chosen based on mean ± 2 standard deviations. Any rat with a pubertal onset below this range was considered to have had an early pubertal onset and any rat with a pubertal onset above this range was considered to have had a delayed pubertal onset. The proportion of rats with pubertal abnormality was computed from the total number of rats evaluated for puberty onset.

A table of the incidence of individual disease/abnormalities in rats from each group was created and the proportions of individual disease, total disease/abnormality and multiple disease incidences were computed. For the individual disease/abnormality, only those rats that showed a plus (presence of disease/abnormality) or minus (absence of disease/abnormality) in the table are included in the computation. For the total disease/abnormality, a column with total disease/abnormality is presented and the number of plus signs (indicating the presence of disease/abnormality) was added up for each of the rats and the proportion was computed as the number of rats with one or more diseases (total disease/abnormality) out of all listed rats. For the multiple disease/abnormality, the proportion was computed as the number of rats with more than one disease/abnormality out of all of the listed rats. It should be noted that not all the rats were evaluated for all disease/abnormality due to technical limitations. The computation of the percent incidence of disease data is limiting in this respect and the data presented represent only the minimal incidence of total or multiple disease.

Epididymal Sperm collection

The epididymis was dissected free of connective tissue, the fat pad, the muscles and the vas deferens. A small cut was made to the cauda epididymis and the tissue was placed in 5 ml F12 culture medium containing 0.1% bovine serum albumin for 10 minutes at 37°C and then kept at 4°C to immobilize the sperm. The epididymal tissue in the buffer was put in a petri dish and minced with a blade to release the sperm into the buffer. The sperm released into the buffer was placed into a 1.5 ml centrifuge tube and then centrifuged at 13, 000 × *g* to pellet the sperm. Sperm were stored in fresh NIM buffer (Nucleus Isolation Medium: 123.0 mmol/l KCl, 2.6 mmol/l NaCl, 7.8 mmol/l NaH_2PO_4, 1.4 mmol/l KH_2PO_4 and 3 mmol/l EDTA) at −20°C until processed further.

Sperm DNA isolation and methylated DNA immunoprecipitation (MeDIP)

Sperm heads were separated from tails through sonication following previously described protocols (without protease inhibitors) [38] and then purified using a series of washes and centrifugations [39] from a total of nine F3 generation rats per lineage (control or jet fuel) that were 120 days of age. DNA was extracted from purified sperm heads as previously described [4]. Equal concentrations of DNA from individual sperm samples were then used to produce pools of DNA material. Three DNA pools were produced in total per lineage, each one containing the same amount of sperm DNA from each of three different animals. Therefore a total of 18 animals were used for building three different DNA pools per lineage (control or jet fuel) making the following groups: C1–C3 and J1–J3. These DNA pools were then used in three different methylated DNA immunoprecipitation (MeDIP) experiments.

MeDIP was performed as follows: 6 μg of genomic DNA was subjected to a series of three 20 pulse sonications at 20% amplitude (Sonic dismembrator model 300, Fisher Scientific) and the appropriate fragment size (200–1000 ng) was verified on 2% agarose gels. The sonicated genomic DNA was resuspended in 350 μl TE buffer and denatured for 10 min at 95°C and then immediately placed on ice for 5 min. One hundred μl of 5X IP buffer (50 mM Naphosphate pH 7, 700 mM NaCl, 0.25% Triton X-100) was added to the sonicated and denatured DNA. An overnight incubation of the DNA was performed with 5 μg of antibody

anti-5- methylCytidine monoclonal from Diagenode (Denville, NJ) at 4°C on a rotating platform. Protein A/G beads from Santa Cruz were prewashed with PBS-BSA 0.1% and resuspended in 40 µl 1X IP buffer. Beads were then added to the DNA-antibody complex and incubated 2 h at 4°C on a rotating platform. Beads bound to DNA-antibody complex were washed 3 times with 1 ml 1X IP buffer. Washes included incubation for 5 min at 4°C on a rotating platform and then centrifugation at 6000 rpm for 2 min. Beads-DNA-antibody complex were then resuspended in 250 µl digestion buffer (50 mM Tris HCl pH 8, 10 mM EDTA, 0.5% SDS) and 3.5 µl of proteinase K (20 mg/ml) was added to each sample and then incubated overnight at 55°C on a rotating platform. DNA purification was performed first with phenol and then with chloroform:isoamyl alcohol. Two washes were then performed with 70% ethanol, 1 M NaCl and glycogen. MeDIP selected DNA was then resuspended in 30 µl TE buffer.

Tiling Array MeDIP-Chip Analysis

Roche Nimblegen's Rat DNA Methylation 3x720K CpG Island Plus RefSeq Promoter Array was used, which contains three identical sub-arrays with 720,000 probes per sub-array, scanning a total of 15,287 promoters (3,880 bp upstream and 970 bp downstream from transcription start site). Probe sizes range from 50–75 bp in length with the median probe spacing of 100 bp. Three different comparative (control-lineage MeDIP vs. jet fuel-lineage MeDIP) hybridization experiments were performed. MeDIP DNA samples from jet fuel lineage were labeled with Cy3 and MeDIP DNA samples from the control lineage were labeled with Cy5. Therefore, three different biological sperm DNA pooled samples, each containing 3 different animals, from both control and jet fuel F3 generation lineage were analyzed on different MeDIPChip comparative hybridizations. The MeDIP-Chip data bioinformatics and statistics protocol previously described [4, 34] are presented in the Supplemental Methods section.

Only the differential methylation regions (DMR) found to be statistically significant ($p<10^{-7}$) and similar/present in all three different experiments are reported. Therefore, a very stringent criteria was used that involved a statistically significant ($p<10^{-7}$) difference in each experiment and all three experiments had to have the same DMR detected. A simple average of all three experiments for a single statistical significance analysis was not used. The probability analysis of determining the same differential DNA methylation regions (DMR) in all three experiments randomly was determined to be $<10^{-7}$ chance for occurrence. Therefore, the DMR identified could not be randomly derived and were selected with highly stringent criteria described in the Supplemental Methods.

Statistical Analysis of Rat Organ and Pathology Data

For statistical analysis all data on body and organ weights were used as input in the program GraphPad© Prism 5 statistical analysis program (Graphpad Inc., USA) and t-tests were used to determine if the data from the jet fuel lineage differ from those of control lineage. For the number of rats with disease or abnormalities (disease/abnormality incidence) a logistic regression analysis was used to analyze the data (control or jet fuel lineage, and diseased or unaffected). All treatment differences were considered significant if $p<0.05$.

Results

Transgenerational Adult-Onset Disease/Abnormality Analysis

The experimental design included exposure of gestating female rats to daily intraperitoneal injections of DMSO vehicle (control) or JP8 jet fuel during embryonic days 8 to 14 of gestation. The F1 generation rat offspring born to different exposed females were bred to obtain the F2 generation. The F3 generation animals were obtained by breeding non-

littermate females and males of the F2 generation. No sibling or cousin breeding was used to avoid any inbreeding artifacts in generating the different lineages. Randomly selected offspring (male and female each litter) of the F1 and F3 generations were aged to one year and euthanized. Body and organ weights were measured. Testis, prostate, kidney and ovary were examined for histopathology as outlined in the Methods.

Potential toxicity from direct fetal exposure to jet fuel in the F1 generation animals was determined and comparisons were made to the F3 generation animals through analysis of 1-year old rat body weight and organ weights (Supplemental Table S1A). There were negligible statistically significant differences between jet fuel and control lineage female body weights, ovaries, uterine, or kidney weights in F1 and F3 generations (Supplementary Table S1B). There were also negligible statistically significant differences between jet fuel and control lineage male body weights, testis, prostate, seminal vesicle, epididymis, or kidney weights in F1 and F3 generations (Supplementary Table S1A). The exception was an increase in body weights for both males and females of the F3 generation jet fuel lineage compared to the F3 generation control lineage animals. These observations in the F1 generation indicate that there were no overt toxicity effects of the jet fuel exposure.

Adult onset disease or abnormalities of testis, prostate, kidney, ovary, pubertal onset, tumor development and obesity were assessed in 1-year old F1 generation rats of jet fuel lineage. The F1 generation animals had direct fetal exposure to dioxin. A comparison of the control lineage animals with the F1 generation jet fuel lineage rats showed an increase in prostate abnormalities, but no increase in testis disease (Figure 1). Histopathology revealed testis disease in the form of azoospermic and atretic seminiferous tubules and prostate disease/abnormality in the form of atrophic prostatic ductular epithelium. Testis disease in F1 generation males of jet fuel lineage was further investigated by a TUNEL assay that identified apoptotic testicular spermatogenic cells. The number of apoptotic spermatogenic cells in testis was not altered in males of F1 generation jet fuel lineage (Supplemental Figure S1). The kidney disease incidence increased in both females and males of the F1 generation jet fuel lineage (Figure 2). Kidney disease was characterized by the occurrence of proteinaceous fluid filled cysts throughout the kidney sections and an increase in the Bowman's capsule thickness and cyst development in the kidneys. Pubertal abnormalities observed included either early onset or delayed onset of puberty. A significant increase in a delayed pubertal onset (12/12) was observed in F1 generation males of jet fuel lineage (Figure 3). The incidence of pubertal abnormalities in F1 generation females of jet fuel lineage did not reach statistical significance (Figure 3 panel A). Ovarian disease/abnormality was examined as either primordial follicle loss or polycystic ovarian disease in F1 generation females of jet fuel lineage. The incidences of both ovarian diseases increased in F1 generation females of jet fuel lineage (Figure 3 panels C and D). The incidence of tumor development was assessed in both F1 generation females and males of jet fuel lineage and there were no significant changes in either sex (Figure 4 panels A and B). In addition, the incidence of adult onset obesity was examined in both sexes of F1 generation jet fuel lineage and found not to be different from those of control lineage F1 generation (Figure 5 panels A and B). The overall incidence of total disease/abnormality (proportion of animals with one or more diseases) and multiple disease/abnormality (proportion of animals with more than one disease) were assessed in F1 generation rats of jet fuel lineage. The incidence of total disease/abnormality in both females and males increased in F1 generation jet fuel lineage (Figure 4 panels C and D). Likewise, the incidence of multiple disease/abnormality in both females and males increased in the F1 generation jet fuel lineage (Figure 4 panels E and F). Therefore F1 generation animals of jet fuel lineage experienced an increase in the incidence of adult-onset prostate disease, kidney disease, ovarian abnormality of primordial follicle loss and polycystic ovarian disease, as well as pubertal abnormalities and an overall increase in total and multiple disease/abnormality.

Adult onset disease or abnormalities of testis, prostate, kidney, ovary, pubertal onset, tumor development and obesity were assessed in 1-year old F3 generation rats of control and jet fuel lineages. The F3 generation rats of the jet fuel lineage showed fewer adult onset disease/abnormalities (Figure 1–3). The predominant diseases in F3 generation jet fuel lineage were ovarian disease and obesity. The incidence of ovarian primordial follicle loss and polycystic ovarian disease were increased in F3 generation females of jet fuel lineage (Figure 3 panels C and D). The ovaries of F3 generation jet fuel lineage rats presented both small and large luteal cysts that were not observed in control lineage ovaries. The incidence of adult onset obesity in F3 generation females and males increased 10–15% relative to those of control lineage (Figure 5 panels A and B). Obesity was characterized by the increased body weight (Supplemental Table S1) and excessive deposition of intra-abdominal fat (Figure 5 panels C and D). The incidence of total disease/abnormality increased in females of F3 generation jet fuel lineage (Figure 4 panels C and D). The incidence of total disease/abnormality in males of F3 generation jet fuel lineage did not increase significantly. However, the incidence of multiple disease/abnormalities in both females and males increased in F3 generation jet fuel lineage (Figure 4 panels E and F). Therefore F3 generation animals of jet fuel lineage manifested an increased incidence of adult-onset ovarian disease/abnormality including primordial follicle loss, polycystic ovarian disease (including luteal cysts) and obesity. This increased incidence of disease/abnormality in F3 generation animals of jet fuel lineage involves the transgenerational inheritance of disease.

Potential transgenerational endocrine alterations in serum sex steroid hormone concentrations were measured in the F3 generation control and jet fuel lineage rats. Serum testosterone concentrations in the 1-year-old F3 generation male rats from jet fuel lineage did not differ from those of control lineage (Figure 6). Interestingly, serum estradiol concentrations in female rats during the proestrus phase or diestrus phase were increased in jet fuel lineages compared to control lineage (Figure 6 panels A and B). These results indicate that there were transgenerational endocrine effects in F3 generation females of jet fuel lineage.

The potential epigenetic mechanism behind the ancestral jet fuel exposure promoting transgenerational inheritance of adult onset disease was examined. Transgenerational phenomena require the absence of direct exposure and the transmission of phenotypes through the germline [2, 10]. Jet fuel-induced epigenetic alterations in the sperm epigenome of the F3 generation jet fuel lineage males were previously identified [34] with a methyl cytosine antibody and methylated DNA immunoprecipitation (MeDIP) followed by a genome-wide promoter tiling array chip (MeDIP-Chip) assay [4]. The sperm DNA from F3 generation control and jet fuel lineages were analyzed. Three different experimental pools of control and jet fuel lineage F3 generation MeDIP samples were generated, each containing different sperm DNA from three different animals from different litters for each pool. A comparative hybridization with the MeDIP-Chip assay was performed [34] as described in the Methods to identify differential DNA methylation between control and jet fuel lineage sperm. The analysis previously identified [34] statistically significant differential DNA methylation regions (DMR) in 33 different promoters, each being an average of 800 bp in size, Table 1. The chromosomal locations of these differentially methylated regions are presented in Figure 7. The majority of the different chromosomes contained DMR. The DMR alterations involved both increases and decreases in methylation as indicated in Table 1. The functional gene categories associated with the F3 generation jet fuel lineage sperm DMR are shown in Figure 8. A number of major pathways and cellular processes contained DMR associated genes such as signaling, metabolism and transcription. Since the F3 generation is the first transgenerational progeny lacking any direct exposure, only the F3 generation sperm epigenome alterations were investigated in the current study.

A final analysis of the previously identified jet fuel induced DMR involved a correlation with genes previously identified to be associated with the different transgenerational pathologies identified. From the 33 DMR one was associated with obesity (*Asip*) and none were associated with ovarian disease. This analysis involved genes previously known to be associated with specific disease and listed in the Pathway Studio software suite.

Discussion

The hypothesis was tested that exposure of gestating females to hydrocarbons, specifically JP-8 (Jet Fuel), during fetal gonadal development promotes permanent changes in DNA methylation of the male germline to transmit epigenetic transgenerational inheritance of adult onset disease. Previous studies with hydrocarbons have shown following direct exposure toxicological effects such as adverse effects in lung ventilatory function [17], immune dysfunction [18, 25], increased tumor formation [26], and suppressed immune response to viral infections [27]. In the current study, the F1 generation animals of jet fuel lineage showed an increase in the incidence of adult-onset disease/abnormalities including prostate disease, kidney disease, ovarian primordial follicle loss, polycystic ovarian disease, pubertal abnormalities, and an overall increase in total and multiple disease/abnormalities. The potential transgenerational actions of jet fuel in unexposed F3 generation progeny were also examined in the current study.

The F3 generation females of jet fuel lineage had an overall increase in the incidence of total and multiple disease/abnormalities, and in males the incidence of multiple disease/abnormalities increased. Therefore, ancestral exposure to the environmental toxicant jet fuel increased the overall incidence of total and multiple disease/abnormalities in the unexposed F3 generation progeny. Although these hydrocarbon effects were observed in the F1 and F3 generation animals, the mode of administration and dose do not allow for environmental risk assessment of jet fuel in the current study. The dose used in the current study was based on a 25% fraction of the oral LD50 for jet fuel, JP-8. Future studies with more appropriate mode of administration and dose curves will be required for risk assessment.

Transgenerational phenomena require the absence of any direct exposure to an environmental factor (e.g. compound). This is in contrast to direct exposure toxicological effects. For example, when a gestating female is exposed during the critical period of gonadal development the female, its fetus, and the fetal germ cells are all directly exposed. Therefore, any effects observed in F0 (female), F1 (via fetal exposure) and F2 (via fetal germ cell exposure) generations can be attributed to the direct exposure. The F3 generation is the first generation to clearly demonstrate transgenerational phenotypes [10]. The current study examined the pathologies in the F1 generation animals to observe any direct exposure effects and examined the F3 generation animals to observe the transgenerational phenotypes. Although similarities in phenotype can occur between F1 and F3 generations [1], the distinct mechanisms involved suggest differences may be expected in phenotype between F1 and F3 generation animals. In the current study, the F1 generation jet fuel lineage rats had direct exposure effects on prostate disease, kidney disease and pubertal abnormalities, which were not observed in the F3 generation. The increased incidence of ovarian disease was observed in both the F1 and F3 generation jet fuel lineage females. In contrast, the increased incidence of obesity was only observed in the F3 generation jet fuel lineage animals. Therefore, the F3 generation jet fuel lineage transgenerational disease phenotype is primarily distinct from the F1 generation direct exposure phenotype.

The F3 generation jet fuel lineage females exhibited a dramatic increased incidence (100%) of ovarian disease. The jet fuel lineage F3 generation females had an increased incidence of primordial follicle loss and polycystic ovarian disease. A polycystic condition of the ovary is

the most common ovarian disease prevalent in women of reproductive age [40–45]. Primordial follicle loss is associated with a reduced primordial pool and is a precursor for the disease primary ovarian insufficiency. In addition to the jet fuel hydrocarbon mixture, a number of other environmental toxicants were found to promote the transgenerational inheritance of ovarian disease [5]. In a previous publication, a significant transgenerational alteration in both the transcriptome and the epigenome of the ovarian granulosa cells from rats of the F3 generation after ancestral exposure to the fungicide vinclozolin was observed and associated with ovarian disease [5]. The speculation is other environmental toxicants such as hydrocarbons will also promote transgenerationally altered ovarian cell (e.g. granulosa cell) function. Therefore, the altered sperm epigenome appears to promote somatic cell changes in gene expression during development that can increase susceptibility to lead to adult-onset disease [46]. These epigenetic abnormalities can be induced by exposure to environmental toxicants including jet fuel [5, 34]. Interestingly, epigenetic mechanisms have been suggested to underlie the development of polycystic ovary syndrome-like phenotypes in women [47] and prenatally androgenized rhesus monkeys [48]. Epigenetic modifications are also found in the FMR1 gene in women with fragile X syndrome associated with primary ovarian insufficiency [49, 50]. These studies support the results of the current study suggesting an epigenetic etiology of ovarian disease. Therefore, ancestral exposures to the hydrocarbons may contribute to the development of ovarian disease in our current population. Observations indicate a novel paradigm be considered for the etiology of primary ovarian insufficiency and polycystic ovarian disease in women.

The F3 generation females and males of the jet fuel lineage had an increased incidence (10–15%) of transgenerational adult onset obesity. The obesity phenotype observed involved both an increase in body weight and increase abdominal fat deposition that correlate with the diagnosis of obesity [35]. The increased incidence of obesity as a result of ancestral exposure to the environmental toxicant jet fuel suggests an additional etiology to nutrition be considered as a factor in the occurrence of obesity [51–54]. Obesity is a growing concern in the United States population. According to the Center for Disease Control in 2010, 33% of adults in the United States are obese and 17% of children between the ages of 2–19 are obese. Obesity is a precursor for many clinical conditions such as cardiovascular disease, type 2 diabetes and a diminished average life expectancy [55, 56]. Obesity is a major component of the complex disease trait termed metabolic syndrome. Maternal obesity can have a negative effect on children’s health [57]. Experimental studies in rats indicate that obese dams are responsible for the appearance of obesity in the subsequent generation [58]. Waterland et al., 2008 [59] suggested that epigenetic mechanisms are involved in the transgenerational transmission of maternal obesity. The current observations suggest obesity and the possibility of developing obesity is in part a result of ancestral exposure to the environmental toxicants such as jet fuel. Future studies will be needed to evaluate the adult status of obesity associated conditions such as bone mineralization, adult length and metabolic disease in the F3 generation jet fuel lineage animals to gain insights into the pathogenesis of the transgenerational obesity observed. The suggestion that these different disease phenotypes (ovary disease and obesity) may be linked to a complex disease syndrome that involves a transgenerational inheritance etiology also needs to be considered.

The inheritance phenotype observed does not follow normal Mendelian genetic phenomena. The frequency of a genetic mutation is generally less than a percent and a random event, so similar phenotypes are often not observed. In addition, a reduction in frequency (segregation) at each generation is generally observed. In contrast, the frequency of the phenotype observed is 10–100%, reproducible between animals, and can be acquired in the transgenerational F3 generation. Therefore, the inheritance phenotype appears to follow epigenetic mechanisms rather than genetic [2]. Epigenetic inheritance involves the transmission of epigenetic information or alterations through the germline between

generations in the absence of environmental factors or exposures [1, 2]. Therefore, the jet fuel hydrocarbon mixture induced transgenerational inheritance of disease appears to be through an environmentally induced epigenetic transgenerational adult onset disease mechanism [1, 2]. Although genetic processes are clearly involved in the disease etiology, the generational inheritance appears to involve epigenetic mechanisms.

The molecular mechanism involved in epigenetic transgenerational inheritance of adultonset disease phenotypes is the reprogramming of the germline epigenome during male gonadal sex determination [2, 60]. The modified sperm epigenome (DNA methylation) appears to become permanently reprogrammed and protected from the normal DNA de-methylation and remethylation process that occurs after fertilization. This allows transgenerational transmission of the modified sperm epigenome and subsequent modification of cell and tissue epigenomes and transcriptomes originating from such sperm to promote epigenetic transgenerational inheritance of disease phenotypes [2, 46]. The current study examined the altered sperm epigenome and epimutations induced by the jet fuel hydrocarbon mixture. The F3 generation rat sperm from jet fuel and control lineages were previously [34] used for genome wide promoter DNA methylation analysis utilizing an MeDIP-Chip protocol [4]. Differential DNA methylated regions (DMR), defined both as epimutations and epigenetic biomarkers, were previously identified [34] for the jet fuel lineage F3 generation sperm in comparison with control lineage F3 generation sperm, Table 1. Therefore, the epigenetic analysis identified the development of epimutations in the sperm. Since the F3 generation DMR will be involved in the transgenerational transmission, while the F1 generation are involved with direct exposure, only the F3 generation DMR were examined. Future studies will need to assess the similarities and differences between the F1 and F2 epimutations. These F3 generation epimutations appear to have a role in the epigenetic transgenerational inheritance of the disease phenotypes observed. In addition to DNA methylation, alterations in histone modifications to regulate adjacent gene expression and non-coding RNA to regulate distal gene expression can also be involved in the epigenetic transgenerational inheritance phenomenon [46, 61]. The sperm epigenome will generate an altered epigenome in the embryonic stem cells that then will lead to altered epigenomes and transcriptomes in all the cell types following subsequent embryo development [46]. A recent study demonstrated all adult tissues examined from vinclozolin induced F3 generation animals have altered tissue specific transgenerational transcriptomes [61]. The cascade of epigenetic and genetic (transcriptome) changes associated with adult cell type differentiation will likely show few similarities with the original sperm epigenome. In a different study the environmental toxicant vinclozolin alters the sperm epigenome [4] and promotes an altered pubertal granulosa cell epigenome and transcriptome associated with adult onset ovarian disease [5]. Interestingly, the sperm and granulosa cell epigenome modifications are distinct [5]. Although this previous study suggests a link between the epigenetically altered sperm and somatic cell associated disease, a limitation of the current study is that it is not possible to directly demonstrate a causal role of the altered germline epigenome with the somatic cell linked to the adult onset disease phenotype observed. Future studies will need to examine the cascade of epigenetic and genetic events leading to transgenerational adult onset disease [46]. Jet fuel induced alterations in the F3 generation granulosa cell or adipose cell transcriptome and epigenome needs to be elucidated. Future studies on the various cell types associated with the disease phenotypes will be required to determine potential correlations with the sperm DMR identified. Although observations presented in the current study correlate the sperm DMR associated gene agouti signaling protein (*Asip*) as having a known link to obesity, the causal role of the DMR were not assessed. However, the current study does document the altered germline epigenome is associated with the transmission of the transgenerational phenotypes.

Epigenetic transgenerational inheritance of disease has been shown to be promoted by several environmental compounds. Vinclozolin exposure resulted in F3 generation testis disease, prostate disease, kidney disease, immune system abnormalities, tumor development, uterine hemorrhage during pregnancy and polycystic ovary disease [1, 3, 6, 8]. Alterations in methylation patterns of sperm of F3 generation rats and mice have been reported following exposure of F0 generation females to vinclozolin [1, 3, 4, 62]. Recently, exposures to the plastic compound mixture (bisphenol A and phthalates), dioxin, pesticide mixture (permethrin and DEET) and jet fuel hydrocarbon mixture were found to promote transgenerational disease [34, 63, 64] and unique sperm epigenomes [34]. Other environmental factors such as nutrition [59] also can promote epigenetic transgenerational inheritance of disease phenotypes. Demonstration of epigenetic transgenerational inheritance in worms [65], flies [66], plants [67] and mammals [1, 68–70] suggest this phenomenon will likely be critical in biology and disease etiology in mammals [2]. The current study provides additional support for the ability of an environmental toxicant to induce epigenetic transgenerational inheritance of adult onset disease.

Combined observations demonstrate that exposure of gestating females during the critical development period of gonadal sex determination to the jet fuel hydrocarbon mixture (JP8) promotes epigenetic transgenerational inheritance of adult-onset disease/abnormalities including ovarian disease and obesity. Associated with the occurrence of these transgenerational disease/abnormalities are the epigenetic alterations in the rat sperm DNA. These epimutations may be useful as early stage biomarkers of jet fuel exposure and adult onset disease. These findings have implications for the human and animal populations that are exposed to hydrocarbons such as jet fuel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- First observation that a hydrocarbon mixture (Jet Fuel JP8) promotes epigenetic transgenerational inheritance of disease.
- Ancestral environmental exposures promoted transgenerational sperm epimutations.
- Suggestion ancestral hydrocarbon exposures may be part of the etiology of adult onset disease and obesity.
- Provides additional support for the ability of environmental toxicants to promote epigenetic transgenerational inheritance of disease.

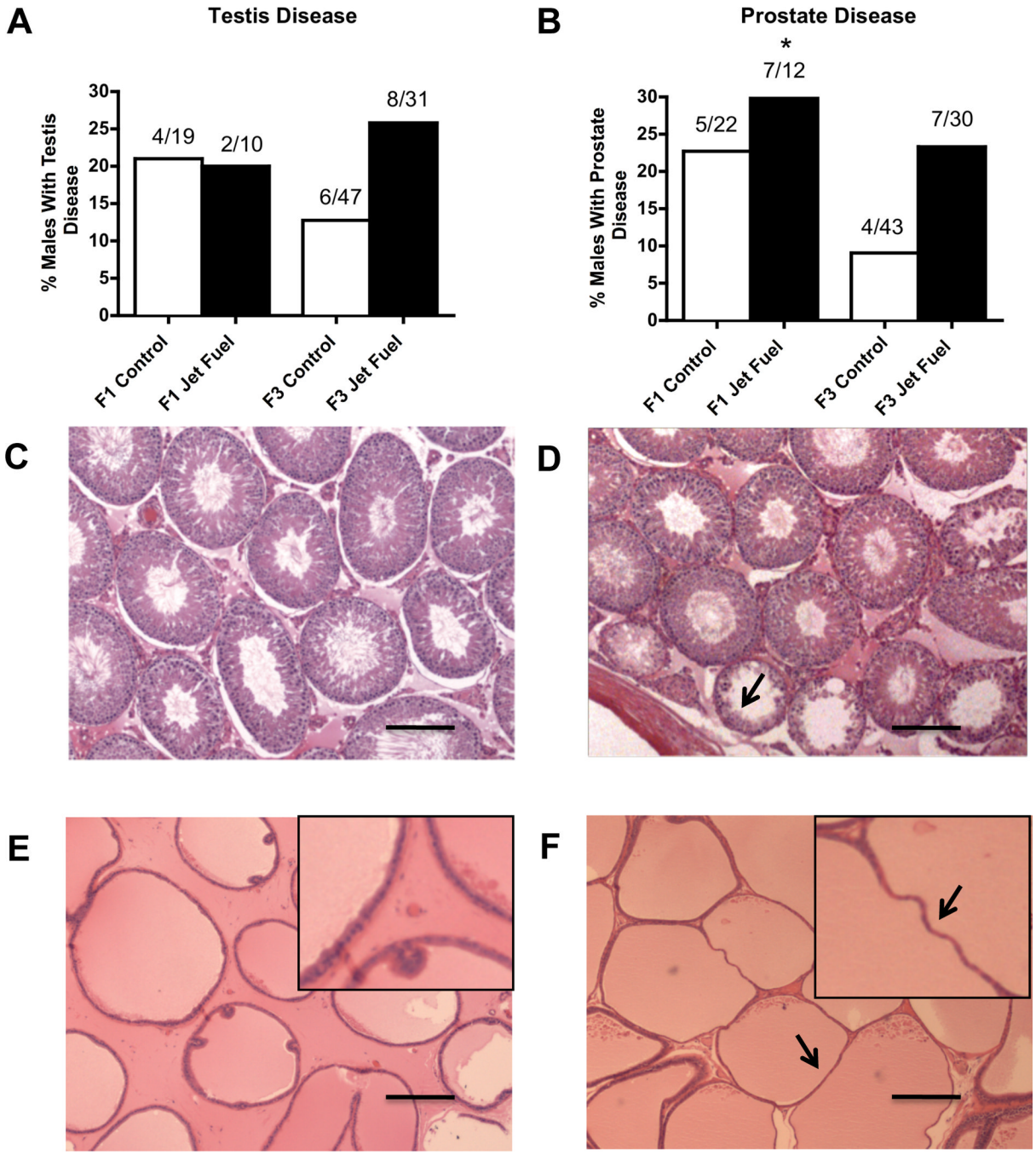

Figure 1.

Ancestral (F0 generation female) exposure to jet fuel (hydrocarbon mixture JP8) and testes or prostate disease in F1 and F3 generation males. Percentages of males with testis (panel A) or prostate (panel B) disease in F1 and F3 generations are presented. The number of diseased rats / total number of rats in each group is shown above the respective bar graphs (* $P<0.05$). Representative micrographs (Scale bar = 200 μm) showing histopathology images of adult-onset transgenerational testis (panel D) and prostate disease (panel F) in jet fuel exposure lineage compared to F3 generation control lineage testis and prostate (panels C and E). Testis sections showed histopathologies including azoospermic and abnormal

seminiferous tubules. Prostate sections (insets) show atrophic ductular epithelium. Arrows indicate the lack of normal spermatogenesis in the testis and prostate epithelial cell atrophy.

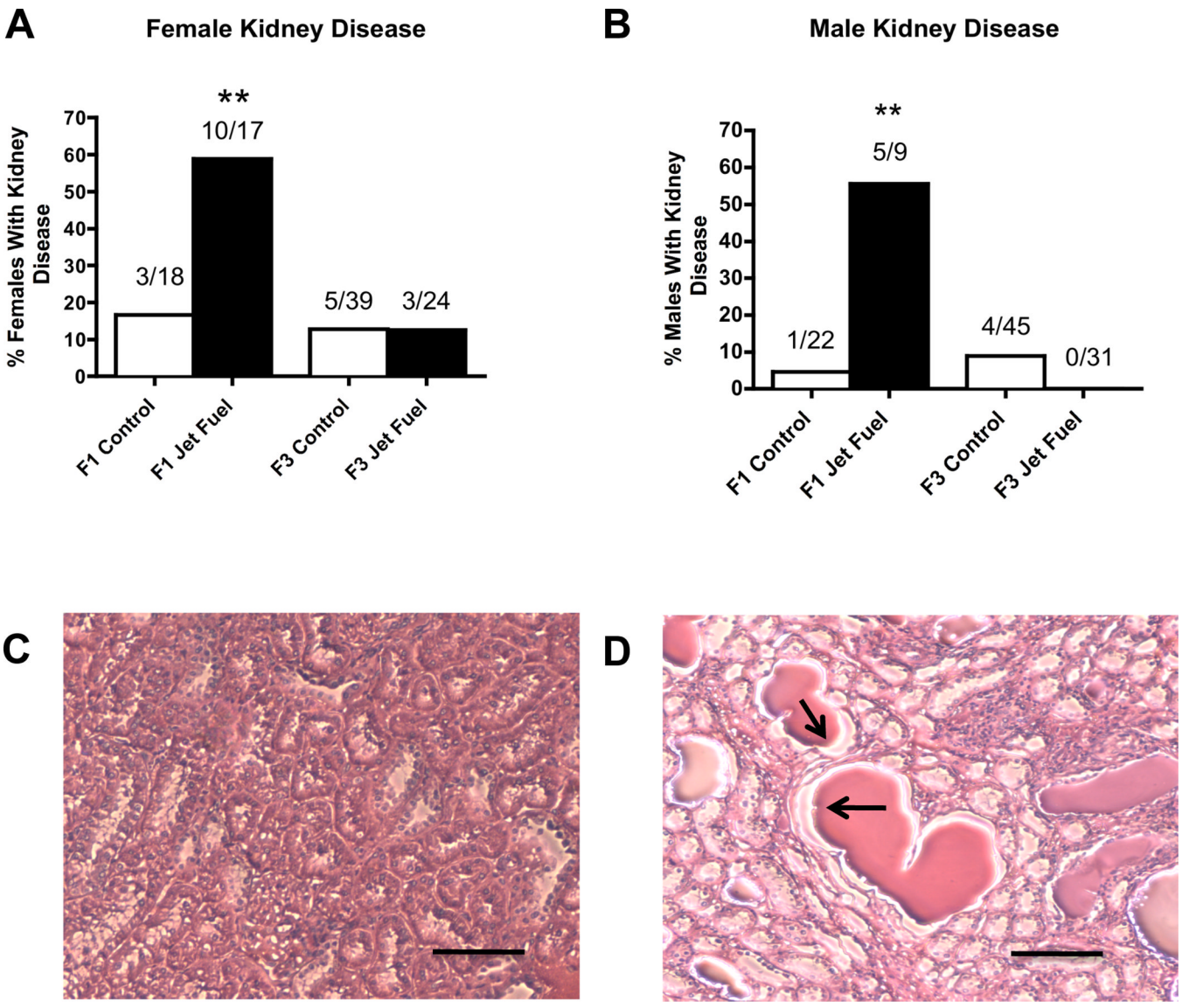

Figure 2.

Ancestral (F0 generation female) exposure to jet fuel (hydrocarbon mixture JP8) and kidney disease in F1 and F3 generation females and males. Percentages of females (panel A) and males (panel B) with kidney disease in F1 and F3 generations are presented. The number of diseased rats / total number of rats in each group is shown above the respective bar graphs (** $P<0.01$). Representative micrographs (Scale bar = 200 μm) showing histopathology images of adult-onset transgenerational kidney disease in jet fuel exposure lineage (panel D) compared to F3 generation control lineage (panel C). Kidney sections showed proteinaceous fluid filled cysts. Arrows indicate cystic structures and abnormal Bowman's capsules.

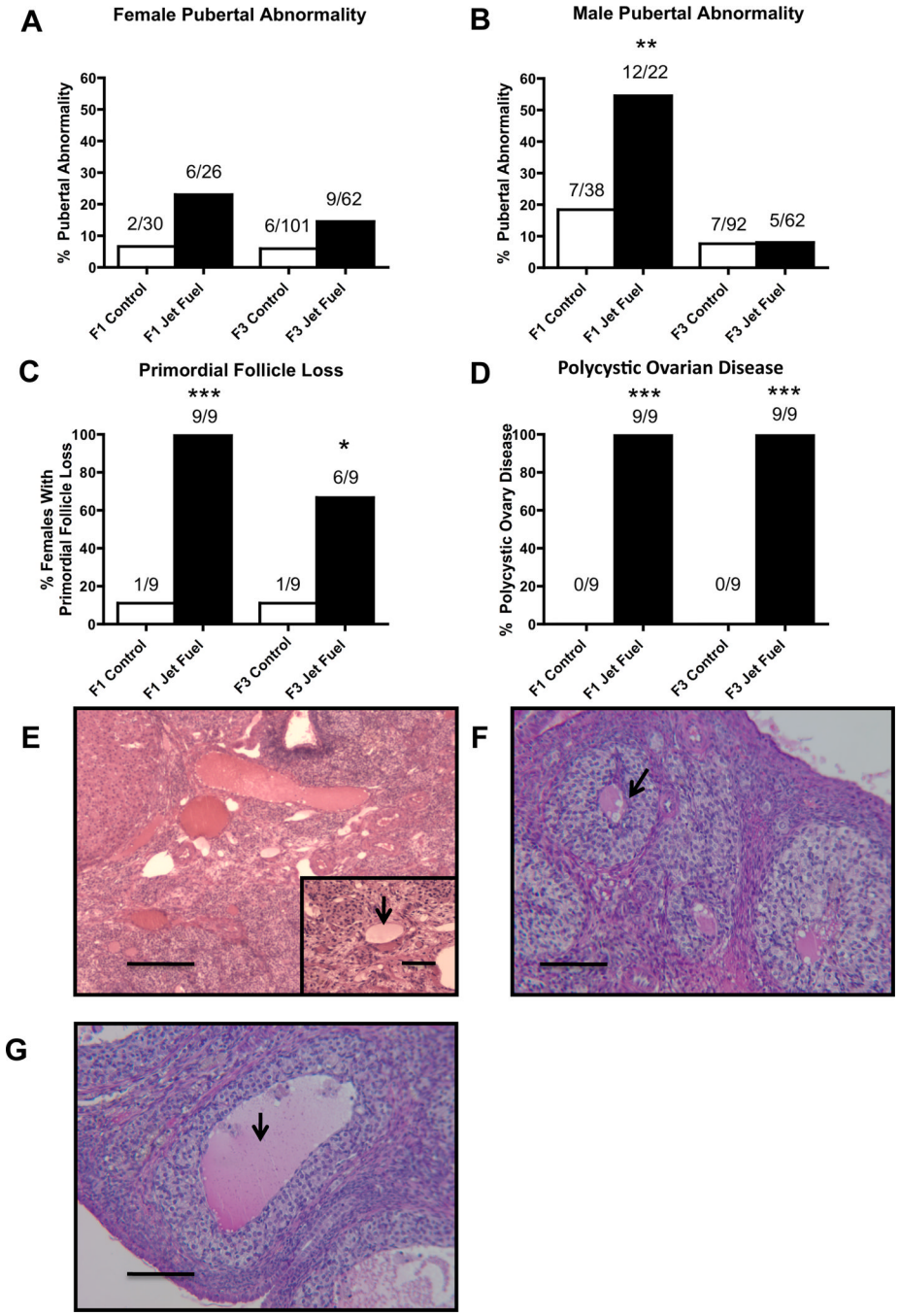

Figure 3.

Ancestral (F0 generation female) exposure to jet fuel (hydrocarbon mixture JP8) and pubertal abnormalities in females and males, and primordial follicle loss and polycystic ovary disease in females. Percentages of females (panel A) and males (panel B) with pubertal abnormality or those with primordial follicle loss (panel C) or polycystic ovary disease (panel D) in F1 and F3 generations are presented. Small follicular cysts (panel E) and luteal cysts (panels F and G) were present in ovaries of jet fuel lineage (Scale bar = 100 μm; 25 μm for inset in panel E). The number of diseased rats / total number of rats in each group is shown above the respective bar graphs (* P<0.05; ** P<0.01; *** P<0.001). Arrows identify ovarian cysts.

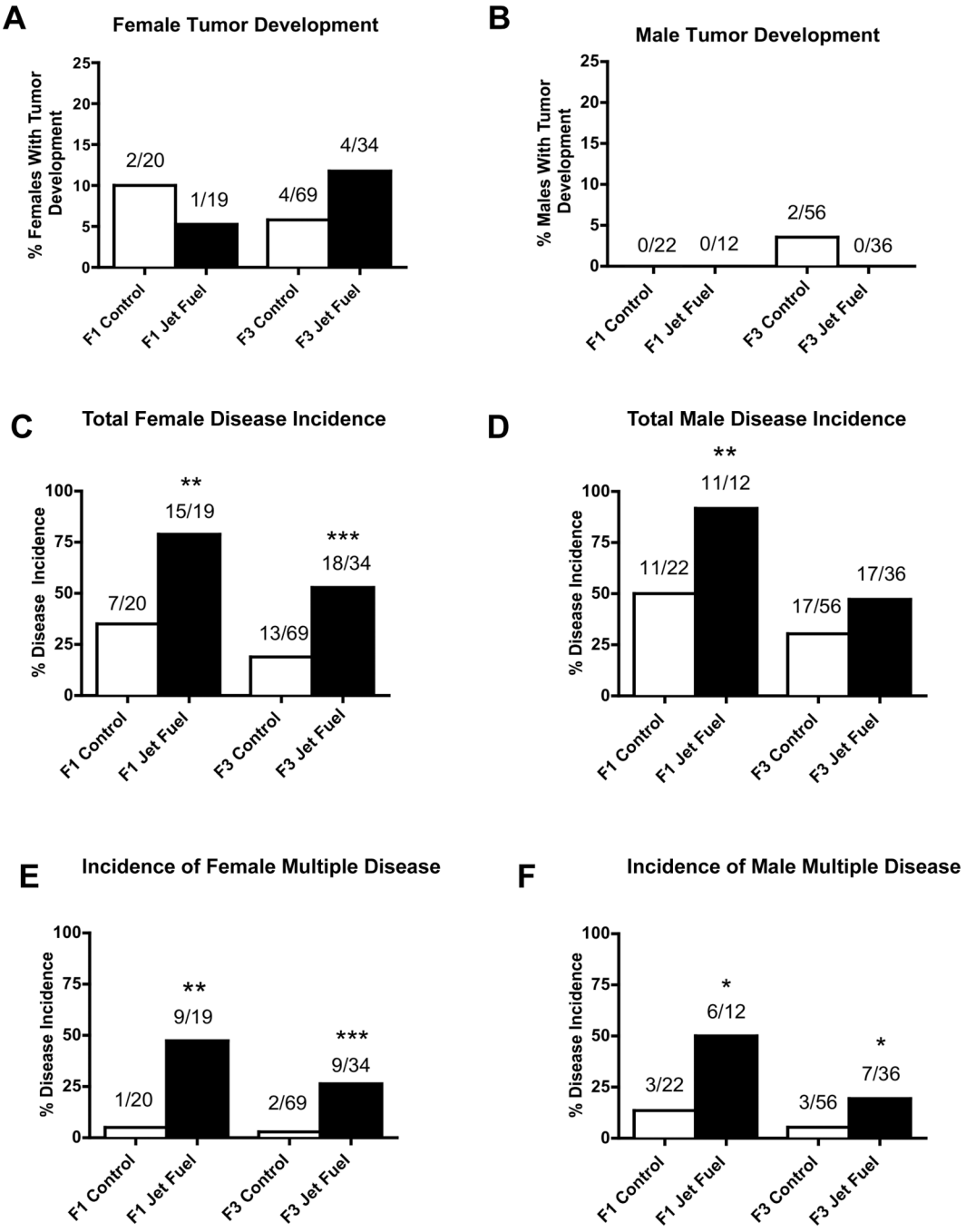

Figure 4.

Ancestral (F0 generation Female) exposure to jet fuel (hydrocarbon mixture JP8) and obesity in females and males. Percentage of females (panel A) and males (panel B) with obesity in F1 and F3 generations are presented. The number of diseased rats / total number of rats in each group is shown above the respective bar graphs (* $P<0.05$; ** $P<0.01$). Abdominal fat deposition in (C) obese F3 generation jet fuel lineage male compared to (D) non-obese F3 generation control lineage male. The pink fat deposition (adiposity) indicated by arrows is observed in panel C on most organs compared to lack of fat deposit in panel D.

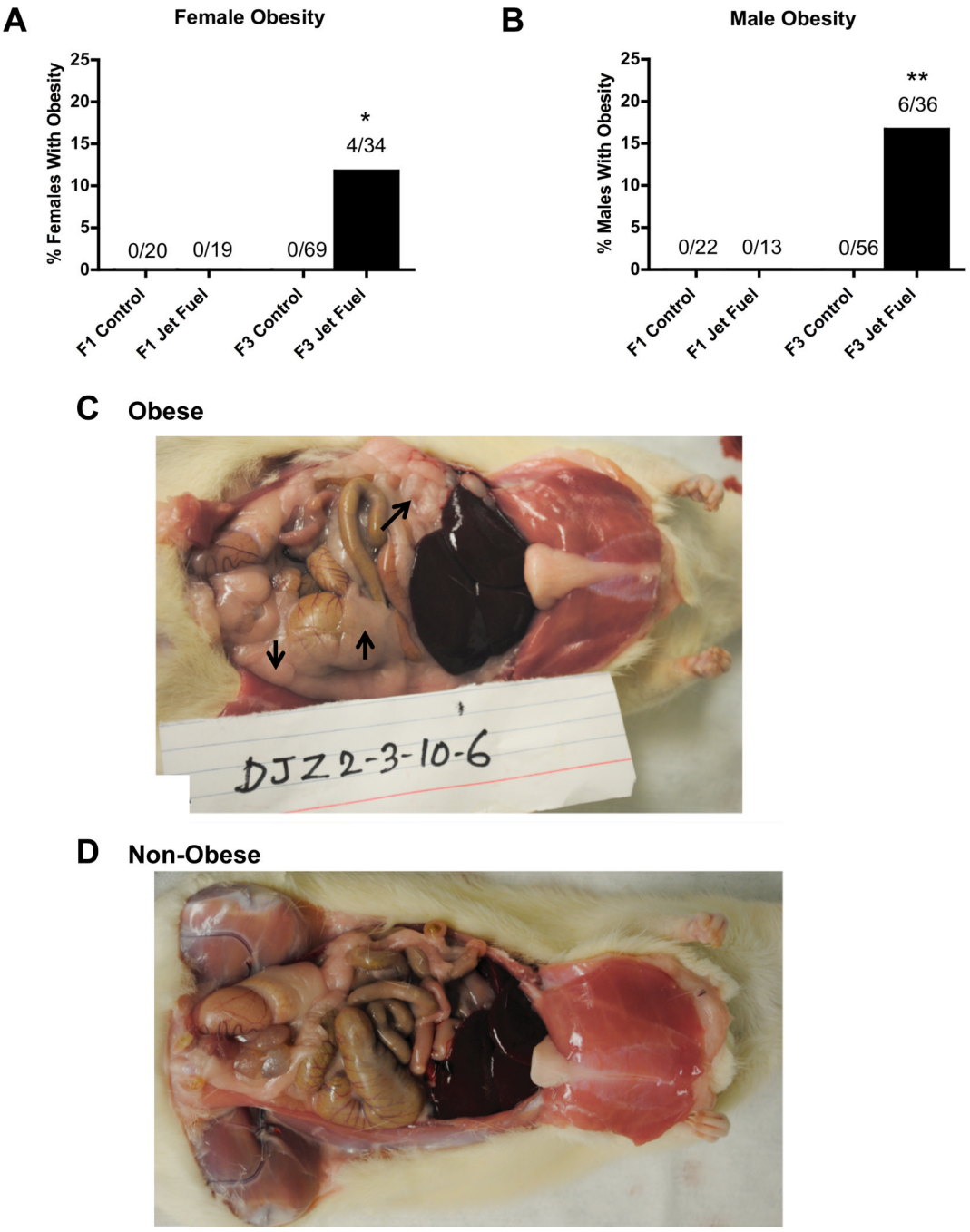

Figure 5.

Ancestral (F0 generation female) exposure to jet fuel (hydrocarbon mixture JP8) and tumor development or disease incidence in rats. Percentages of females (panel A) and males (panel B) with tumor development. Incidences of total disease in females (panel C), total disease in males (panel D), multiple disease in females (panel E) and multiple disease in males (panel F) in F1 and F3 generations are presented. The number of diseased rats / total number of rats in each group is shown above the respective bar graphs (* P<0.05; ** P<0.01; *** P<0.001).

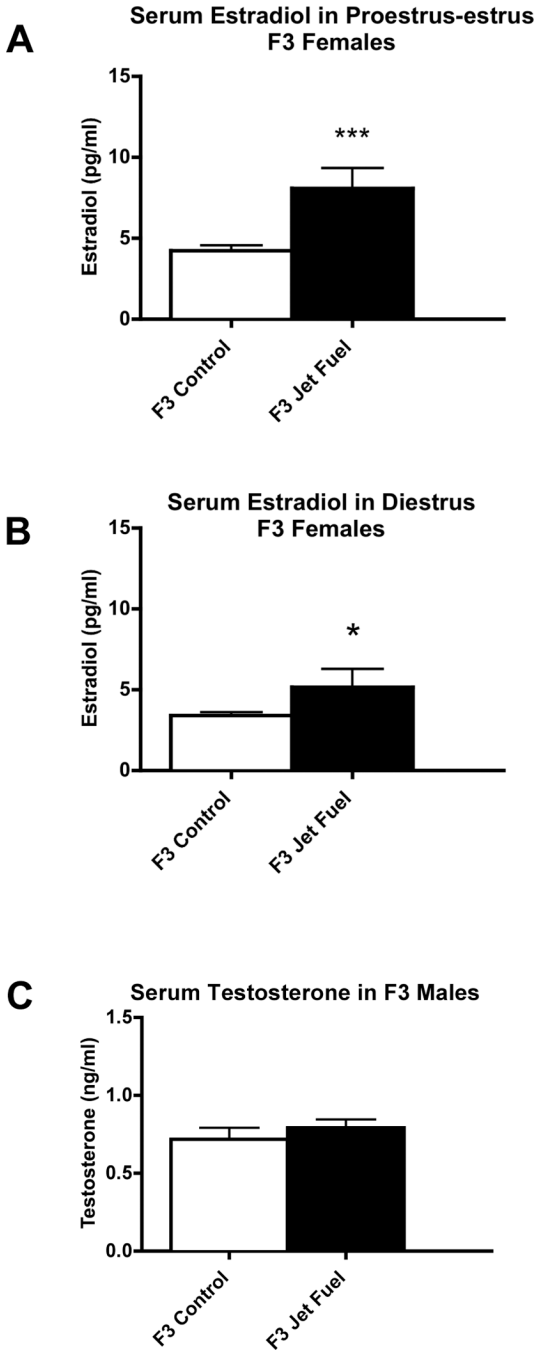

Figure 6.

Serum estradiol concentrations in proestrus-estrus stage in F3 generation control (n=35) and jet fuel (n=21) lineage females (panel A). Serum estradiol concentrations in diestrus stage in F3 generation control (n=36) and jet fuel (n=13) lineage females (panel B). Serum testosterone concentrations in F3 generation control (n=66) and jet fuel (n=40) lineage males (panel C). The mean ± SEM are presented (* $P<0.05$; *** $P<0.001$).

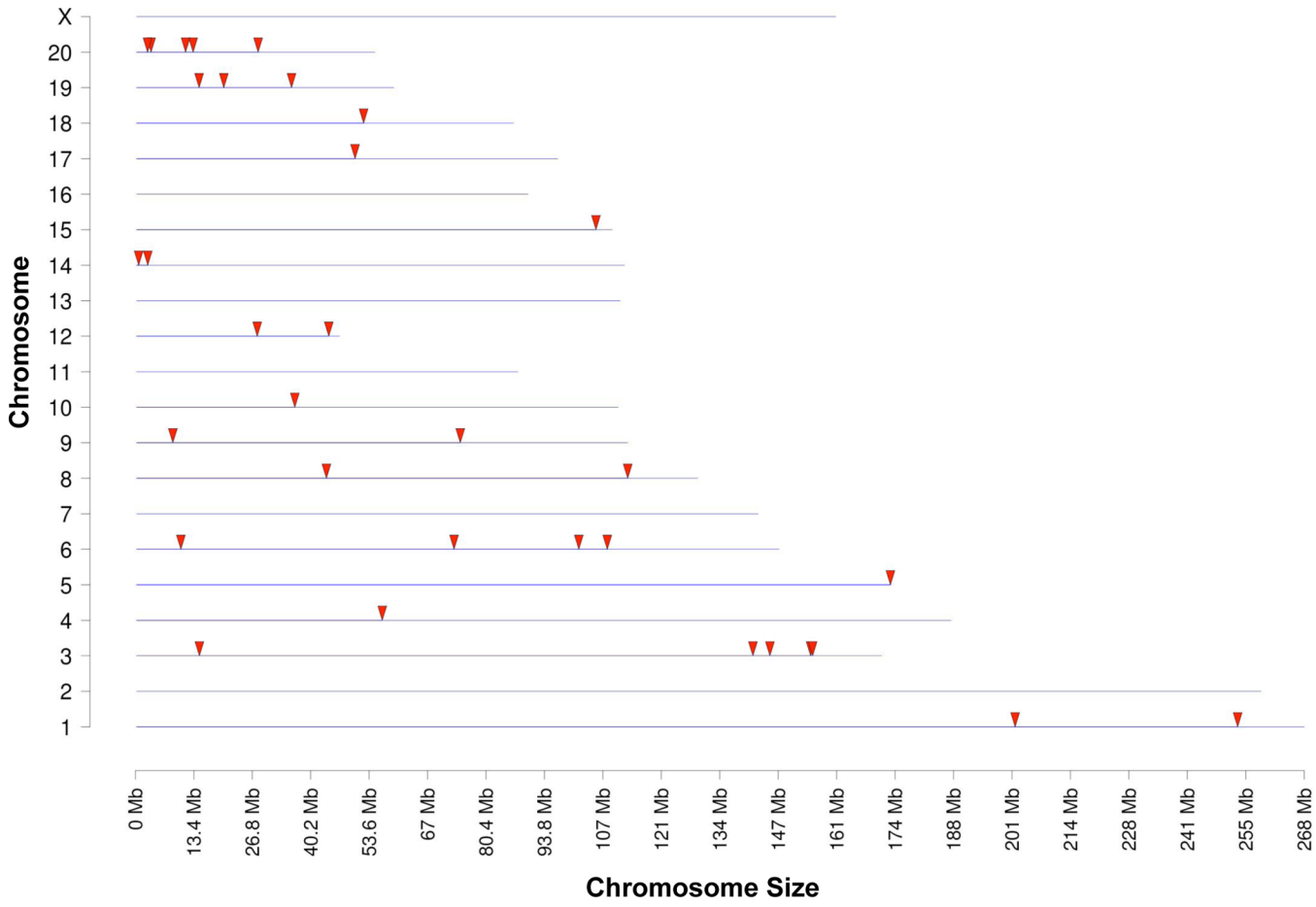

Figure 7.

Chromosome plot (number and size) of differential DNA methylation regions (DMR) in F3 generation jet fuel lineage sperm. Chromosomal locations for DMR are indicated with arrowheads. There were 33 DMR in F3 generation jet fuel lineage sperm DNA compared to control lineage sperm DNA.

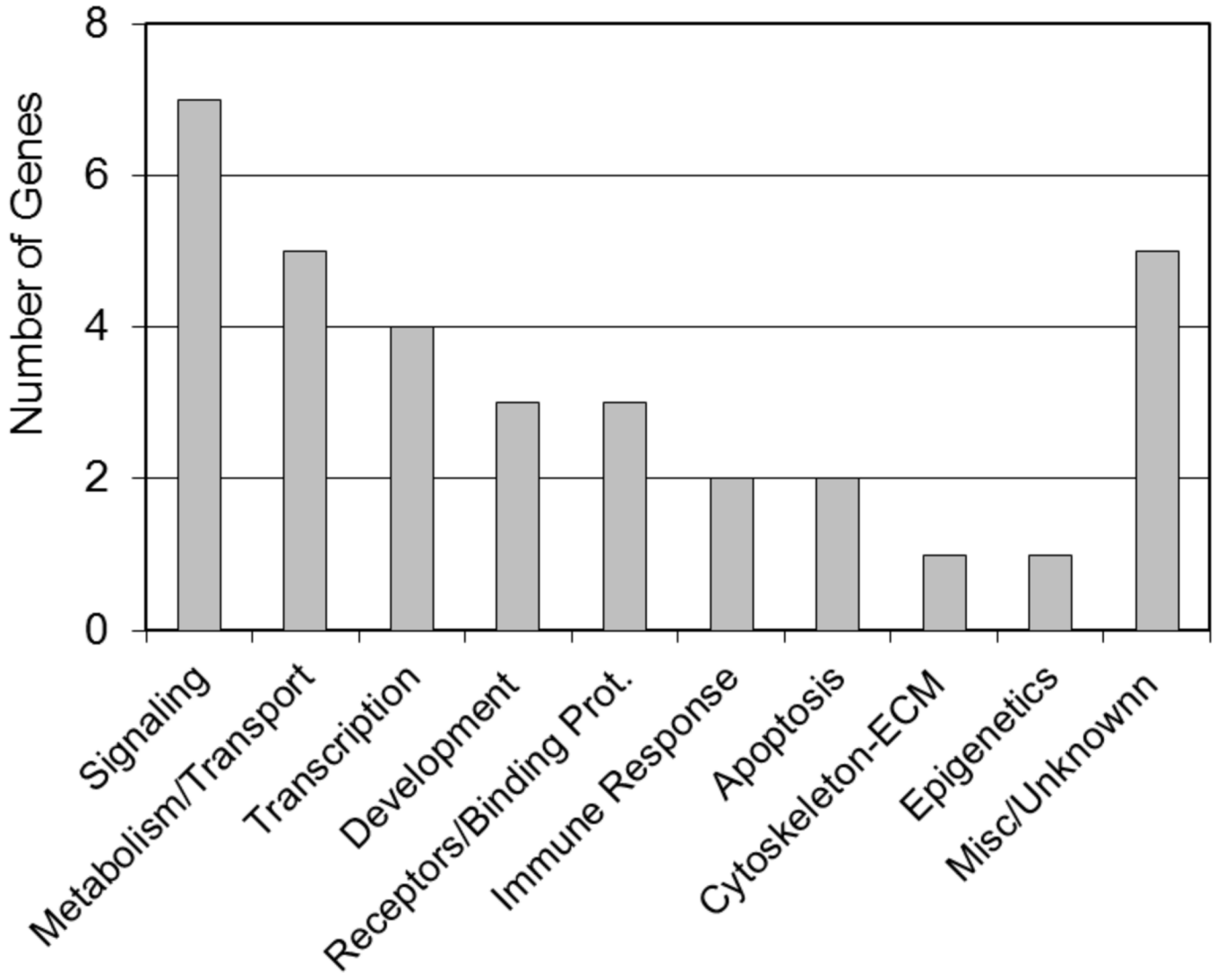

Figure 8.
F3 generation sperm functional gene categories for DMR associated genes. The number of genes per category is indicated.

Table 1

Rat sperm differential DNA methylation regions (DMR) found in F3 generation jet fuel hydrocarbon lineage

Gene Symbol and Category	Chr	Start	Stop	Gene ID	Methylation	min p-value	Gene Title
Apoptosis							
Atg9a	9	74472295	74473090	363254	Down	1.5E-10	ATG9 autophagy related 9 homolog A (S. cerevisiae)
Unc5b	20	28130915	28131715	60630	Down	1.1E-06	unc-5 homolog B (C. elegans)
Cytoskeleton-ECM							
Tspan33	4	56583349	56583949	500065	Up	9.9E-31	tetraspanin 33
Development							
Sh3pxd2a	1	252646829	252647729	309460	Down	9.3E-11	SH3 and PX domains 2A
Svs3	3	155229933	155230533	192239	Up	3.4E-09	seminal vesicle secretion 3
Sema3b	8	112852022	112852622	363142	Down	7.0E-10	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B
Epigenetics							
Hist1h2bn	17	50352153	50352753	291157	Down	9.6E-18	histone cluster 1, H2bn
Immune Response							
RT1-O2-ps	20	2776113	2776813	414983	Up	3.1E-22	RT1 class I, locus O2, pseudogene
RT1-CE2	20	3580516	3581116	414779	Down	4.E-122	RT1 class I, locus CE2
Metabolism & Transport							
Kcnk15	3	154770968	154771568	156873	Down	2.0E-10	potassium channel, subfamily K, member 15
Dnal1	6	108168967	108169567	685664	Up	2.5E-08	dynein, axonemal, light chain 1
Aldh7a1	18	52311273	52311983	291450	Down	2.5E-08	aldehyde dehydrogenase 7 family, member A1
Lcat	19	35787076	35787761	24530	Up	1.4E-16	lecithin cholesterol acyltransferase
Gstt2	20	13218263	13218863	29487	Down	1.7E-09	glutathione S-transferase, theta 2
Receptors & Binding Proteins							
Efcab4a	1	201644972	201645872	309112	Down	1.2E-06	EF-hand calcium binding domain 4A
Olr1404	10	36543212	36544396	405065	Up	5.4E-11	olfactory receptor 1404
Vom2r69	14	740492	741794	289433	Up	2.4E-16	vomeronasal 2 receptor, 69
Signaling							
Asip	3	145443642	145444242	24152	Up	7.2E-17	agouti signaling protein
Dab2ip	3	14667015	14668012	192126	Down	1.8E-10	DAB2 interacting protein
Akap6	6	73042917	73043697	64553	Down	2.E-100	A kinase (PRKA) anchor protein 6

Gene Symbol and Category	Chr	Start	Stop	Gene ID	Methylation	min p-value	Gene Title
Rhoq	6	10413845	10414445	85428	Up	2.6E-11	ras homolog gene family, member Q
Hspa8	8	43783068	43783668	24468	Down	2.3E-09	heat shock protein A8
Sgsm1	12	44329558	44330263	288743	Up	1.2E-16	small G protein signaling modulator 1
Rap2a	15	105569716	105570316	114560	Up	8.0E-16	RAS related protein 2a
Transcription							
Gins1	3	141545679	141546279	499914	Up	1.0E-08	GINS complex subunit 1 (Psf1 homolog)
Klhl17	5	173066362	173067163	246757	Down	4.2E-20	kelch-like 17 (Drosophila)
Foxp4	9	8573569	8574670	363185	Down	8.4E-07	forkhead box P4
Gbas	12	27924888	27925608	498174	Down	1.3E-10	glioblastoma amplified sequence
Miscellaneous & Unknown							
Tmem299b	6	101643488	101644503	503035	Up	4.1E-21	transmembrane protein 189
LOC689986	14	2831796	2832396	689986	Up	3.3E-06	hypothetical protein LOC689986
Tmem188	19	20262077	20262677	291914	Up	9.1E-16	transmembrane protein 299b
LOC679647	19	14597287	14598388	679647	Down	4.8E-18	hypothetical protein LOC679647
RGD1311257	20	11500513	11501113	294333	Down	1.8E-09	similar to C21orf70 protein