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Genomic effects of androstenedione and sex-specific liver cancer susceptibility in mice

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Abstract

Current strategies for predicting carcinogenic mode of action for non-genotoxic chemicals are based on identification of early key events in toxicity pathways. The goal of this study was to evaluate short-term key event indicators resulting from exposure to androstenedione (A4), an androgen receptor agonist and known liver carcinogen in mice. Liver cancer is more prevalent in men compared to women, but androgen-related pathways underlying this sex difference have not been clearly identified. Short-term hepatic effects of A4 were compared to reference agonists of the estrogen receptor (ethinyl estradiol, EE) and glucocorticoid receptor (prednisone, PRED). Male B6C3F1 mice were exposed for 7 or 28 days to A4, EE, or PRED. EE increased and PRED suppressed hepatocyte proliferation, while A4 had no detectable effects. In a microarray analysis, EE and PRED altered >3000 and >670 genes, respectively, in a dose-dependent manner, whereas A4 did not significantly alter any genes. Gene expression was subsequently examined in archival liver samples from male and female B6C3F1 mice exposed to A4 for 90 days. A4 altered more genes in females than males and did not alter expression of genes linked to activation of the mitogenic xenobiotic receptors AhR, CAR, and PPAR α in either sex. A gene expression biomarker was used to show that in female mice, the high dose of A4 activated the growth hormone-regulated transcription factor STAT5b, which controls sexually dimorphic gene

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expression in the liver. These findings suggest that A4 induces subtle age-related effects on STAT5b signaling that may contribute to the higher risk of liver cancer in males compared to females.

Keywords

mode of action; key events; liver carcinogenesis; androstenedione; ethinyl estradiol; prednisone; nuclear receptor; sexual dimorphism; STAT5b; aryl hydrocarbon receptor; constitutive activated receptor; peroxisome proliferator-activated receptor α

INTRODUCTION

Pathway information organized by mode of action (MOA) has played a central role in the assessment of chemical carcinogens for over a decade (Boobis *et al.*, 2006; EPA 2005). In practice, however, use of the MOA framework has been largely retrospective, following identification of a cancer signal in long-term carcinogenicity or epidemiologic studies. An alternative prospective approach to the classical two-year rodent bioassay was proposed by Cohen (2004), in which potential carcinogenicity of a chemical would be based in part on short-term exposures and measurement of cancer risk indicators such as DNA-reactivity and increases in cell proliferation. These evaluations may also include *in vitro* assays, computerized models, and short-term (up to 13 weeks) bioassays as needed. A number of more recent reviews have expanded on opportunities for integrating alternative methods into screening for cancer hazard (Luijten *et al.*, 2016; Simon *et al.*, 2014). Moving forward, a central challenge of toxicological science is to identify shorter-term biomarkers that can be used to predict potential health hazards and inform risk assessment in the absence of traditional toxicity data. This type of approach relies strongly on the mapping of early chemical effects to key events in known biological constructs, called adverse outcome pathways (AOPs) (Villeneuve *et al.*, 2014a; Villeneuve *et al.*, 2014b), which are intended to integrate newer types of mechanistic information into chemical safety assessments and facilitate the transition toward more prospective molecular endpoints.

In the current case study, we examined early markers of steroid effects in the liver mediated by androgen, estrogen, and glucocorticoid receptors. Our primary focus was growth factor pathways mediated by androgen activity. Androgenic agents include natural steroid hormones, hormonal therapies, off-market athletic supplements, and intermediates in the production of various chemical and pharmaceutical products (Dodge and Hoagland, 2011). The primary health effects of androgens are mediated through the androgen receptor (AR), a classical nuclear receptor that regulates transcriptional responses in the liver following ligand binding and transactivation (Davey and Grossman, 2016). An important but poorly understood health concern for androgenic agents is increased risk of liver cancer (Giannitrapani *et al.*, 2006; IARC 2007). Liver cancer is among the leading causes of cancer mortality worldwide, and although a variety of factors contribute to its development, including hepatitis B or hepatitis C virus infections, alcohol consumption, smoking and obesity, the sex differences in disease rate cannot be ignored (Giannitrapani *et al.*, 2006; Zhang *et al.*, 2015). Epidemiologic studies indicate that the incidence of hepatocellular

carcinoma is more common in men compared to women (>2:1) and correlates positively with serum androgens (De Maria *et al.*, 2002; NTP 2010). There is also evidence, albeit limited, for increased risk of liver cancer with use of androgenic steroids, for example, among bodybuilders and patients with anemia (IARC 2007; NTP 2010). While gender differences in liver cancer in humans may be impacted by social or behavioral factors (e.g. differences in alcohol consumption or life style choices that lead to higher incidence of hepatitis infection), these factors are not sufficient to explain the sex differences observed in animal models (Shepard *et al.*, 2005; Wilsnack *et al.*, 2010). In experimental studies, there is a higher background incidence of liver cancer in many strains of male mice compared to female mice; decreased incidence of liver cancer in male mice following castration, knockout of the AR gene, or treatment with an AR antagonist; and increased incidence of liver cancer in ovariectomized female mice treated with AR agonists (e.g., Ma *et al.*, 2014). In addition, the androgenic agents androstenedione (A4) and oxymetholone both resulted in increased incidence of liver cancer in long-term rodent carcinogenicity studies of the National Toxicology Program (NTP) (Blystone *et al.*, 2011; NTP 1999; NTP 2010). A4 increased hepatocellular adenomas or carcinomas in both male and female B6C3F1 mice and preneoplastic hepatocellular foci in male F344/N rats (Blystone *et al.*, 2011; NTP 2010). Based on the 2-year gavage studies, the NTP concluded that there was clear evidence of carcinogenicity for A4 in male and female mice. The carcinogenic effects in mice were observed in the absence of overt cytotoxicity or mutagenicity, suggesting a mitogenic role for AR signaling. Currently, however, there is limited understanding of growth factor pathways induced by AR activity in the liver

Other steroid receptors may also influence proliferative responses in the liver. Estrogen receptor (ER) activity in particular may interact with AR signaling and contribute to sexually dimorphic effects (Giannitrapani *et al.*, 2006; Li *et al.* 2012). The role for ER in hepatocellular carcinogenesis is complex, however. Epidemiologic studies of estrogen-containing hormone therapies in menopausal women have reported lower risk of liver cancer (e.g., Fernandez *et al.*, 2003), while other reports indicate higher liver cancer risk for oral contraceptive use in premenopausal women (Giannitrapani *et al.*, 2006). Experimental evidence on estrogens and liver cancer in rodents is also mixed. Early studies reported promotional effects of estrogens on liver cell growth (dependent on the type of estrogen and study model) (e.g., Reznik-Schuller 1979), while more recent work has pointed to protective effects of estrogen mediated by anti-inflammatory mechanisms (reviewed in Shi *et al.*, 2014). In this study, we evaluated early effects of ethinyl estradiol (EE), which is widely prescribed as part of many oral contraceptive regimens. EE was negative for liver tumors in previous mouse carcinogenicity studies (IARC 2007; Schuppler and Gunzel, 1979) and reduced the incidence (Lee *et al.*, 1989; Standeven *et al.*, 1994) and cell proliferation (Moser *et al.*, 1996) of preneoplastic foci in mice initiated with diethylnitrosamine (DEN), indicating that it is not a liver carcinogen in mice and suggesting that it may have anti-proliferative effects. For this work we predicted that EE would interact with AR-mediated pathways and potentially oppose androgenic effects in the liver.

A third major steroid hormone pathway in the liver is mediated by the glucocorticoid receptor (GR), which has broad roles in energy metabolism, immunity, and cell fate determination (Cain and Cidlowski, 2015). In the liver, there is limited evidence that GR

may have a protective role in cancer risk. First, Dillberger *et al.* (1992) found that prednisone (PRED), a GR agonist, significantly decreased the incidence of hepatocellular tumors in male and female mice. Second, combined deletion of mouse hepatic GR and the transcription factor STAT5, which controls growth hormone-regulated gene expression, increased incidence of hepatocellular carcinomas (Mueller *et al.*, 2011). Third, a recent study by Matthews *et al.* (2015) found that an intact GR ligand-binding domain was necessary for mitotic progression, that GR heterozygous mice compared to wild-type mice had an increase in liver tumors, and that GR gene expression was reduced in a panel of human tumors, including those from the liver. Collectively, these findings suggest that expression and activation of GR may have protective effects on liver cancer incidence.

In the current study, we evaluated short-term effects of steroidal agents in the liver and investigated pathways by which A4 may induce liver cancer. We compared A4 effects to those induced by the reference agonists for ER (EE) and GR (PRED). Endpoints included liver histopathology, cell proliferation, markers of xenobiotic receptor activation, and transcriptomic profiles. Prior studies of A4 have shown a lack of mutagenicity in genotoxicity assays, and inconsistent evidence of liver cytotoxicity at carcinogenic doses (NTP 2010). Based on this evidence, we hypothesized that A4 would induce a direct mitogenic effect and a male-specific transcriptomic profile.

MATERIALS AND METHODS

Chemicals.

Androstenedione (A4) (Steraloids, Newport, RI; 98% purity, lot number B1141) was suspended in 0.5% aqueous methylcellulose (Sigma-Aldrich, St. Louis, MO) and administered by gavage. Ethinyl estradiol (EE) (Sigma-Aldrich, >98% purity, lot number SLBF2546V) and prednisone (PRED) (Sigma-Aldrich, >98% purity, lot number BCBK19224V) were added to AIN93G rodent diet (TestDiet, Richmond, IN). Control AIN93G diet was processed in the same way as diets with chemicals added. Phenobarbital (PB) (Sigma-Aldrich, 99.8% purity, lot number SLBF7347V) was administered in the drinking water as a non-steroidal mitogenic positive control.

A4 purity determination.

The purity of an A4 solution prepared from the Steraloids standard was confirmed to be at least 98.9% by comparison of the HPLC/UV peak area obtained on an Agilent 1200 Series HPLC (Santa Clara, CA) to the peak area from an A4-certified reference standard (Cerilliant, Round Rock, TX, 99.8% purity, lot number FE08201302) at the same concentration. HPLC method parameters are provided in Supplemental Table 1.

Study design.

The study design is analogous to that used by Lake *et al.* (2016). Weanling B6C3F1 male mice were acquired at ~21 days of age due to the difficulty of obtaining 60-day old age-matched mice. Mice were obtained from Charles River Laboratories (Raleigh, NC) and were maintained according to the guidelines of the National Research Council Guide for the Care and Use of Laboratory Animals (NRC, 2011) at the U.S. EPA AAALAC-accredited animal

facility (Research Triangle Park, NC). Mice were group-housed to fulfill social housing recommendations from the NRC guideline (NRC, 2011), AAALAS, and our Institutional Animal Care and Use Committee. All procedures involving animals were conducted under a protocol approved by the Institutional Animal Care and Use Committee of the U.S. EPA National Health and Environmental Effects Research Laboratory (NHEERL). Upon receipt, mice were randomly placed (4 per cage) in solid-bottom cages on Alpha-dri® bedding (Sheppard Specialty Papers) with nestlets (Anacare) and nylabones for enrichment. Mice were allowed *ad libitum* access to feed and water and housed under standard conditions (21 ± 1°C room temperature, 50 ± 10% humidity, and 12 hr on/12 hr off light cycles). AIN93G was used as the control and base diets for incorporation of chemicals administered in the feed, because it is free of soybean meal and soy protein constituents and thus has low to no phytoestrogens (Thigpen *et al.*, 1999; Thigpen *et al.*, 2013).

Body weight and consumption of feed were monitored during acclimation and treatment periods. At the time of experimentation, cages were randomly assigned to treatment groups and total body weight per cage was analyzed to ensure treatment groups did not differ significantly at the start of dosing. Mice were aged to approximately 60 days before the experiment began.

Mice were exposed for 7 days to A4 administered in 0.5% aqueous methylcellulose by gavage (10 ml/kg) at the following dose levels (0, 2, 10, 20, 50 mg/kg/day) (n=16/dose group). Control mice (0 mg A4/kg/day) received the appropriate amount of gavage vehicle. Other groups received either control diet or diets containing EE (1, 2.5, 5, 10 ppm) or PRED (1.25, 2.5, 5, 25 ppm) at one of four doses (n=16/dose group). The levels in the diet correspond to nominal intake levels of 0.2, 0.5, 1, and 2 mg/kg/day for EE and 0.25, 0.5, 1, and 5 mg/kg/day for PRED, respectively (Dillberger *et al.*, 1992; Tyl *et al.*, 2008). Dose ranges were established based on previous rodent carcinogenicity studies, which showed a significant increase in liver tumor incidence for A4 but not EE or PRED (Dillberger *et al.*, 1992; IARC 2007; NTP 2010). Additional dose groups of mice (n=16/group) were exposed for 7 days to PB in the drinking water (with AIN93G diet control feed) at 600 ppm as a reference non-steroid positive mitogenic control group. Separate groups of mice were treated for 28 days. These mice were (1) fed either a control diet or diets containing high doses of EE (10 ppm) or PRED (25 ppm), (2) administered A4 (50 mg/kg/day in methylcellulose) or methylcellulose, or (3) maintained on PB in the drinking water at 600 ppm (n=16–24/group).

Sample sizes varied depending on the assay measured and sample availability. Group sample sizes for body and liver weights and clinical chemistry were n=10–16/group. Subsets of liver samples were analyzed for liver cell proliferation (n=8–10/group), histopathology (n=6–14/group), global gene expression (n=4/group), targeted gene expression (n=6–8/group), and hepatic cytochrome P450 (CYP) enzyme activity (n=5–8/group).

Tissue collection.

Necropsies were carried out on unfasted mice in 4 blocks at 7 days of exposure and 2 blocks at 28 days following euthanasia by carbon dioxide. Mice were randomly selected for termination, with necropsies starting at ~9:30 a.m. and ending no later than ~1:30 p.m. Blood was collected by cardiac puncture, and serum was harvested from the blood and

frozen at -80°C . After weighing the livers, the left lateral, caudate, and right medial liver lobes were trimmed, and portions of each lobe were either snap-frozen in liquid nitrogen or fixed in fresh 4% paraformaldehyde. After 18–24 hrs, fixed samples were transferred to 70% ethanol and used for histopathology and immunohistochemistry (IHC). Frozen samples were stored at -80°C and used for gene expression and enzyme activity assays.

Hepatic cytochrome P450 enzyme activity.

Frozen liver samples were thawed on ice and homogenized in buffer. Microsomes were isolated for enzyme activity analysis according to Guengerich *et al.* (1982). Centrifugation steps were conducted at 4°C and total protein was quantitated with bovine serum albumin (BSA) standards. Enzyme activity was measured, without reference to treatment group identification, using O-deethylation of ethoxyresorufin (EROD) for CYP1A, pentoxyresorufin O-depentylation (PROD) for CYP2B, and benzyloxyresorufin O-debenzylation (BROD) for CYP3A. A Spectramax Gemini SX plate reader (Molecular Devices, Sunnyvale, CA) was used to measure the fluorescence activity of the enzyme reaction. The excitation and emission wavelengths were set at 544 and 590 nm, respectively. Stock solutions of substrates were prepared in methanol at $420\ \mu\text{M}$ benzyloxyresorufin and ethoxyresorufin and $4200\ \mu\text{M}$ for pentoxyresorufin. A stock solution of 1 mM resorufin, the enzymatic product, was also prepared in methanol. Stock solutions were stored at -20°C . The reaction mixture consisted of 0.05 M Tris buffer, pH 8, 0.05–0.1 mg microsomal protein, and either $4.5\ \mu\text{M}$ ethoxyresorufin, $4.5\ \mu\text{M}$ benzyloxyresorufin, or $0.9\ \mu\text{M}$ pentoxyresorufin. Samples were run in triplicate in an opaque 96-well plate. The sample plate was pre-incubated in the plate reader at 37°C for 3–5 min. The reaction was initiated by the addition of 25 μl of stock NADPH (100 mM NADPH was prepared in 0.05 M Tris buffer, pH 8). The total volume of incubate was 235 μl . The production of resorufin was monitored for 5 min at 37°C . Data were collected at 36 sec intervals. Because of the large number of samples, analysis was conducted over 3 days (7-day Blocks 1 and 2; 7-day Blocks 3 and 4; 28-day Blocks 1 and 2). A standard curve of 0 – 50 pmol resorufin was developed for each analysis. The standard curve was forced through zero. The r^2 for the standard curve was: 7-day Blocks 1 and 2, 0.9986; 7-day Blocks 3 and 4, 0.9994; 28-day Blocks 1 and 2, 0.9993. Results were calculated as pmol of resorufin formed per mg of protein per min. A Bio-Rad (Richmond, CA) protein assay kit with BSA as the protein standard was used to measure the concentration of microsomal protein.

Serum liver enzyme chemistry

Alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) activity levels were measured blindly as serum indicators of liver cytotoxicity using automated procedures in the NHEERL Clinical Chemistry Research Core Laboratory on a Konelab Arena 30 Clinical Chemistry Analyzer (ThermoFisher Scientific, Waltham, MA).

Liver histopathology.

Fixed liver samples were embedded in paraffin, sectioned at 6 μm , and stained with hematoxylin and eosin (H&E) using standard histologic procedures. Liver sections were then evaluated via light microscopy by a board-certified study pathologist at Experimental Pathology Laboratories, Inc. (EPL) (Research Triangle Park, NC). Histopathological review

was performed according to best practices for nonclinical safety studies, as described by Crissman *et al.* (2004). Histopathological diagnoses were based on standard criteria and nomenclature (Thoolen *et al.*, 2010). Severity of non-neoplastic lesions was qualitatively scored using a 0–5 scale (0=absent, 1=minimal, 2=mild, 3=moderate, 4=moderately severe, 5=severe). Severity index was calculated as the average severity score per group for a given change. All pathology data, tabulations, and observations were recorded under GLP-compliant conditions.

Ki67 IHC and labeling indices.

Liver cell proliferation was evaluated by IHC staining for the Ki67 antigen (Wood *et al.*, 2015). The labeling procedure included a rabbit monoclonal primary antibody for Ki67 (Clone SP6, ThermoFisher Scientific), antigen retrieval with citrate buffer, biotinylated goat anti-rabbit secondary antibody as a linking reagent (BA-6100, Vector Laboratories, Burlingame, CA), peroxidase-conjugated avidin as the label (ABC Complex Vector Kit, Vector Laboratories), and 3,3'-diaminobenzidine (DAB) as the chromogen (Biocare Medical, Concord, CA) (Lake *et al.*, 2016). Anti-Ki67 antibodies were diluted 1:500 with diluent buffer (S0809, Dako, Carpinteria, CA), and immunostained slides were counterstained with hematoxylin (QS, Vector Laboratories). Negative and positive control slides were run with each IHC batch. Negative staining slides used the same protocol as for study slides except with Tris-buffered saline in place of the primary antibody; positive control slides used mouse intestine tissues in place of liver.

Nuclear labeling for Ki67 was quantified by a computer-assisted counting technique using an automated procedure of cell selection (Lake *et al.*, 2016). At least three fields per liver (1 per lobe) were digitally imaged at 20× objective magnification using a Nikon DS-Fi2 color camera and Nikon Ti inverted microscope (Nikon Instruments, Inc.). Following image capture, nuclei were counted using NIS Elements software (v4.13), and counting was performed in a blinded manner. Binary layers generated from thresholding individual color components were used to identify liver cell nuclei based on size, shape, and intensity criteria. Customized macros were used to sort cells into four categories based on label (positive or negative) and size/shape (large round nuclei indicating mature hepatocytes or “other” smaller cells). At least 1000 cells were counted per liver, and counts were analyzed as a percentage of the total number of cells examined to generate a labeling index (LI) for each sample.

Gene expression microarrays.

Global gene expression was evaluated by microarray analysis. Total RNA was isolated from ~20 mg of frozen liver tissue homogenized in RNazol®RT (Molecular Research Center, Cincinnati, OH), purified with the RNeasy MinElute column protocol (Qiagen GmbH, Hilden, Germany), evaluated for integrity using an Agilent RNA 6000 Nano chip on an Agilent 2100 Bioanalyzer (Agilent Technologies GmbH, Berlin, Germany), and quantitated using the NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). RNA samples were isolated and purified at Expression Analysis Genomic Services, Q2 Solutions (Durham, NC). After initial randomization, samples were hybridized in a blinded manner onto Mouse Ref-8 V2.0 Expression BeadChip arrays (Illumina, San Diego, CA) in the

NHEERL Genomics Research Core Laboratory using standard Illumina protocols. Arrays were scanned, and raw data (.idat files) were obtained using Illumina iScan software (v3.3.28) and analyzed using Illumina GenomeStudio® Data Analysis Software. Array data are publicly available at Gene Expression Omnibus (GEO), accession number GSE84590.

Targeted gene expression.

Relative expression of 11 gene targets indicative of sex-specific expression (*Ahnak*, *Cyp2c29*, *Elov13*, *Hsd3b5*, *Mup4*, *Mup5*, *Nat8*, *Prkaa2*, *Scara5*, *Stbd1*, and *Susd4*) were measured using reverse transcriptase quantitative PCR (qPCR). Genes were derived from the STAT5b biomarker (Oshida *et al.*, 2016a) and from the literature (Clodfelter *et al.*, 2006). RNA was isolated as described for the array analysis with additional steps using TurboDNase treatment (ThermoFisher Scientific,) and Qiagen MinElute column purification before cDNA preparation. Total RNA was quantitated using the Qubit RNA assay (ThermoFisher Scientific). The cDNA was prepared using the BioRad iScript™ cDNA synthesis kit (BioRad, Hercules, CA). qPCR was carried out using BioRad PrimePCR SYBR assays. Three reference genes (*Hprt*, *Ppia*, and *Tbp*) and PCR quality controls were also assayed for each sample. Samples were loaded in technical duplicate for each gene target using the Qiagen QiAgility robotic workstation and measured on a BioRad CFX384 real-time qPCR machine using manufacturer's recommendations for PrimePCR assays. Melt-curve analysis was used to verify single products for each amplified target. During analysis, cycle threshold (Ct) values were determined using the BioRad CFX Manager software (v3.0.12224.1015). Relative quantification of gene expression to controls, normalized to reference gene expression, was assessed using the 2^{-Ct} method (Livak and Schmittgen, 2001). Statistical significance of expression values for 7-day control *vs.* treatment samples were determined by one-way ANOVA and Tukey-Kramer post hoc analysis with multiple test correction. Significantly different expression in 28-day control *vs.* treatment samples were determined by a two-sided, non-parametric Mann Whitney test using qBase+ software (Biogazelle, Zwijnaarde, Belgium).

TempO-Seq analysis of archival liver samples.

Changes in gene expression patterns were analyzed using targeted RNA-sequencing (TempO-Seq, BioSpyder Technologies, Inc., Carlsbad, CA) of formalin-fixed paraffin-embedded (FFPE) liver samples from study TR-560 of the NTP (<http://ntp.niehs.nih.gov/results/pubs/longterm/reports/longterm/tr500580/listedreports/tr560/index.html>), in which male and female B6C3F1 mice were treated by gavage with 0, 10, 20, and 50 mg/kg of A4 for 90 days. A total of 32 samples were analyzed, 16 each from males and females (n=4/dose group). The TempO-Seq platform measures a predefined set of 3099 mouse genes that represent a surrogate whole transcriptome assay called the "S1500" (<https://federalregister.gov/a/2015-08529>). Two sections 10 µm in thickness were collected from each tissue block, placed in a microfuge tube, and processed according to BioSpyder recommendations. Briefly, 76 µL of lysis buffer was added to each tube and overlaid with 150 µL mineral oil. Samples were heated to 75°C until all paraffin had melted (~30 min), and the paraffin separated into the mineral oil was removed. Proteinase-K was added to the remaining tissue/lysis buffer at a final concentration of 1 µg/mL, incubated for 1 hr at 37°C, and inactivated at 95°C for 15 min. Any remaining tissue was pelleted by centrifugation at

13,000 × g for 15 min. To hybridize detector oligonucleotides to mRNA targets, 2 µL of lysate was transferred to a well in a 96-well plate and combined with 2 µL of annealing mix (1× annealing buffer and 1 nM detector oligonucleotide pool). Samples were incubated at 75°C for 10 min, then ramped down to 45°C at a rate of 0.5°C per min, and held overnight at 45°C. Excess oligonucleotides were digested by adding 24 µL of nuclease mix (1× nuclease buffer and 3'exo-nuclease enzyme) to each sample and incubated at 37°C for 1.5 hr. Detector oligonucleotides were ligated to generate a pool of amplification templates that share PCR primer annealing sites by adding 24 µL of ligation mix (1× ligation buffer and ligase) to each sample and incubating at 37°C for 1 hr, followed by 80°C for 30 min to inactivate ligase. Ten µL of each sample was amplified in a pre-made qPCR plate as follows: 37°C for 10 min, 95°C for 2 min, 6 cycles of 95°C for 30 sec, 54°C for 30 sec, 72°C for 2 min, 16 cycles of 95°C for 30 sec, 66°C for 30 sec, 72°C for 2 min, and a final step of 72°C for 2 min. During product amplification, each sample well was assigned a specific barcoded primer pair, allowing for sample identification following sequencing. Sample amplicons were pooled and purified using a PCR clean-up kit (Clontech, Mountain View, CA). Libraries were sequenced at MOGene, LC (St. Louis, MO) using a NextSeq Mid-Output Sequencing System (Illumina, San Diego, CA).

Statistical analyses.

Total body weights by cage were analyzed by analysis of variance (ANOVA) using the SAS GLM procedure (SAS v9.4) prior to the start of dosing. Terminal measurements for body weight, relative liver weight, clinical chemistries (ALT and SDH), body weight change, and enzymatic activity data were analyzed as dependent variables in 7- and 28-day two-way ANOVA models using the SAS MIXED procedure. To determine the scale of analysis for each variable, model residuals for both linear and log₁₀-scaled dependent variables were evaluated for normality (Shapiro-Wilk test in SAS UNIVARIATE) and homogeneity of variance (Levene's test in SAS GLM). Separate two-way main-effects ANOVA with factors for block and dose group were performed for each dependent variable for each of the four chemicals for each dosing duration time. A Bonferroni multiple-comparison adjustment was used for the ANOVA F-tests for the EE, PRED, and PB treatments which shared control animals but not for the A4 study which had its own control animals. Where an F-test was declared significant, Dunnett's pairwise tests for differences in least-squares mean between each dose group and the corresponding control group are also reported using a statistical significance threshold of $\alpha=0.05$. The P450 activity values from one mouse treated for 7 days with prednisone at 1.25 ppm were dropped from the analysis because it was an outlier.

Histopathology incidence data were analyzed by comparing results for each treatment group to the respective control group at 7 or 28 days using a one-sided Fisher's exact test. Pairwise p-values for all incidence data were adjusted using a Bonferroni multiple test correction for the number of direct comparisons within each treatment arm. An adjusted α level of 0.05 was used to determine a significant difference between groups. A Cochran-Armitage trend test with Bonferroni adjustment was used to evaluate dose response trends for incidence of histopathology outcomes. Severity scores of non-neoplastic histopathology outcomes are presented only as descriptive data.

For microarray data, raw gene expression intensities were quantile-normalized using Illumina GenomeStudio®. Principal Component Analysis (PCA) was performed to examine within and across group profiles in Partek Genomics Suite. ANOVA analysis was also performed in Partek Genomics Suite, with false discovery rate testing (Benjamini–Hochberg test) and a $p < 0.05$ for significance. For TempO-Seq data, sequencing readouts were demultiplexed to generate FASTQ files. Genes that were differentially expressed between each A4 dose group and control, and between the male and female control groups were identified with a Wald test followed by a Benjamini-Hochberg multiple test correction (false discovery rate q -value < 0.1) in the DESeq2 R package (Love *et al.*, 2014). Heat maps were generated using Eisen Lab Treeview software (<http://rana.lbl.gov/EisenSoftware.htm>). Pathway analysis was performed on differentially expressed genes in Ingenuity Pathway Analysis (Qiagen Bioinformatics, Redwood City, CA).

Gene expression correlation analysis.

Lists of statistically significant, differentially expressed genes (DEGs) were compared to each other using tools in a commercially available database provided by Illumina BaseSpace Correlation Engine, also called NextBio (www.nextbio.com). This database contains over 128,000 lists of statistically filtered genes from over 20,200 microarray studies carried out in 16 species (as of June, 2016). The normalized ranking approach used by NextBio enables comparability across data from different studies, microarray platforms, and analysis methods by removing dependence on absolute values of fold-change, and minimizing some of the effects of the normalization methods used, while accounting for the level of genomic coverage by the different platforms. The Running Fisher algorithm using a Fisher's exact test generates a p -value and correlation direction (positive or negative) for each pairwise comparison. A description of criteria used to identify datasets in GEO suitable for analysis and details of the microarray processing steps in the NextBio pipeline are summarized in Oshida *et al.* (2015c) and are discussed in greater detail in Kupershmidt *et al.* (2010). All statistically filtered gene lists generated as part of this study were uploaded into NextBio after filtering for $|\text{fold-change}| \geq 1.2$. We have previously used the database and computational tools to accurately identify factors (e.g., chemicals, genes, diets) that activate or suppress other transcription factors important in chemical-induced liver carcinogenesis, such as aryl hydrocarbon receptor (AhR), constitutive activated receptor (CAR), peroxisome proliferator-activated receptor (PPAR) α , and STAT5b (Oshida *et al.*, 2015a-c, 2016a,b).

RESULTS

Liver weight and serum markers.

Treatment with A4 did not significantly alter terminal body weight, the ratio of liver weight (LW) to body weight (BW), or serum activity of ALT or SDH at 7 or 28 days (Table 1, Supplemental Table 2). EE resulted in lower terminal body weight at 7 and 28 days and higher LW/BW and ALT at 28 days suggestive of an effect on the liver. PRED resulted in decreased body weight gain at the two highest doses at 7 days and decreased body weight gain, increased relative LW/BW, and increased ALT at 28 days. PB resulted in higher LW/BW at 7 and 28 days and higher ALT and SDH at 28 days. As these modest increases in serum ALT and/or SDH were observed in the absence of cytotoxicity (based on

histopathological analysis), they are likely associated with microsomal enzyme induction, which can occur earlier in the disease process and be a more sensitive indicator of liver cell injury compared to histopathology (Hall *et al.*, 2012).

Liver histopathology.

No significant histopathological effects of A4 or PRED were observed at 7 or 28 days (Supplemental Table 3). Treatment with EE for 7 days resulted in bile duct proliferation at the three lowest doses, but not the high dose, and oval cell proliferation at doses ≥ 2.5 ppm. Treatment with EE for 28 days resulted in midzonal to periportal hypertrophy and bile duct and oval cell proliferation. PB induced minimal to mild centrilobular hypertrophy at 7 and 28 days, consistent with the effects observed in a prior study by our group using similar methods (Lake *et al.*, 2016). Representative images of select histopathological findings in the liver are provided in Supplemental Figure 1.

Liver cell proliferation.

Steroid effects on liver cell proliferation were determined by Ki67 labeling. A4 did not alter cell proliferation at 7 days at any dose (Figure 1A). For EE, higher LIs were observed at all doses at 7 days, but this effect was statistically significant only for small cells at 2.5 and 10 ppm (Figure 1B). PRED resulted in lower LIs (large cells only) at 7 days at the 1.25, 5, and 25 ppm doses (Figure 1C), while PB resulted in significantly higher LIs at 7 days (large, small, and all cells) (Figure 1A). At 28 days, there were no effects of A4 or PRED (Figure 1D), while EE resulted in significantly higher cell proliferation (large, small, and all cells) which was comparable in magnitude to that seen at 7 days, suggesting a sustained compensatory proliferative effect secondary to cytotoxicity. PB also resulted in higher Ki67 LI at 28 days, but this effect (4.8% LI, all cells) was diminished compared to 7 days (8.2% LI), consistent with an early mitogenic burst (Wood *et al.*, 2015). Representative images of Ki67 immunolabeling are provided in Supplemental Figure 2.

CYP450 enzyme activity.

We measured the activities of EROD, PROD, and BROD, which provide indicators of AhR, CAR, and Pregnane X Receptor (PXR) activation, respectively (Burke and Mayer, 1974; Burke *et al.*, 1985; Hagemeyer *et al.*, 2010; Lehmann *et al.*, 1998; Ma and Lu, 2007; Yoshinari *et al.*, 2001). No significant changes were observed in enzyme activities following A4 exposure at 7 or 28 days (Table 2). EE significantly decreased BROD (0.4- to 0.6-fold), EROD (0.5- to 0.7-fold), and PROD (0.3- to 0.4-fold) activities following 7 days of exposure and EROD (0.7-fold) and PROD (0.4-fold) activities following 28 days of exposure. EE has been shown to inactivate CYP2B (i.e., PROD) and CYP3A (i.e., BROD) in a mechanism-based manner (Guengerich, 1988; Kent *et al.*, 2002). PRED at 25 ppm significantly increased BROD (9.9- and 16.4-fold, respectively) and PROD (4.2- and 13.3-fold) activities after 7 and 28 days, respectively. PRED effects corresponded to increases in genes encoding CYP2B and CYP3A family members (Supplemental Table 4). PRED has been shown to induce CYP3A (Deb *et al.*, 2012; Pichard *et al.*, 1992). In contrast, PRED decreased EROD (0.5- to 0.8-fold and 0.8-fold) activity following 7 and 28 days, respectively, at doses >1.25 ppm. PB exposure for 7 and 28 days resulted in significant increases in BROD (6.1- and 15.4-fold, respectively), EROD (3.0- and 4.0-fold), and PROD (5.1- and 11.1-fold) activities.

Global gene expression.

PCA showed no clear separation in global gene expression between the control and A4 dose groups at 7 or 28 days (Figure 2A), in contrast to EE and PRED (Supplemental Figure 3A-C). Microarray analysis did not identify any genes to be significantly altered by A4 at any dose at 7 or 28 days (Figure 2B), while EE and PRED significantly altered up to 3070 and 671 genes in a dose-dependent manner (Supplemental Table 4). A4 effects on known target genes of androgens in the mouse liver (Clodfelter *et al.*, 2006; Oshida *et al.*, 2016a) were also measured by qPCR. Out of the 11 genes measured, *Hsd3b5* and *Cyp2c29* both expected to increase in expression after androgen exposure exhibited decreased expression at 7 or 28 days, respectively (Supplemental Figure 4). These minor changes inconsistently observed across the treatment times including a 90-day time point (discussed below) likely are not due to a direct effect of A4 (discussed in legend of Supplemental Figure 4). One-dimensional clustering of DEGs showed a consistent direction of change across dose groups for EE and PRED at 7 and 28 days (Figure 2C). The pattern of expression was remarkably consistent across all doses and time points for EE, despite the fact that at 28 days, the 10 mg/kg/day and the 25 mg/kg/day doses for EE and PRED, respectively, exhibited differences in indicators of toxicity (terminal body weights and ALT). The extent of the overlap in the regulated genes by the two compounds was examined at 7 days (highest dose) or 28 days (Supplemental Figure 5). Out of the 205 genes that were regulated at 7 days by both EE and PRED, 167 (81%) had concordant regulation (i.e., genes were regulated in the same direction). Similarly, at 28 days, EE and PRED commonly regulated 335 genes, of which 296 (88%) had concordant direction of change. Genes regulated by EE significantly overlapped with those regulated by an ER α agonist in an ER α -dependent manner and genes regulated by PRED overlapped with those regulated by a GR agonist in a GR-dependent manner (Supplemental Figure 6). In summary, these results indicate that prototypical agonists of ER and GR result in the modulation of an overlapping set of genes. The biological basis for regulation and the impact on liver effects of the overlapping genes remains to be determined.

Genomic effects of A4 at 90 days.

Given the lack of any A4 effects at 7 or 28 days, we used targeted RNA sequencing to examine A4 responses in FFPE liver samples from male and female mice treated for 90 days with three doses of A4 (NTP study TR-560). All samples passed standard TempO-Seq quality control metrics, including a signal-to-noise ratio cutoff of 20:1, greater than 80% of reads mapped, and average reads per probe greater than 250. PCA showed a clear separation of control males and control females (Figure 3A). Genes significantly altered between control males and females were highly similar in expression differences to male *vs.* female comparisons from other studies examining liver gene expression in control mouse liver (Figure 3B). There was no obvious separation between the control and A4 groups for either sex. Only a small number of genes were altered in male mice (8 genes, or 0.26% of the total, at any dose, and only 1 gene, *Fabp5*, at all 3 doses). In contrast, there were greater numbers of genes altered in female mice at the low and high dose groups (Figure 3C). The complete list of genes altered by A4 at 90 days is found in Supplemental Table 5.

The genes regulated in females at the high dose of A4 were subjected to an analysis in IPA (Supplemental Table 6). The top two canonical pathways were “Complement System” and “Acute Phase Response Signaling” and included a number of overlapping genes that were increased in expression (*C1ra*, *C1s*, *C5*, *C8a*, *C9*, *Hp*, *Serpina3k*). Other top 5 most significant canonical pathways included “Melatonin Degradation I”, “LPS/IL-1 Mediated Inhibition of RXR Function”, and “Nicotine Degradation II” due to changes in a number of phase I and II metabolism genes. The upstream activator analysis hypothesized that the transcription factor interferon regulatory factor 7 (IRF7) was activated due to modulation of target genes (*C5*, *Ifi44*, *Ifitm3*, *Igtp*, *Irgm2*). While it appears that A4 may be regulating genes which overlap with an inflammatory response, there is no evidence from histopathology in the present study or in the NTP bioassay for an inflammatory component in A4-induced liver tumors.

Marker genes for AhR, CAR, or PPAR α were examined as alternate pathways of xenobiotic-induced liver cancer. Out of the 3099 genes examined, there were 18, 26, and 14 genes from the respective AhR, CAR, and PPAR α gene expression biomarkers used to accurately predict receptor activation in previous studies (Oshida *et al.*, 2015a-c). These genes included targets for AhR (*Cyp1a1*), CAR (*Cyp2b10*), or PPAR α (*Pdk4*) activation. None of the genes in the AhR or CAR biomarkers were altered by A4, while only *Hsd17b12*, which is induced by PPAR α activation, was increased in expression by A4 (data not shown). Of note, testosterone treatment of female mice for three weeks (from GEO study GSE13388) also did not activate AhR, CAR, or PPAR α based on lack of responsiveness of target genes (data not shown). The gene expression profiles from EE and PRED groups did not significantly correlate with AhR, CAR, and PPAR α biomarkers (p-values > 1E-4) (Supplemental Figure 7).

A4 masculinized the gene expression pattern in female mice.

We predicted that the gene expression pattern after A4 exposure in females from the 90-day study would resemble not only sexually dimorphic differences (males *vs.* females), but also the genomic profile induced by testosterone in female mice in reference datasets. For this analysis, we used the A4 profile observed at the high dose in females from the NTP study and compared the expression of these DEGs to male *vs.* female comparisons. Twelve male *vs.* female comparisons from 9 studies were randomly selected from the mouse liver compendium described in Oshida *et al.* (2016a). All male *vs.* female comparisons exhibited highly significant positive correlation to the A4 comparison (p-values = 6.4E-6 to 4.2E-14) (Figure 4A, left). The positive correlations reflected both increased expression of male-predominant genes and decreased expression of female-predominate genes by A4. Male-predominant genes included a cluster of major urinary protein (*Mup*) genes (*Mup3*, *Mup12*, *Mup15*, and *Gm2083* (*LOC100048885*)), which are expressed in a sexually dimorphic (and often androgen-dependent) manner, with male mice expressing 2- to 8-fold higher protein levels than female mice (Cheetham *et al.*, 2009). As testosterone treatment of female mice masculinizes the pattern of gene expression in the liver (Oshida *et al.*, 2016a), we compared the A4-regulated genes with two testosterone *vs.* control comparisons in which female mice were treated twice weekly with 0.9 mg testosterone (GSE13388). There was significant

positive correlation between A4 and testosterone in both comparisons (p-values = 1.5E-7 and 1.4E-8) (Figure 4A, middle).

Differences in sex-dependent gene expression in the rodent liver are regulated by the transcription factor STAT5b, which is activated by the male-specific pulsatile pattern of growth hormone release from the pituitary (described in Waxman and O'Connor, 2006). To determine if the A4-regulated genes were dependent on STAT5b, the A4 comparison was compared to the STAT5b gene expression biomarker consisting of genes that are differentially expressed between males and females and dependent on STAT5b for sex differences. This biomarker has been previously found to accurately predict STAT5b activation or suppression in a mouse liver gene expression compendium (Oshida *et al.*, 2016a,b). The p-value of the positive correlation was 5.2E-11 (Figure 4A, right). There was also highly significant negative correlation between the STAT5b biomarker and all of the EE treatment groups, consistent with suppression of the male-specific STAT5b activation pattern and feminization in male mice by estrogen treatment (discussed in Oshida *et al.*, 2016a,b) (Figure 4B). There were no consistent correlations between PRED comparisons and the STAT5b biomarker. Overall, these results indicate that A4 exerts effects on expression of sexually dimorphic genes that are dependent on STAT5b.

DISCUSSION

Androgens induce liver cancer in mice and likely contribute to the higher risk of liver cancer in men compared to women (Zhang *et al.*, 2015). Mechanistic drivers and early biomarkers for these effects are not well defined. In this study, we evaluated short-term effects of the androgen A4 in reference to other steroid hormones and investigated potential growth factor pathways mediated by androgenic signaling. Treatment of male mice with A4 did not induce key events for common chemical-induced modes of action (MOA) of liver cancer (Holsapple *et al.*, 2009). A4 did not increase liver cell hypertrophy, cytotoxicity, or proliferation, alter enzymatic markers of xenobiotic receptor activation, or result in significant transcriptional changes at 7 or 28 days. Further analysis of dose-matched archival samples from the 90-day NTP study of A4 showed no consistent changes in genes regulated by AhR, CAR, or PPAR α , which are common mitogenic targets of liver carcinogens. There were modest effects of A4 on expression of sexually dimorphic genes in female mice that are dependent on STAT5b and suppressed by estrogen. These findings indicate that liver cancer induced by A4 does not occur through the typical targets of liver carcinogens or through direct AR-mediated mitogenesis but rather by subtle long-term effects on male-predominant growth factor pathways that may be coordinated by AR or STAT5b.

The lack of short-term morphologic effects is unusual among liver carcinogens. A previous analysis of NTP studies by Allen *et al.* (2004) showed that 93% (25/27) of mouse liver carcinogens (genotoxic or not) exhibited increased liver weight and/or hepatocyte hypertrophy, cytomegaly, or necrosis in the corresponding pre-chronic 90-day studies. For A4, the absence of histopathological effects at 7 or 28 days is consistent with prior mouse NTP studies at 14 and 90 days, which found no effects in either sex across the same dose range (NTP 2010). A4 also had no effects on liver cell proliferation, in contrast to PB, the positive mitogenic control. This latter finding is supported by the NTP 14-day studies, which

found no effect of A4 on liver cell proliferation markers as assessed by enzymatic assays in male and female mice. Most known non-genotoxic chemical carcinogens induce cancer through mitogenic effects, either directly or secondary to cytotoxicity (Wood *et al.*, 2015), so the clear lack of a short-term proliferative effect here is notable. A4 and possibly other androgens may induce liver cancer by a MOA that requires an interaction with age-related factors. At least in male mice, A4 may maintain activation of androgen-dependent genes that normally decline with age (Oshida *et al.*, 2016a).

Given that A4 is a direct precursor of testosterone and an AR agonist, we hypothesized that A4 increases liver cancer incidence through an androgen-dependent mechanism that may also underlie the greater susceptibility of males to liver cancer. Liver cancer incidence and mortality is higher in men than women in almost all populations examined (Ferlay *et al.*, 2010), and in the U.S., the incidence is approximately three-fold higher in men compared to women (Altekruse *et al.*, 2009). Sex hormones are thought to play a key role in this male-predominant pattern, although the molecular pathways are largely unknown. Male mice have higher incidences of both spontaneous and chemically-induced liver tumors compared to female mice (Smith *et al.*, 1973; Toh, 1981; Vesselinovich and Mihailovich, 1967), and castrated male mice have a lower incidence of spontaneous and chemically-induced liver cancer compared to intact mice (Bugni *et al.*, 2001; Kemp *et al.*, 1989a; Poole and Drinkwater, 1996; Toh, 1981; Vesselinovich and Mihailovich, 1967). Conversely, ovariectomized female mice develop greater numbers of liver tumors compared to intact female mice (Bugni *et al.*, 2001; Poole and Drinkwater, 1996; Vesselinovich and Mihailovich, 1967; Yamamoto *et al.*, 1991). In the A4 carcinogenicity studies, female mice also exhibited a more statistically significant liver tumor induction than male mice (NTP 2010) (Supplemental Figure 8). These studies collectively support the idea that androgens enhance and ovarian hormones suppress liver carcinogenesis.

If A4 is acting through AR-mediated pathways, genomic responses should be consistent with sexually dimorphic patterns. After 90 days of A4 exposure, gene expression changes were seen primarily in female mice, which had a larger set of genes at the highest dose tested (a total of 60 genes or 1.9% of the total) compared to male mice. The genes altered by the high dose of A4 in female mice were found to have significant positive correlations to 12 male vs. female comparisons across 9 different microarray studies. The A4-regulated gene set also exhibited significant positive correlation to the genes altered by a 3-week testosterone treatment in the livers of female mice. For these comparisons, the alterations included both increased expression of male-predominant genes as well as decreased expression of female-predominant genes. These comparisons support the idea that A4 is inducing a more male-like gene expression pattern.

A central candidate for regulating A4-induced genes is STAT5b, a master regulator of sexually dimorphic gene expression in the liver controlled by the male-specific pulsatile pattern of growth hormone secretion from the pituitary (Clodfelter *et al.*, 2006, Zhang *et al.*, 2012). We determined if the genes regulated by A4 overlapped with those regulated by STAT5b. Expression of A4-regulated genes was compared to a biomarker that accurately predicts STAT5b activation or suppression (Oshida *et al.*, 2016a,b). This comparison showed significant positive correlation (p -value = $5.2E-11$), providing strong support that A4 is

activating STAT5b. In contrast, there were highly significant negative correlations between the STAT5b biomarker and the EE comparisons (p-value range = 4.8E-14 – 5.9E-29), consistent with the idea that estrogens suppress the growth hormone pulsatile pattern and, secondarily, STAT5b activation (Waxman and O'Connor, 2006). These results demonstrate that A4 causes masculinization of gene expression, most likely through STAT5b.

Previous studies have identified a role for both STAT5b and AR in liver carcinogenesis. STAT5b signaling was examined in three mouse strains by assessing liver cancer in the presence (wild-type) or absence of STAT5b (*Stat5b*-null mice) following diethylnitrosamine (DEN) exposure (Oberly *et al.*, 2014). *Stat5b*-null mice on the C57BL/6J background developed more DEN-induced liver tumors than wild-type mice, while loss of STAT5b had no effect on the BALB/cJ background, and led to resistance to liver cancer on the C3H/HeJ background. It should be noted that the strain used in the NTP study was B6C3F1, a cross between female C57BL/6 and male C3H mice. Thus, STAT5b appears to act either as an oncogene or a tumor suppressor depending on the background strain. AR is another candidate target of A4 carcinogenesis. By comparing effects between wild-type and hepatocyte-specific AR-null mice, AR has been shown to be required for DEN- and hepatitis B virus-mediated hepatocellular carcinoma (Ma *et al.*, 2008; Wu *et al.*, 2010). In males, studies using mice lacking a functional AR (testicular feminization (*Tfm*) mice) have also revealed the importance of AR in promotion of liver tumors, as DEN treatment resulted in >25-fold more tumors in wild-type male mice than their *Tfm/Y* counterparts (Kemp *et al.*, 1989b). Further studies are required to determine if STAT5b and AR are involved in A4-induced liver cancer, particularly as mice age and endogenous androgens decline (Oshida *et al.*, 2016a).

Increased cellular proliferation is a central key event in the MOA for many non-genotoxic chemical carcinogens (Wood *et al.*, 2015). In our study, exposure to EE led to increases in hepatocyte proliferation at 7 and 28 days. This finding is notable given that four of the five chronic bioassays of EE resulted in no significant increases in liver tumors in mice, and the one statistically significant increase has been considered equivocal because of the relatively low spontaneous liver tumor incidence and the small increase over controls in the treated animals (Schuppler and Gunzel, 1979). There are several differences in study design that might explain these apparent discrepancies. While we used B6C3F1 mice, CF-LP, and BDH-SPF strains were used in the chronic bioassays. We cannot rule out that B6C3F1 mice exhibit different short- and long-term responses to EE compared to the strains used in the bioassays or that B6C3F1 mice might be susceptible to increases in liver tumorigenesis resulting from EE. In addition, our cell proliferation results contrast with three studies that showed suppression of liver foci growth by EE in initiation-promotion assays (Lee *et al.*, 1989; Standeven *et al.*, 1994; Moser *et al.*, 1996). None of these studies measured cell proliferation at time points that overlapped with our 28-day window. One study found that there was an approximate 2-fold (not significantly significant) increase in cell proliferation in the non-foci hepatocytes in female B6C3F1 mice upon treatment with EE for 16 weeks compared to non-foci hepatocytes from untreated mice (Moser *et al.*, 1996). In a similar study from the same laboratory using B6C3F1 mice, there were increases in liver to body weights of ~70% upon EE exposure (Standeven *et al.*, 1994), indicating that the liver increased in size due to hypertrophy and/or hyperplasia. The behavior of EE may be similar

to that of peroxisome proliferators in which short-term exposures transiently induce cell proliferation but act to promote and induce cell proliferation in only a subset of foci, in this case basophilic foci, while apparently inhibiting growth in eosinophilic foci (Cattley and Popp, 1989; Marsman and Popp, 1994). It cannot be ruled out that EE under the conditions that increase acute cell proliferation might increase liver tumor induction only in a subset of foci that were not observed in the initiation-promotion studies.

One difference between our study and that of the NTP 2010 study is the composition of the diets. Diet is an important factor in the growth, body weight, survival, and development of age-related diseases in rodent chronic studies (Rao and Crockett, 2003). In the present study, AIN-93G was used, while in the NTP study, the NTP-2000 diet was used. It is possible that differences in diets could account for differences in responses to A4. However, we feel that this is unlikely based on the uniform lack of responses after short-term exposures in both studies. The fact that we did not see any morphologic effects in our 7 and 28 day studies is completely consistent with the original NTP study in which short-term effects on liver histopathology were not observed in male or female mice at 14 or 90 days. The results of the gene expression analysis of the FFPE livers from the 90-day NTP study were also consistent with the lack of histopathological effects in those same livers. We observed changes in a relatively low number of genes (~60 out of 3099 examined). Thus, our results are consistent with the NTP study and argue that differences in diet do not contribute to differences in responses to A4.

In summary, the short-term molecular effects of A4 exposure are not consistent with classical mitogenic or cytotoxic pathways of liver carcinogenicity. After 90 days of treatment, we identified genomic effects of A4 similar to sexually dimorphic patterns between males and females. We propose that A4 is acting via growth factor pathways that drive age-dependent increased susceptibility of males to liver cancer through an as yet unidentified mechanism. STAT5b activation in the livers of chemically-treated mice could be used as an indicator of such risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Disclaimer

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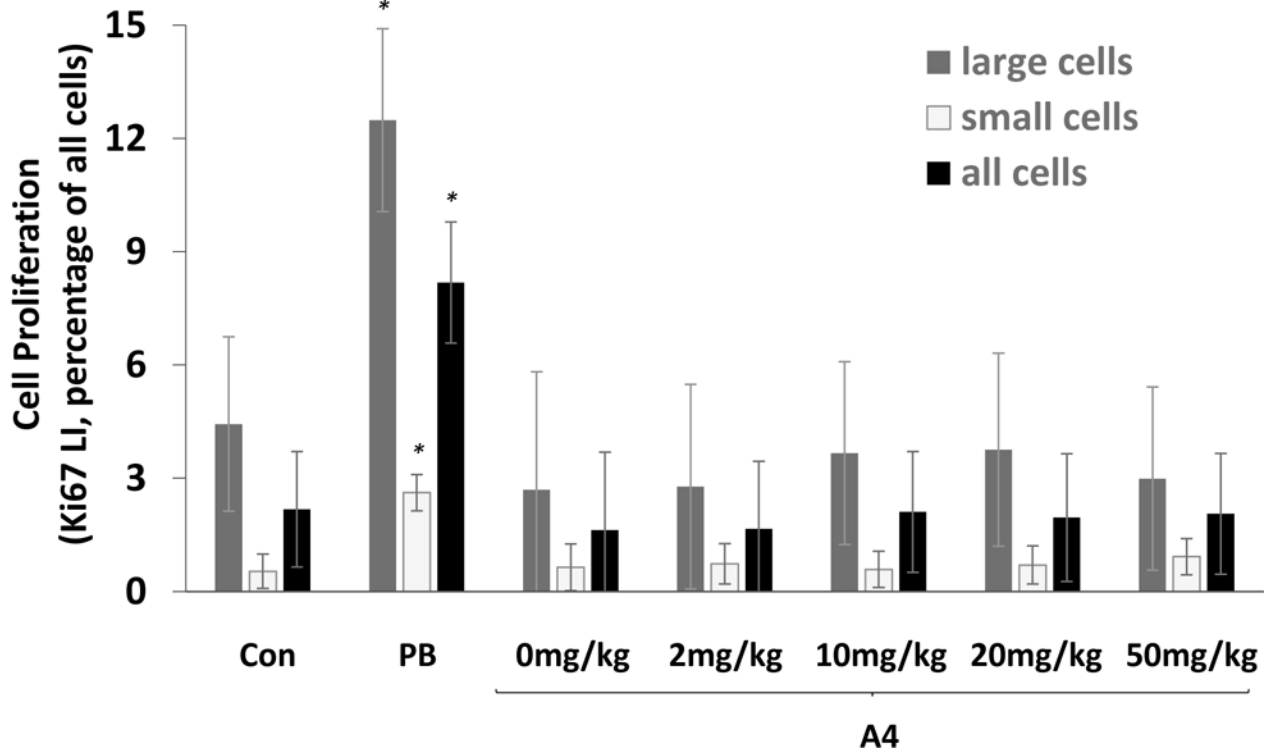
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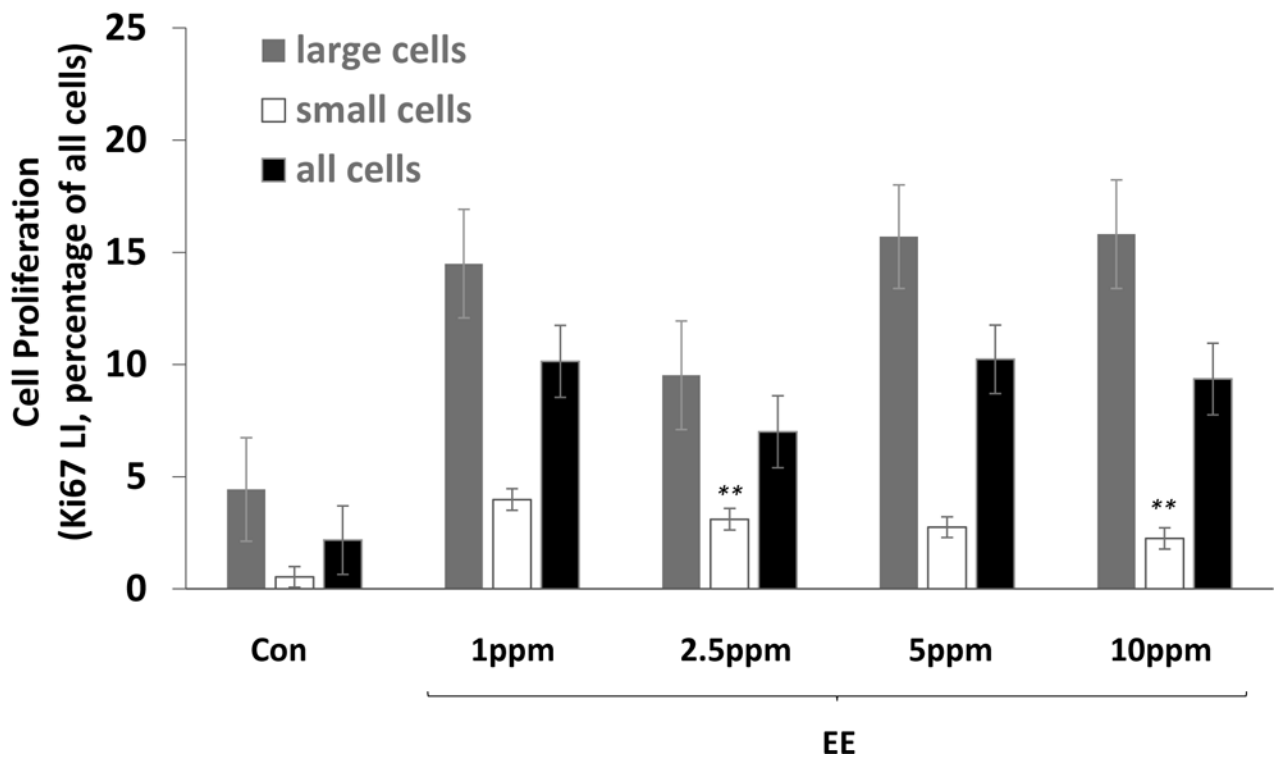
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A



B



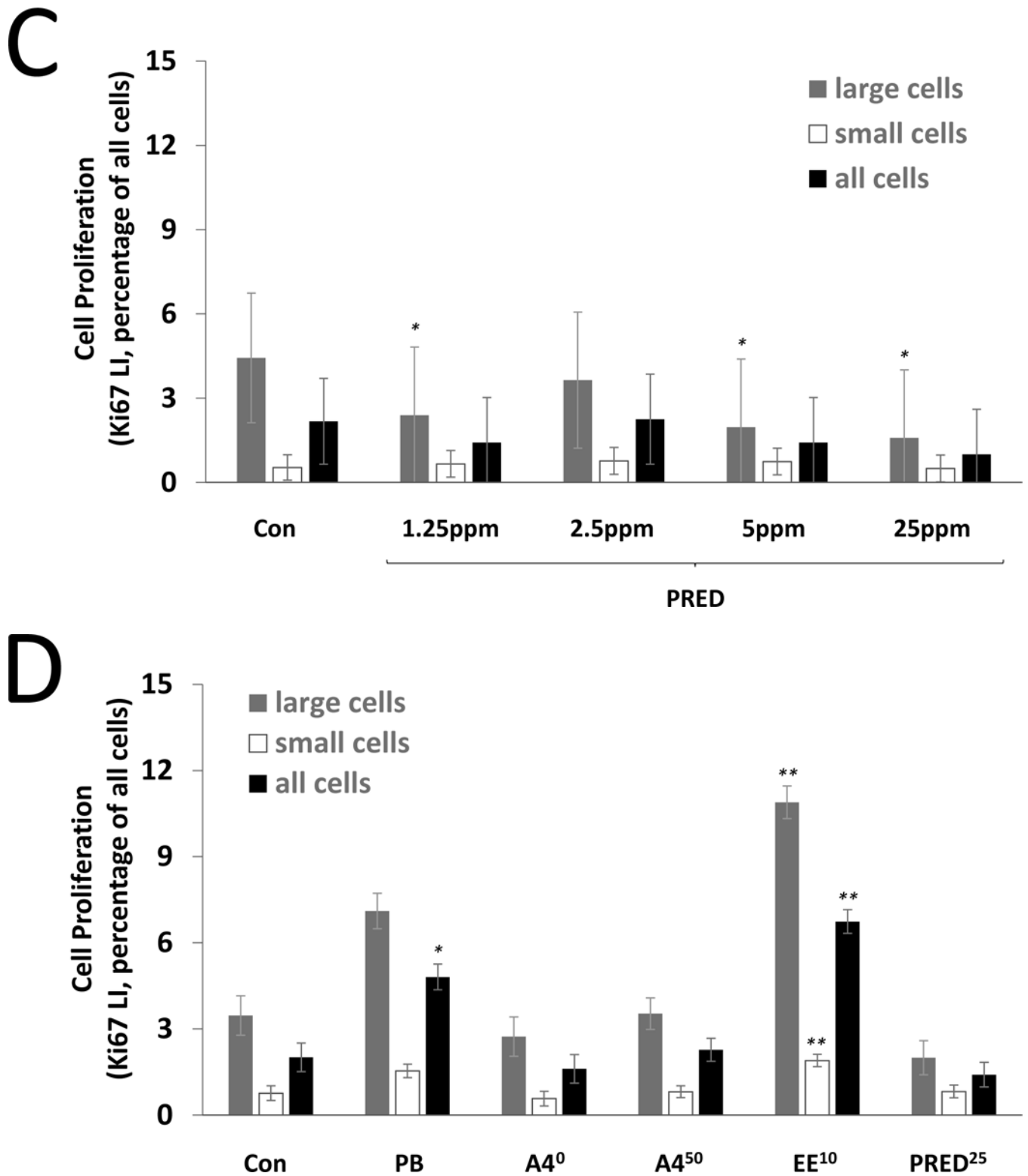
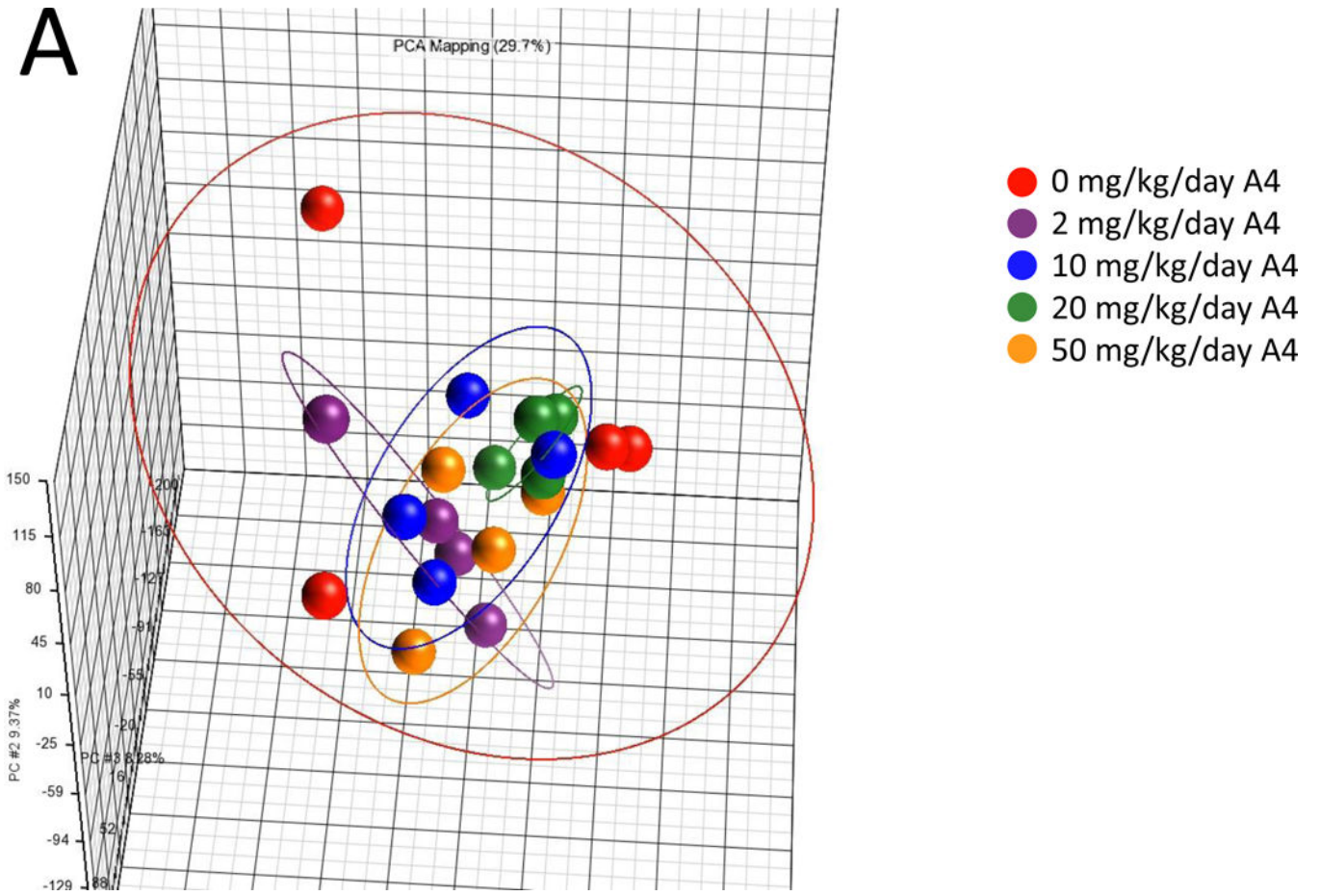
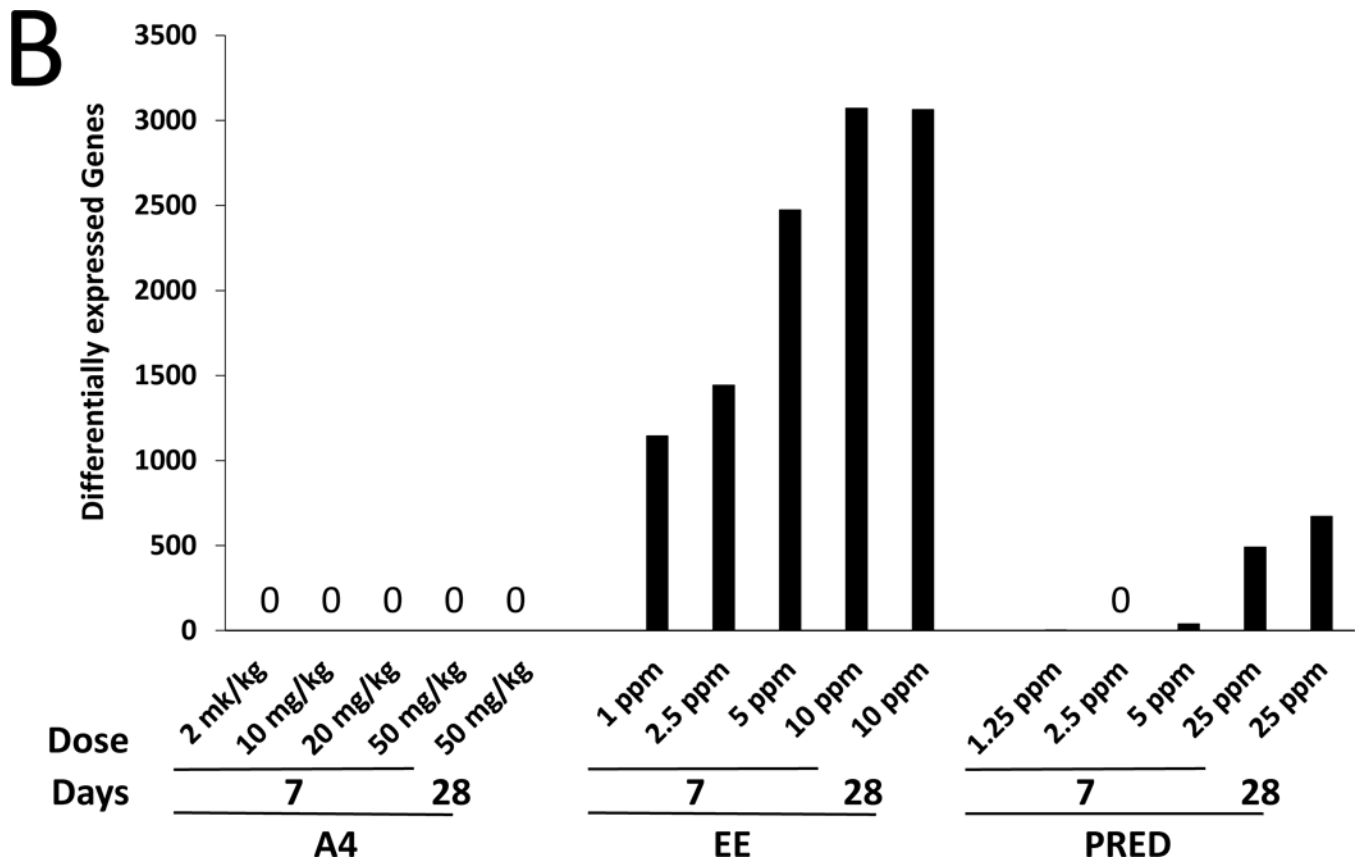


Figure 1. Labeling index (LI) for the proliferation marker Ki67 in the livers of mice treated with A4, EE, or PRED.

Ki67 LI is shown for large cells (grey), small cells (white), and all cells combined (black) after 7 days (A-C) and 28 days (D) of exposure. Significance is indicated by * $p < 0.05$ and ** $p < 0.01$ compared to the respective control (Con) group. The 0 mg/kg A4 group indicates gavage control.





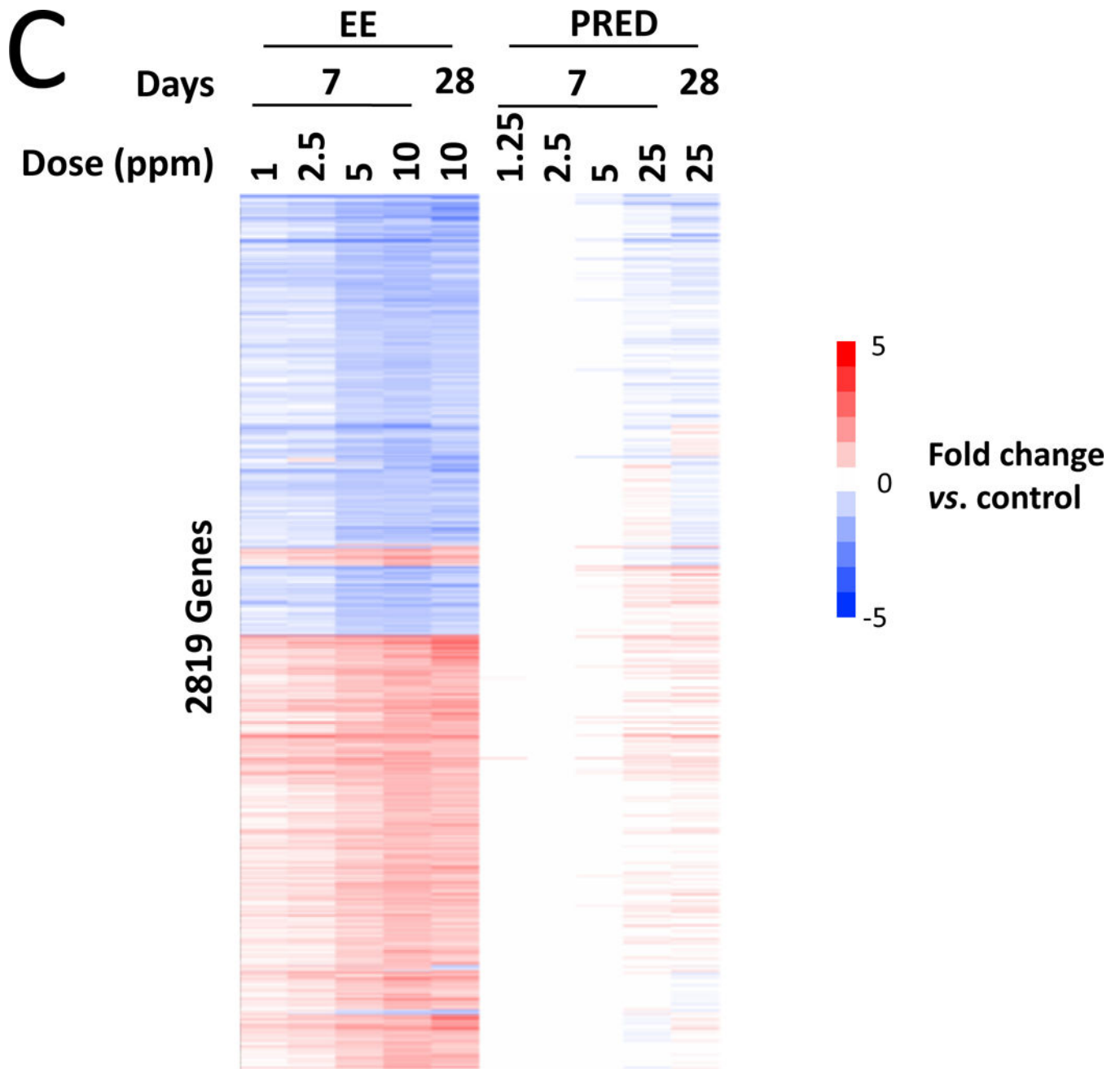
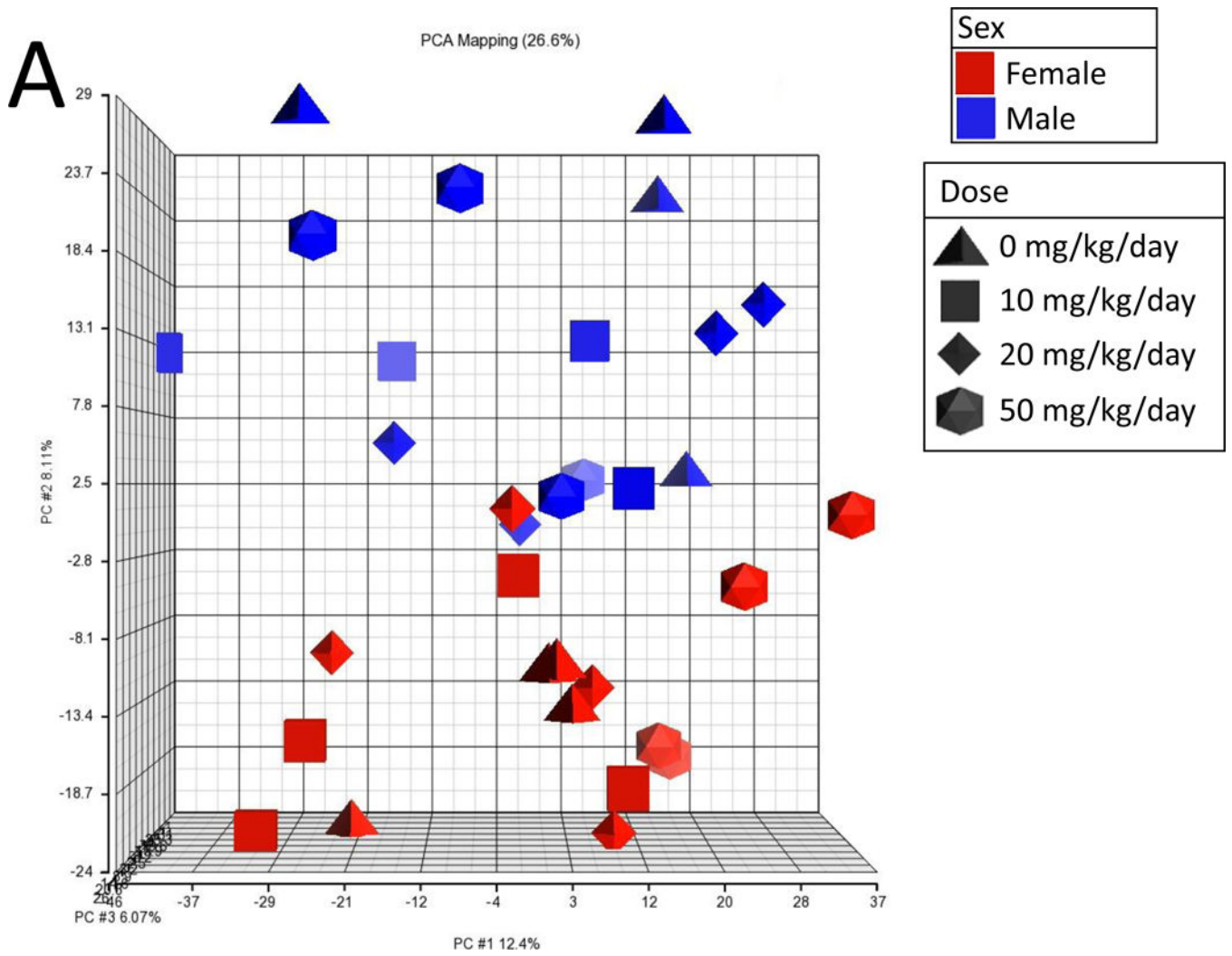


Figure 2. Steroid effects on global gene expression at 7 and 28 days.

A. Principal component (PC) plot of microarray profiles for A4 samples at 7 days. B.

Number of significant differentially expressed genes in each dose group. C. One-

dimensional hierarchical clustering analysis of all significantly altered genes. (Clades are not shown.)

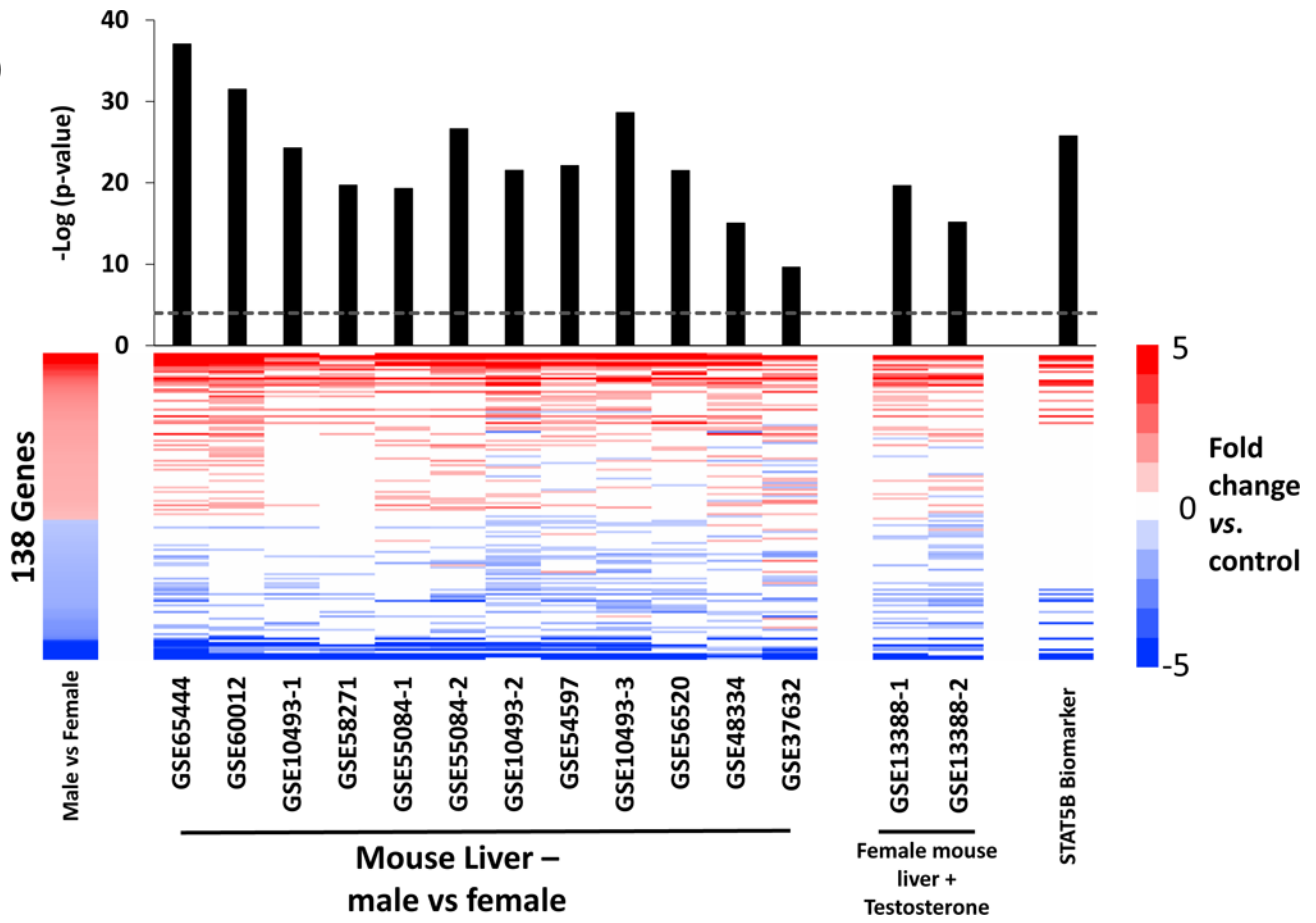


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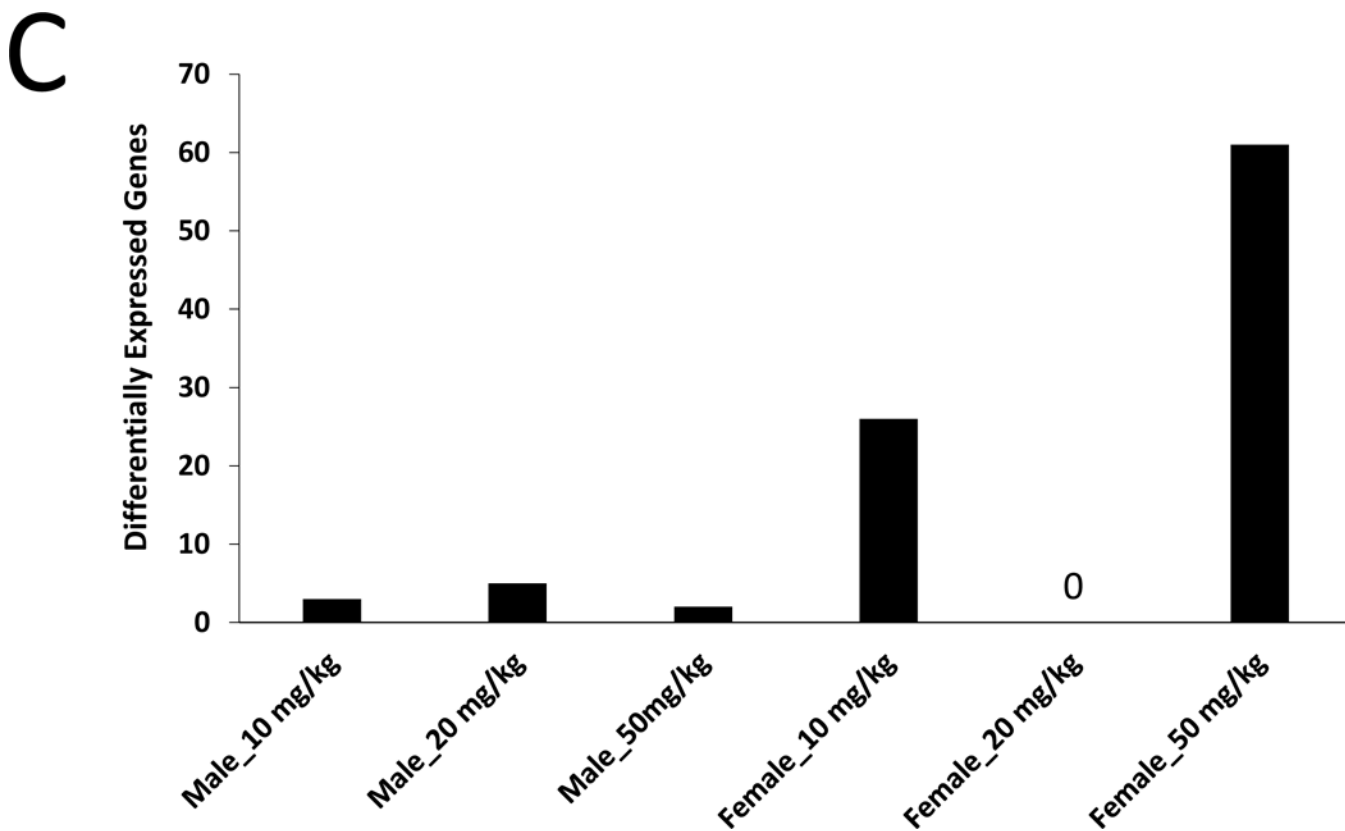
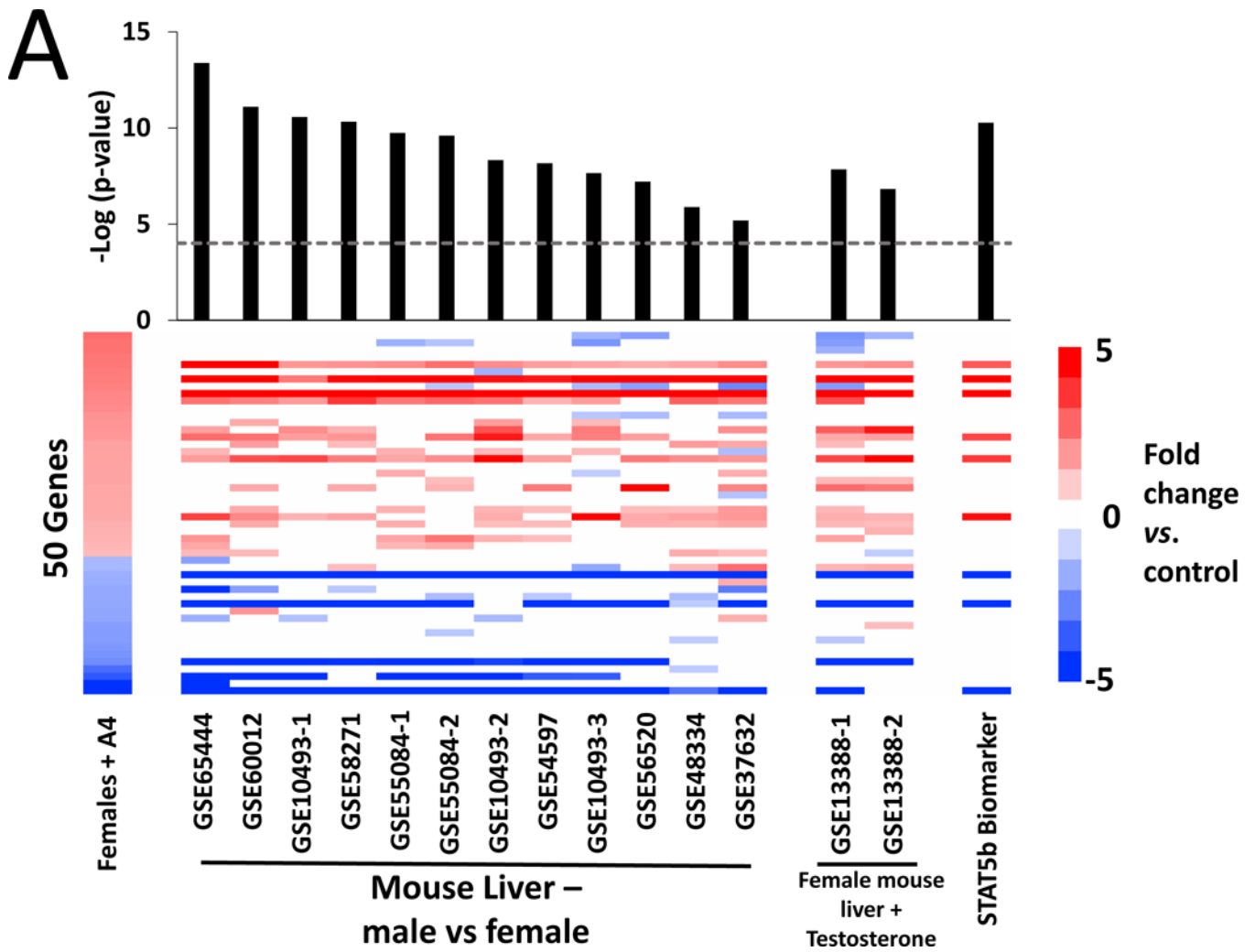


Figure 3. Gene expression analysis of the archival NTP liver samples from mice treated with A4 for 90 days.

Sections of FFPE liver from male and female mice treated by gavage with 0, 10, 20, and 50 mg/kg A4 for 90 days were used to examine the expression of 3099 genes as described in the Methods. A. Principle component analysis of TempO-Seq profiles. B. Genes that were differentially expressed between control males and control females were identified as described in the Methods. The genes were compared to male vs. female comparisons from wild-type adult mice fed normal diets. The GEO number associated with each of the studies is indicated. Comparisons were made using the Running Fisher's test in the NextBio environment as described in the Methods. The $-\text{Log}(p\text{-value})$ of each comparison is shown; positive values represent positive correlations. C. Number of significantly altered genes in each dose group.



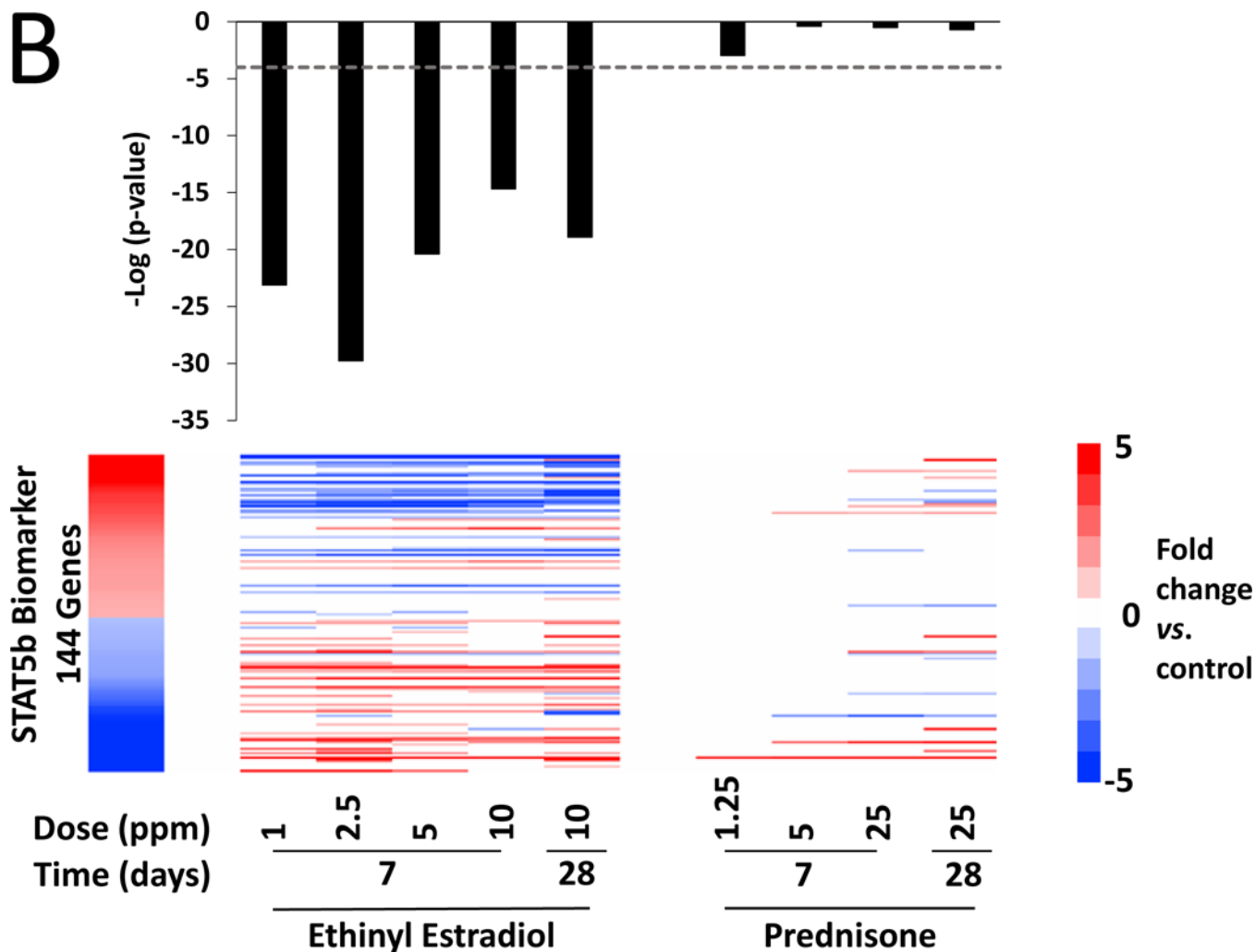


Figure 4. A4 effects on sexually dimorphic genes.

A. Significance of correlations between the A4 high dose group (left) and reference datasets for male *vs.* female mice, female mice treated with testosterone, and the STAT5b biomarker. Fifty genes from the 60 that were altered in the high dose group were also significantly altered in at least one of the comparisons. The male *vs.* female comparisons were from wild-type adult mice on standard diets. The GEO number associated with each study is indicated. Female mice were treated with testosterone (0.9 mg) or sesame oil (vehicle) twice a week for three weeks. Gene expression in the liver was analyzed either directly after treatment (GSE13388-1) or after three weeks of hormone withdrawal (GSE13388-2). The STAT5b biomarker was described previously (Oshida *et al.*, 2016a,b). All of the indicated comparisons were compared to the A4 bioset using the Running Fisher's test. The $-\text{Log}(\text{p-value})$ s of the comparisons are shown. B. Significance of the correlations between the genes altered by EE and PRED and the STAT5b biomarker. The $-\text{Log}(\text{p-value})$ s with negative values represent pairwise comparisons with negative correlation.

Table 1.

Terminal Body Weight, Relative Liver Weights and Clinical Chemistries Following 7 and 28 Days of Exposure to Androstenedione (A4), Ethinyl Estradiol (EE), Phenobarbital (PB) and Prednisone (PRED).

Day	Chemical	Dose ^a	Terminal Body Weight gm		Relative Liver Weight %		ALT ^b IU/L		SDH ^c IU/L	
			n	LS-Mean (SE)	n	LS-Mean (SE)	n	LS-Mean (SE)	n	LS-Mean (SE)
7	A4 ^f	0	11	31.30 (0.79)	11	4.17 (0.11)	6	52.57 (28.95)	6	19.54 (12.54)
		2	13	31.80 (0.72)	13	4.24 (0.10)	7	55.21 (27.08)	8	36.05 (10.91)
		10	15	31.09 (0.67)	15	4.22 (0.09)	10	80.49 (22.36)	10	39.02 (9.68)
		20	15	30.62 (0.67)	15	4.38 (0.09)	9	44.17 (23.84)	9	15.82 (10.33)
		50	15	31.99 (0.67)	15	4.26 (0.09)	10	37.20 (22.36)	10	17.57 (9.68)
		EE	0	16	33.25 (0.52)	16	4.48 (0.36)	9	56.99 (73.94)	10
	1	16	24.38*** (0.52)	16	4.56 (0.36)	10	52.96 (70.22)	10	20.78 (24.37)	
	2.5	16	24.28*** (0.52)	16	4.48 (0.36)	10	87.75 (70.22)	9	14.84 (25.94)	
	5	16	24.09*** (0.52)	16	4.66 (0.36)	11	321.32 (66.87)	10	68.91 (24.37)	
	10	16	25.04*** (0.52)	16	4.81 (0.36)	8	29.82 (78.95)	8	65.05 (27.13)	
PB	0	16	33.25 (0.52)	16	4.48 (0.10)	9	69.69 (26.15)	10	30.70 (7.14)	
	600	16	32.43 (0.52)	16	6.03*** (0.10)	10	105.73 (24.86)	10	33.19 (7.14)	
PRED	0	16	33.25 (0.53)	16	4.48 (0.09)	9	69.99 (13.76)	10	30.86 (5.17)	
	1.25	16	31.94 (0.53)	16	4.05** (0.09)	9	68.77 (13.77)	10	24.29 (5.17)	

		Terminal Body Weight gm	Relative Liver Weight %	ALT ^b IU/L	SDH ^c IU/L					
28	2.5	16	32.44 (0.53)	16	4.22 (0.09)	10	58.92 (13.07)	10	23.95 (5.17)	
	5	16	30.31 ^{***} (0.53)	16	4.18 (0.09)	10	70.47 (13.07)	10	23.33 (5.17)	
	25	16	29.62 ^{***} (0.54)	16	4.55 (0.09)	10	92.01 (13.07)	10	35.85 (5.17)	
28	A4 ²	0	13	34.73 (0.76)	13	4.12 (0.14)	9	37.97 (4.29)	9	28.75 (16.95)
		50	20	34.43 (0.61)	20	4.21 (0.11)	13	37.21 (3.49)	14	45.21 (13.38)
BE	0	16	38.43 (0.61)	16	4.02 (0.13)	10	43.14 (28.34)	10	29.15 (8.47)	
	10	21	21.77 ^{***} (0.54)	21	6.73 ^{***} (0.11)	12	125.58 ^{**} (25.63)	13	41.99 (7.38)	
PB	0	16	38.43 (0.70)	16	4.02 (0.21)	10	36.54 (8.08)	10	26.52 (5.02)	
	600	18 ³	35.81 [*] (0.67)	18	6.17 ^{***} (0.20)	11	79.02 ^{**} (7.65)	11	45.51 [*] (4.75)	
PRED	0	16	38.43 (0.69)	16	4.02 (0.11)	10	36.36 (5.13)	10	26.65 (2.83)	
	25	19 ⁴	31.45 ^{***} (0.64)	19	4.42 [*] (0.10)	12	72.37 ^{***} (4.64)	12	33.03 (2.56)	

¹ n reduced to less than 16 per dose/group due to deaths over the course of the 7 dosing period.

² 7 mice in the control group and 4 mice in the 50 mg/kg/day dose group died over the course of 28 day dosing period.

³ 2 mice died over the course of the 28 day dosing period.

⁴ 1 mouse died over the course of the 28 day dosing period.

Least-squares (LS) means and standard errors (SE) by dose for 7- and 28-day steroid exposed mice.

* p < 0.05,

** p < 0.01,

*** p < 0.001.

^aDose is the gavage dose in mg chemical/kg body weight for A4, dietary concentration (ppm in diet) for EE and PRED, and ppm in drinking water for PB.

^bAlanine aminotransferase

^cSorbitol dehydrogenase

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Table 2.

17Hepatic P-450 Enzyme Activity Levels and Fold Changes Following 7 and 28 Days of Exposure to Androstenedione (A4), Ethinyl Estradiol (EE), Phenobarbital (PB) and Prednisone (PRED).

Day	Chemical	Dose ^a	n	BROD pmole/mg protein/min			EROD pmole/mg protein/min			PROD pmole/mg protein/min		
				Least Square Mean (SE)	Fold Change	n	Least Square Mean (SE)	Fold Change	n	Least Square Mean (SE)	Fold Change	n
7	A4	0	5	3.63 (0.41)		5	22.75 (1.49)		5	2.62 (0.31)		
		2	5	3.64 (0.42)	1.0	5	23.53 (1.54)	1.0	5	2.59 (0.32)	1.0	
		10	5	3.28 (0.41)	0.9	5	21.54 (1.49)	0.9	5	2.65 (0.31)	1.0	
		20	6	4.14 (0.37)	1.1	6	25.50 (1.36)	1.1	6	2.39 (0.29)	0.9	
		50	5	3.61 (0.40)	1.0	5	28.27 (1.48)	1.2	5	2.50 (0.31)	1.0	
7	EE	0	6	3.83 (0.26)		6	25.68 (1.85)		6	2.67 (0.20)		
		1	6	2.41** (0.26)	0.6	6	16.00** (1.85)	0.6	6	0.94*** (0.20)	0.4	
		2.5	6	2.09*** (0.26)	0.5	6	17.49* (1.85)	0.7	6	0.96*** (0.20)	0.4	
		5	5	2.04*** (0.28)	0.5	5	16.80* (2.02)	0.7	5	0.92*** (0.22)	0.3	
		10	6	1.48*** (0.26)	0.4	6	13.91*** (1.85)	0.5	6	0.90*** (0.20)	0.3	
7	PB	0	6	4.50 (3.31)		6	28.22 (10.41)		6	3.24 (2.13)		
		600	6	27.44** (3.31)	6.1	6	86.01** (10.41)	3.0	6	16.44** (2.13)	5.1	
7	PRED	0	6	4.30 (3.63)		6	25.45 (1.05)		6	3.04 (1.46)		
		1.25	5	8.20 (4.07)	1.9	5	21.77 (1.17)	0.9	5	2.87 (1.64)	0.9	

Day	Chemical	Dose ^a	n	BROD pmole/mg protein/min			EROD pmole/mg protein/min			PROD pmole/mg protein/min		
				Least Square Mean (SE)	Fold Change	n	Least Square Mean (SE)	Fold Change	n	Least Square Mean (SE)	Fold Change	n
28	A4	2.5	6	4.14 (3.63)	1.0	6	20.05 ^{**} (1.05)	0.8	6	3.45 (1.46)	1.1	6
				5.38 (3.63)	1.3	6	16.09 ^{***} (1.05)	0.6	6	2.63 (1.46)	0.9	6
				42.74 ^{***} (4.02)	9.9	6	11.67 ^{***} (1.16)	0.5	6	12.89 ^{**} (1.62)	4.2	6
28	A4	0	4	4.17 (0.55)		4	22.63 (3.96)		4	2.82 (0.47)		4
				4.53 (0.44)	1.1	6	32.64 (3.18)	1.4	6	2.32 (0.37)	0.8	6
				4.33 (0.58)		6	25.50 (1.67)		6	2.57 (0.12)		6
28	EE	10	8	4.17 (0.53)	1.0	8	17.31 ^{**} (1.53)	0.7	8	1.03 ^{***} (0.11)	0.4	8
				3.79 (2.84)		6	26.87 (4.04)		6	2.57 (1.62)		6
				58.31 ^{***} (2.70)	15.4	7	108.03 ^{***} (3.84)	4.0	7	28.63 ^{***} (1.54)	11.1	7
28	PRED	0	6	4.04 (2.18)		6	25.20 (1.32)		6	2.33 (1.88)		6
				66.39 ^{***} (2.07)	16.4	7	18.98 ^{**} (1.25)	0.8	7	30.99 ^{**} (1.79)	13.3	7
				4.04 (2.18)		6	25.20 (1.32)		6	2.33 (1.88)		6

Asterisks indicate significant difference at

* p < 0.05,

** p < 0.01,

*** p < 0.001 compared to control group.

BROD: Benzyloxresorufin O-debenzylase; PROD: Pentyloxresorufin O-depenty/lase; EROD: Ethoxyresorufin O-deethylase

^aDose is the gavage dose in mg chemical/kg body weight for A4, dietary concentration (ppm in diet) for EE and PRED, and ppm in drinking water for PB.