Gene expression profiling of cultured mouse testis fragments treated with ethinylestradiol

Noriko Nakamura¹, Daniel T. Sloper¹ and Pedro L. Del Valle²

¹Division of Systems Biology, National Center for Toxicological Research, Food and Drug Administration, Jefferson AR 72079, USA
²Center for Drug Evaluation and Research, Food and Drug Administration, Silver Spring, MD 20993, USA

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ABSTRACT — The assessment of xenobiotic-induced testicular toxicity is important in drug development. Nonetheless, in vitro models to test drugs and chemicals that may cause testicular toxicity are lacking, requiring the continued use of animal models for those studies. We previously evaluated an in vitro mouse testis organ culture system using ethinylestradiol (EE), a well-studied testicular toxicant, and demonstrated a dose-dependent relationship between adverse effects to germ cell differentiation and increasing EE concentrations. However, we terminated that study after 20 days of culture due to oxygen deficiency during germ cell differentiation. Therefore, in the current study, we aimed to identify gene(s) with potential for supporting the histopathological evaluations of testicular toxicity using in vitro testis organ culture system. We cultured testis fragments obtained from mice at postnatal day (PND) 5 in α-Minimal Essential Medium containing 40 mg/mL AlbuMAX™ I and treated them with 0.01 or 1 nM EE on day 1 of culture. On day 20, we collected testis fragments for RNA sequencing analysis and quantitative polymerase chain reaction (qPCR). We found that phospholipase C, zeta 1 and testis-specific serine kinase 4 genes, that are involved in spermatogenesis and predominantly expressed in the testis, were significantly reduced in testis fragments treated with the highest concentration of EE. Also, cytochrome P450, family 26, subfamily b, polypeptide 1 (Cyp26b1) and interleukin 16 (Il16) were up-regulated in the highest EE-treated groups. Further studies are needed to confirm the variations of these gene expression using other testicular toxicants.

Key words: Ethinylestradiol, Gene expression, In vitro mouse testis organ culture, Poly(A) RNA-seq sequencing

INTRODUCTION

General toxicity testing is fundamental in drug development and assessment of testicular toxicity is crucial to support adequate prescription information. Testicular toxicity may alter the normal development of male reproductive organs, reduce reproductive performance and cause infertility (Sasaki et al., 2011; ICH Draft guideline S5(R2), 2005; Semet et al., 2017; Sousa et al., 2017). However, reliable in vitro testing methods to measure drug toxicity in the testis are lacking. In recent years, in vitro toxicity models have reduced the use of experimental animals and saved considerable time and resources (Jain et al., 2018). Evaluating spermatogenesis is a common and important endpoint for testicular toxicity. Currently, two models are used to culture testis tissue: one consists of culturing of the whole testis, or fragments of the testis (Steinberger and Steinberger, 1970); the other consists of culturing of isolated testicular cells (Adhikari et al., 2000; Yu et al., 2005, 2009). The lack of a reliable in vitro model limits our options to the use of in vivo studies for obtaining data to support identifying biomarkers of testicular toxicity. We previously evaluated an in vitro mouse testis organ culture system established by Sato et al. (2011) who showed that viable, mature mouse sperm was produced with culturing the whole testis. In our evaluation, the goal was to ascertain whether the model could be used to assess drug- and chemical-induced toxicity in testicular tissue. We used ethinylestradiol (EE), a well-studied testicular toxicant (Iwase et al., 1995; Kaneto et al., 1999; Naciff et al., 2005; Shimomura et al., 2005) and evaluated histopathology without completely disturb-
ing the seminiferous tubule (ST) structure. Testis fragments from mice at postnatal day (PND) 5 were treated with EE. Resulting histological analysis showed that the culture system was limited in treatment time (for 20 days) due to germ cell degeneration and necrosis from oxygen deficiency. Further histopathology analysis was successful in evaluating the percentage of the number of STs with germ cell differentiation vs. any dead germ cells and showed a significant dose dependency in the highest treatment group (Nakamura et al., 2019). As a complement to histological examination in our previous study, the aim of the present study was to identify gene(s) that could be used as a supplement for assessing testicular toxicity in the testis fragments. Identified genes, showing variation in gene expression, may provide a more accurate assessment of toxicity than histology alone. In this study, the gene expression variations were measured using RNA sequencing analysis (Poly(A) RNA-seq sequencing), followed by quantitative polymerase chain reaction (qPCR) validation.

MATERIALS AND METHODS

Materials
All reagents and chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA) and Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

Animals
All animal procedures were approved by the NCTR Institutional Animal Care and Use Committee and followed the guidelines set forth by the National Research Council Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). Briefly, 7–8-week-old C57BL/6J breeders were obtained from Jackson Laboratory. Mice were housed and maintained under a 12:12-hr light/dark cycle with controlled room temperature (23°C ± 3°C) and humidity (50% ± 20%). Water and chow (low-phytoestrogen chow, Purina 5K96; Purina Mills LLC, St. Louis, MO, USA) were provided ad libitum. Low phytoestrogen chow was chosen to minimize estrogenic background signals in the animals. Testis samples were collected from postnatal C57BL/6J mice and separated into four similarly sized pieces as described by Nakamura et al. (2017).

In vitro testis organ culture and chemical treatment
Male pups were euthanized on PND 5 (day of birth = PND 0) by carbon dioxide inhalation for 4–5 min, followed by decapitation. Testes were removed from the abdominal area and placed in α-Minimal Essential Medium containing AlbuMAX™ I (Albumax) (Life Technologies, Carlsbad, CA, USA). After removing the tunica albuginea, each testis was subsequently divided into four pieces. Two to three fragments from the same testis were collected and placed onto a section (approx. 1 cm²) of 1.5% agarose gel (Dojindo Molecular Technologies, Rockville, MD, USA). Two to three gels were each placed in six-well plates and cultured at 34°C in an atmosphere of 5% CO₂. After 24 hr, the testis cultures were exposed to two different concentrations (0.01, and 1 nM) of EE (Cat #: E4876; CAS #: 57-63-6; Sigma-Aldrich, St. Louis, MO, USA). The vehicle (control) was a 1-mM stock of DMSO (Life Technologies). The media were changed every 4–7 days. On culture day 20 (corresponding to PND 25) (Nakamura et al., 2019), testis fragments were collected and used for histology, RNA sequencing, and qPCR analysis.

Quantification of histology evaluation for testicular toxicity
Cultured testis fragments were placed in Bouin’s fixative (IMEB Inc., San Marco, CA, USA) for 2 hr at 4°C, then held in 70% ethanol at 4°C until processing. Testis fragments were then paraffin embedded following a standard procedure. Serial sections (4 µm) were then cut and mounted onto slides. After deparaffinization, sections were stained with hematoxylin QS (Vector Laboratories, Burlingame, CA, USA) for 30 sec, washed under running water, and stained with a 1% eosin/alcohol solution (Sigma-Aldrich) for 3 min. The sections were then dehydrated and mounted with Poly-Mount® (Polysciences, Warrington, PA, USA). Images were acquired with Leica/Aperio ScanScope AT2 whole slide scanner and Leica ImageScope software (Leica Biosystems; Buffalo Grove, IL, USA). Using light microscopy, STs with any dead germ cells or round spermatids were counted in hematoxylin and eosin (H&E)-stained testis fragments. For quantification, the number of STs with any dead germ cells or round spermatids were divided by the number of STs exhibiting germ cell differentiation on a cross-section. STs without germ cell differentiation were excluded from the analysis. When STs exhibited partial germ cell differentiation, we counted the STs as those with germ cell differentiation.

RNA extraction
Approximately 6–8 cultured testis fragments were required to achieve a detectable RNA concentration. Total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Valencia CA, USA). The RNA concentra-
RNA sequencing analysis in testis fragments in vitro

Poly(A) RNA-seq sequencing and data analysis

Due to low RNA sample concentrations, RNA sequencing was chosen because it can detect novel genes and/or transcripts and has a higher sensitivity than microarray analysis (Wang et al., 2009). To identify gene(s) to supplement the histopathological evaluations of testicular toxicity using in vitro testis organ culture system, poly(A) RNA-seq sequencing, including cDNA library preparation and data analysis, was performed by LC Sciences (Houston, TX, USA). Briefly, total RNA (n = 4 per group) was qualified using a Bioanalyzer 2100 and a Total RNA Nano Chip (Agilent, CA, USA). Poly(A) enrichment with poly-T magnetic beads (Invitrogen) was performed using 1 µg of total RNA, with an RIN value of 9 or higher. Following purification, the poly(A) mRNA fraction was fragmented using divalent cations at an elevated temperature. Cleaved RNA fragments were then reverse transcribed to create a final cDNA library in accordance with the TruSeq Stranded mRNA Sample Preparation Guide (Illumina, Catalog # RS-122-9004DOC, Part # 15031047 Rev. E). The insert size for the paired-end libraries was 70–370 bp. Paired-end, 2 × 150-bp sequencing was performed on an Illumina HiSeq 4000 platform according to the vendor’s recommended protocol.

Cutadapt (Martin, 2011) and Perl scripts were used to remove reads containing adaptor contamination, low-quality bases, and/or undetermined bases. Sequence quality was then verified using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). HISAT2 (Kim et al., 2015) was then used to map reads to the genome of Mus musculus (Version: v88). The mapped reads of each sample were assembled using StringTie (Pertea et al., 2015). All transcriptomes from the 12 samples were then merged to reconstruct a comprehensive transcriptome using Perl scripts and gffcompare (https://github.com/gpetea/gffcompare/). After the final transcriptome was generated, StringTie and Ballgown (Frazee et al., 2015) were used to estimate the expression levels of the transcripts. StringTie was used to determine the expression levels of mRNAs by calculating FPKM (FPKM = [total_exon_fragments/ mapped_reads(millions)×exon_length(kB)]). Differentially expressed mRNAs were selected with log2 (fold change) > 1 or log2 (fold change) < -1, and with parametric F-tests comparing nested linear models (p < 0.05) by R package Ballgown.

**cDNA synthesis and qPCR**

cDNA was synthesized using Superscript IV VILO Master Mix (Thermo Fisher Scientific, Carlsbad, CA, USA) and 0.8 µg of RNA. qPCR analysis was performed using an ABI PRISM 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA), with PowerUp SYBR Green Master Mix (Thermo Fisher Scientific Inc., Rockville, MD, USA) according to the manufacturers’ protocols. Specific primer pairs are shown in Table 1. The synthesized cDNA template was used in a 10 µL reaction mixture. Reaction conditions were as follows: initial denaturation steps at 50°C for 2 min and 95°C for 2 min, followed by 40–45 amplification cycles (95°C for 15 sec, 60°C for 1 min) and a dissociation step (95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec). Relative steady-state transcript levels were calculated using threshold cycle (Ct) values and the following equation: relative quantity = 2^(-ΔΔCt) (Livak and Schmittgen, 2001). Expression levels were normalized using Rnl18s as an internal control for each sample. Relative ratios of the transcript levels in each sample were then calculated using the value for the control as 1. qPCRs for each sample were run in triplicate.

**Statistical analysis**

Values are presented as the mean ± standard error of the mean. Statistical analyses of the pathology results were performed using one-way ANOVA analysis, followed by Tukey’s test. Statistical analyses for the qPCR results were performed using t-tests with Bonferroni adjustment (Hochberg, 1988; Shaffer, 1995). For all comparisons, the significance level was set at p < 0.05.

**RESULTS**

**Histology analysis**

Damage to the testicular tissue by EE exposure was determined by counting the number of STs containing dead germ cells and round spermatids in H&E-stained sections (Fig. 1). Dead germ cells (condensed nucleus; pyknotic changes) were observed in 1 nM EE treated group (arrows, Fig. 1A, right panel) when compared to the control group (Fig. 1A, left panel). The percentages of the number of the STs with any dead germ cells were as follows: control, 48.2%; 0.01 nM EE, 50.4%; and 1 nM EE, 77.8% (Fig. 1B, left). Only the 1 nM EE-treated group resulted in a significantly higher number of STs with dead cells compared to the control (Fig. 1, left panel). The percentages of STs with round spermatids were as follows: control, 18.0%; 0.01 nM EE, 7.1%; and 1 nM EE, 5.1%. These results were statistically significant (Fig. 1B, right).
Gene ontology analysis of testis fragments treated with EE

Gene ontology (GO) analysis showed a number of genes related to three domains: biological process, followed by cellular components, and, finally, molecular function using the differentially expressed genes (Supplemental Fig. 1). Membrane, cytoplasm, and nucleus categories were related to the cellular component, while molecular function and protein-binding categories were considered higher categories within the molecular function domain (Supplemental Fig. 1). The GO enrichment scatterplot shows that the sperm principal piece, spermatogenesis, spermatid differentiation and development, sex determination, motile cilium, inner dynein arm, epithelial cilium movement, cilium movement and cilium were significantly altered \((p < 1e^{-4})\). However, the actual number of altered genes were small (gene number of GO enrichments was less than 200) (Fig. 2A). This was followed

| Genes       | Primer sequences                                                                 | Amplified size (bp) | GenBank Accession |
|-------------|----------------------------------------------------------------------------------|---------------------|-------------------|
| mCyp26b1    | For: 5’-TGC CCA TAC CCC ATC GCC-3’<br>Rev: 5’-AGA TGG CAA GAT GTA GGA A-3’         | 84                  | NM_001177713.1    |
| mAkr1c14    | For: 5’-AAG TCT CAA GAC CTG CGT GG-3’<br>Rev: 5’-CCT TAT CGG GCA CAG TGG TT-3’     | 123                 | NM_134072.1       |
| mRad9a      | For: 5’-AGG CTG TCC ATT CGC TAT CC-3’<br>Rev: 5’-CAG AAG GGC ACG AGT TCA CA-3’    | 96                  | NM_011237.2       |
| mGrebl1     | For: 5’-GTC CAG AGG GTG ACA TTG ACA T-3’<br>Rev: 5’-TCT GGG TGC TGG AAT TTG TGC-3’ | 150                 | NM_001252071.1    |
| mTssk4      | For: 5’-GTG CAC CGG GAT TTA AAG TTG-3’<br>Rev: 5’-CAT TTT GCG GTA AGA GC-3’       | 132                 | NM_027673.3       |
| mPlcz1      | For: 5’-CTG GCA TTT ATA GGT TTG TT-3’<br>Rev: 5’-ACA CGA CGA TAA CCT TCG A-3’     | 113                 | NM_054066.4       |
| mCyt1l      | For: 5’-GCC AGC AGC ATT AGA GTG C-3’<br>Rev: 5’-GCA TGG ATC CTG TTT GGC C-3’      | 83                  | NM_001360087.1    |
| mNdufa6     | For: 5’-AGG CAG CGG ACA CAC GTT AT-3’<br>Rev: 5’-CAT GCA CCT TCC CAT CAG GT-3’     | 120                 | NM_025987.3       |
| mAcrv2a     | For: 5’-TAC GCA CAC CCT ATT ACA-3’<br>Rev: 5’-TGA AAG GCC AGT TGA GGT GGA-3’      | 148                 | XM_00649723.3     |
| mNtn4       | For: 5’-GTC GAG AGG AGG TGT ATG TG-3’<br>Rev: 5’-CGT TAG GAG CCC CTG TTG CG-3’     | 118                 | NM_021320.3       |
| mSpef2      | For: 5’-CTG CAC CTG CAG CCA CCA ATC-3’<br>Rev: 5’-TCC CAG ACC CTG CCA CTC TCC-3’  | 81                  | NM_001305042.1    |
| mVsp36      | For: 5’-GGA GAG AGA GAA CCA CAG TGA GA-3’<br>Rev: 5’-GTC AGT CCG TCG GTG TTA-3’    | 123                 | NM_007488.3       |
| mArnt2      | For: 5’-GAC GCT TGA GCC TCG GGT G-3’<br>Rev: 5’-TGC TTT GAC TGA ATG ATA-3’        | 129                 | NM_009292.2       |
| mHspa4l     | For: 5’-GAA GCC GGA GAA CCA CAG GGTT TTT-3’<br>Rev: 5’-TGG GTA GCC CTA CCT TGA-3’  | 142                 | XM_006500766.3    |
| mGpat4      | For: 5’-GGT GCC TAA GAG GAG GTG TG-3’<br>Rev: 5’-AGG CAG CAG AAA ACT GA-3’<br>Rev: 5’-ACG CTG CAC TGA TGG TTT-3’ | 182                 | NM_018743.4       |
| mMtxs1      | For: 5’-GTC GGG CTT GGG GTT GGG GG-3’<br>Rev: 5’-CTT CCG TGG TCC TTC TCC-3’       | 129                 | NM_144800.2       |
| mDnah7a     | For: 5’-ACT GCG TGG TTT TTT TTT TTT-3’<br>Rev: 5’-AAC GGC GGT GTG TTT TTT TTT-3’   | 133                 | NM_001252070.1    |
| mLuzp4      | For: 5’-GT TAA AGA GAC ACC ACC ACA GC-3’<br>Rev: 5’-CAC GGT TGG TTA CTA CCT TTG-3’ | 88                  | NM_001114383.1    |
| mRn18s      | For: 5’-GAC CGG GGG AGG TAG TGA CAA-3’<br>Rev: 5’-GGA GCT GGA ATT ACC GGG CTC-3’   | 141                 | NR_003278.3       |

Table 1. Primer pairs for qPCR.
by GO enrichment: regulation of cellular protein localization, Ras GTPase binding, inactivation of MAPK activity, and protein tyrosine/threonine phosphatase activity, with cellular differentiation and positive regulation of GTPase activity were significantly changed ($p < 2e^{-4}$). KEGG pathway analysis ($p < 0.01$) showed significantly altered pathways accompanied by over 20 altered genes related to the cell cycle, pyrimidine and purine metabolism ($p < 0.01$), followed by ubiquitin-mediated proteolysis, thyroid hormone signaling pathways, oocyte meiosis, and Huntington’s disease ($0.01 < p < 0.03$) (Fig. 2B).

**Gene expression profiles of testis fragments treated with EE**

Hierarchical clustering analysis identified 100 genes that were significantly altered in the testis fragments treated with EE (Figs. 3, 4). Among them, we identified three gene sub-groups: (1) down-regulated genes in the 1 nM treated group, (2) up-regulated gene in the 1 nM treated group, and (3) up-regulated genes in the 0.01 nM group (gene information in Supplemental Table 1). Of the 45 down-regulated genes, 26 genes have an unknown function, while 16 genes were testis-specific or known to be abundantly expressed in the testis.

Of the 55 up-regulated genes, ten have unknown function, while four were known to be abundantly expressed in the testes. In down-regulated genes in the 1 nM treated group, testis-specific serine kinase 4 ($Tssk4$), dynein, axonemal, heavy chain 14 ($Dnah14$), sperm flagellar 2 ($Spef2$) and phospholipase C, zeta 1 ($Plcz1$) genes that are related to spermatogenesis or sperm structure (Vaughan et al., 1996; Sironen et al., 2010, 2011; Schrimpf et al., 2011).

**Fig. 1.** Evaluation of testis morphology in cultured testis fragments treated with ethinylestradiol (EE). (A) Testis fragment sections treated with 0, 0.01, and 1 nM EE were stained with hematoxylin and eosin (left, middle and right panels, respectively). Dead cells were observed in 1 nM EE group (arrows). Bars: 60 μm. (B) The percentage of seminiferous tubules (STs) with dead germ cells (left graph) and round spermatids (right graph) in mouse testis fragments. Values represent the mean ± standard error (n = 8–12 testis fragments). Percentages are based on the number of STs with germ cell differentiation in whole testis fragment sections. *$p < 0.05$ compared with the control group; **$p < 0.01$ compared with the control group.
2014; Wang et al., 2015a, 2015b). The up-regulated gene in the 1 nM treated group, leucine zipper protein (Luzp4), testis expressed gene 11 (Tex11), stimulated by retinoic acid gene 8 (Stra8) have testis-specific expression (Supplemental Table 1). Interleukin 16 (IL16), netrin 4 (Ntn4), vacuolar protein sorting 36 (Vps36), RAD9 checkpoint clamp component A (Rad9a), gene regulated by estrogen in breast cancer protein (Greb1), and activin receptor IIA (Acrv2a) were identified. Up-regulated genes in the 0.01 nM group, myeloid derived growth factor (Mydgf) and asparagine synthetase domain containing 1 (Asnsd1) were identified. 

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**Gene expression validation using qPCR**

Selected gene function groups were validated using qPCR. The gene function groups in the testis fragments treated with or without EE included spermatogenesis, sperm flagellum, cell cycle (including cancer-regulated genes), cytokines, osmotic stress response, tumor suppressors, mitochondria, endocytosis, steroidogenesis, and estrogen-responsive genes/transcripts (Table 2). Down-regulated gene and transcript levels were measured for Plcz1, Tssk4, dynein, axonemal heavy chain 7A (Dnah7a), cytokine-like 1 (Cyt1l), heat shock protein 4 like (Hspa4l), phospholipase C, and NADH:ubiquinone oxidoreductase subunit A6 (Ndufa6). Up-regulated genes and transcript levels were measured for Acrv2a, cytochrome P450, family 26, subfamily b, polypeptide 1 (Cyp26b1), Luizp4, Ntn4, II-16, metastasis suppressor 1 (Msis1), glycerol-3-phosphate acyltransferase 4 (Gpat4), Vps36, aldo-keto reductase family 1, member C14 (Akr1c14), Rad9a, Spef2, Stra8, Arnt2, and Greb1.

The Plcz1 gene expression was reduced in a dose-dependent manner; this reduction was statistically significant at 1 nM EE (Fig. 5). Tssk4 gene was significantly lower in the 1 nM EE treated group than the control group (0 nM) (Fig. 5). Expression level of Cyp26b1 and Il16 was higher in 0.01 and 1 nM treated groups than in the control group; however, no statistical significance was observed between the groups because of variations among samples (Fig. 5). The expression of the Cyt1l and...
Gpat4 genes decreased dose-dependently, and transcript levels of the Akr1c14 and Ndufa6 genes were dose-dependently increased, but no statistical significances were observed. In addition, no significant differences in the expression of the remaining genes were observed (Supplemental Fig. 2).
DISCUSSION

The aim of this study was to identify and evaluate the expression of genes that could be used as supplements to assess testicular toxicity in vitro. We cultured testis fragments obtained from PND 5 mice, treated them with 0 (control), 0.01, and 1 nM EE for 20 days, as previously described, and collected the cultured fragments as samples for gene expression profiling. Using RNA sequencing analysis including a hierarchical clustering analysis (HCA) of transcripts in cultured testis fragments treated with ethinylestradiol (EE). For statistical HCA, differentially expressed mRNAs were selected with log2 (fold change) > 1 or log2 (fold change) < -1 and with the parametric F-test comparing nested linear models ($p < 0.05$) by R package Ballgown (Frazee et al., 2015). In HCA, up-regulated genes are represented in red, while down-regulated genes are represented in blue.
RNA sequencing analysis in testis fragments *in vitro*

**Fig. 5.** Relative transcript levels of the cultured testis fragments treated with ethinylestradiol (EE). Expression levels of Plcz1, Tssk4, IL16, and Cyp26b1 genes were determined by qPCR. Data are expressed as the mean fold change ± standard error (n = 6–7 per group). *p < 0.05 compared with the control group.

Using qPCR, we found that transcript levels of the Plcz1 and Tssk4 genes were decreased at 1 nM with significant differences in the 1 nM treated group in this study. This decrease in genetic expression corresponded to an increased percentage of STs with dead cells, and a decreased percentage of STs with round spermatids. Although no statistic significances were observed, the transcript levels of Cyp26b1 and IL16 genes were increased in the testis fragments treated with EE compared to the control group.

Other *in vivo* and *in vitro* studies of gene expression after testis exposure to endocrine-disrupting chemicals, EE, or phthalates have reported the involvement of genes related to steroidogenesis, apoptosis, stress response, or changes in cholesterol metabolism and mitochondria (Lahousse et al., 2006; Yu et al., 2009). The remaining genes related to stress responses, steroidogenesis and so on, did not result in any significant expression differences between control and treatment groups (Supplemental Fig. 2). This may be due to the variability of testis fragments caused by the delay of testis/germ cell development compared to *in vivo* in the first wave of spermatogenesis (Pence et al., 2019) or their lower expression in the testis fragments at day 20 of culture.

The Plcz1 and Tssk4 genes were found to be significantly reduced in EE-treated testis fragments. Although no previous studies have reported gene expression profiling of *in vitro* testis fragments treated with EE, Naciff et al., (2005) examined gene expression profiling using gestation day (GD) 20 rat testes exposed to 0.001–10 µg/kg/
Table 2. Functional categories for genes and transcripts on heatmaps.

| Function                        | Genes                                      | References |
|---------------------------------|--------------------------------------------|------------|
| Spermatogenesis                 | Acvr2a↑; Cyp26b1↓; Plce1↓; Tssk4↓; Stra8↑  | Zhou et al., 2008; Tan et al., 2005; Schrumpf et al., 2014; Hogarth et al., 2015; Wu et al., 2017; Wang et al., 2015a; 2015b; |
| Sperm flagellum                 | Dnah7a↓; Lceu4↑; Spef2↓                   | Neesen et al., 1997; Sironen et al., 2010, 2011 |
| Cell cycle including cancer related genes | Nid4↑                                      | Eveno et al., 2011 |
| Cytokine                        | Cyl1↓; Il16↑                               | Chao et al., 2011; Purzycka-Bohdan et al., 2016 |
| Stress response                 | Hspa4L↓                                    | Held et al., 2006 |
| Tumor suppressor                | Mis1↑; Amt2↑                               | Zeleniak et al., 2017; Yang et al., 2015 |
| Mitochondria                    | Gpat4↑; Cyp26b1↓; Ndufa6↓                  | Vijay et al., 2016 |
| Membrane trafficking            | Vps36↑; Cyp26b1↓                           | Wang and Hong, 2006 |
| Steroidogenesis                 | Akr1c14↑                                  | Ge et al., 1999; Di-Luoffo et al., 2016 |
| Estrogen responsive genes/transcripts | Rad9a↑; Amt2↑; Akr1c14↑; Greb1↑          | Sun et al., 2007; Kretzschmar et al., 2010; Karlsson et al., 2011 |

The up-arrow indicates up-regulated and the down-arrow indicates down regulated.

day EE from GD 11 through 20 in vivo. In addition, some in vivo and in vitro studies have reported gene expression profiles of animal testis or cultured isolated testicular cells exposed to phthalates (Li et al., 2016; Lahousse et al., 2006). However, Plce1 and Tssk4 genes were not identified in those studies. As experimental designs (end-point and chemicals) may differ from study to study, the results of gene expression profiles may also vary.

The function of Cyp26b1 gene is thought to aid germ cell survival and control retinoic acid levels in testis development (MacLean et al., 2007). This study found that Cyp26b1 gene expression are increased at 0.01 or 1 nM treated group. The increased Cyp26b1 gene expression may protect germ cells from apoptosis induced by EE treatments. Il16 cytokine gene has an important role of the responses to inflammation, immune response (Hessel et al., 1998); however, Il16 gene’s function in testis remains unclear. Anastasiadou and Michailidis (2016) reported that Il16 gene expression was increased in rooster testis after Salmonella infection, suggesting that cytokines, including Il16, may be related to immune response to infection. Testicular toxicity induced by the higher concentration of EE may have caused an increase in cytokine gene expression via immune response. Similar statistical significances were not observed in Cyp26b1 and Il16 genes expression in the testis fragments treated with EE; possibly due to lower expression levels or variation in the testis fragments.

Using RNA sequencing analysis, we found that 16 genes of the 45 down-regulated genes (26 genes were unknown) had testis-specific expression or were predominantly expressed in the testis according to NCBI database searching. Our finding of changes of the transcript levels of the genes that express testis-specific or abundant in testis are similar to a report of gene expression profiles in the testis of rats exposed to the highest dose of the sunscreen compound oxybenzone (Nakamura et al., 2018). As testis-specific genes have been previously reported to play a role in spermatogenesis (Dix et al., 1996), the exposure to highest dose of chemicals probably affects testis growth, resulting in altered gene expression changes that are related to spermatogenesis. In addition, as the GO terms via GO analysis in this study are thought to be related with spermatid and flagellum formation, these identified genes were constant and EE exposure may affect spermatid differentiation. The Plce1 gene is expressed from secondary spermatocytes through elongated spermatids and spermiogenesis (Rengaraj et al., 2008; Aarabi et al., 2012). As round spermatids were observed in testis fragments treated with EE for 20 days, EE exposure may alter transcript levels of the Plce1 gene, resulting in disturbed round spermatid differentiation. The TSSK4 protein is related to apoptosis and plays an important role in sperm flagellum development (Wang et al., 2015a, 2015b). As these genes are related to spermatogenesis and have testis-specific expressions, these genes may be used as potential biomarkers to assess testicular toxicity.

This study identified four genes (Plce1, Tssk4, Cyp26b1 and Il16 genes) as potential supplemental genes for assessing testicular toxicity in vitro. This in vitro mouse testis organ culture system was limited to 20 days of culture because of germ cell degeneration in the seminiferous tubules. When histological evaluation of testis fragments is not performed using enough seminiferous tubules with germ cell differentiation or the criteria of histological evaluation is not consistent, these gene expression
profiles may be useful in determining testicular toxicity in vitro. Plcz1 and Tsks4 genes can be used for examining the effects of testicular toxicity to germ cell differentiation; Cyp26b1 gene will be used for testicular cell survival; and Il16 gene will be used for immune response.

Yu et al. (2009) reported that gene expression profiles of rat testicular cells exposed in vitro to various phthalates identified 12 genes with significant variations: e.g., steroidogenic enzyme genes [steroidogenic acute regulatory protein (Star), cytochrome P450, family 19, subfamily a polypeptide 1 (Cyp19a1)], and aldo-keto reductase family genes [aldo-keto reductase family 1, member B8 (Akr1b8)]. Our previous study validated Cyp11a1 genes that were significantly reduced at the highest dose of EE via qPCR (Nakamura et al., 2019). In this study, we identified the Akr1c14 gene that encodes 3α-hydroxysteroid dehydrogenase which catalyze the conversion of dihydrotestosterone reversibly in mouse Leydig cells (Ge et al., 1999). We consider that the genes related to steroidogenesis may be potential candidates for biomarker(s) for assessing testicular toxicity using in vitro models. Further experiments are necessary to examine gene expression profiles in vitro using a variety of well-known testicular toxicants.

In conclusion, this study found that transcript levels of the Plcz1 and Tsks4 genes, both expressed abundantly in the testis, were significantly reduced in testis fragments treated with the highest concentration of EE for 20 days. This reduced expression was accompanied by an increase in the percentage of seminiferous tubules with dead germ cells. Also, Cyp26b1 and Il16 genes were up-regulated in the testis fragments treated with EE. Our present and previous studies validated the genes related to steroidogenesis and spermatogenesis to be altered underlying EE treatment using in vitro mouse organ culture system. However, of the 100 genes identified by RNA sequencing analysis, only 2 genes were validated using qPCR that resulted in statistically significant differences in testis fragments treated with EE. As the limited results may have been due to a wide-variety of germ cell differentiation in testis fragments, the efforts are necessary to develop improved in vitro models with testis tissues. Furthermore, future testicular culture studies using other testicular toxicants are needed to i) confirm this gene expression profile by increasing sample number and to ii) identify additional genes able to assess testicular toxicity in vivo and in vitro.

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Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Aarabi, M., Yu, Y., Xu, W., Tse, M.Y., Pang, S.C., Yi, Y.J., Sutovsky, P. and Oko, R. (2012): The testicular and epididymal expression profile of PLCζ in mouse and human does not support its role as a sperm-borne oocyte activating factor. PLoS One, 7, e33496.

Adhikari, N., Sinha, N. and Saxena, D.K. (2000): Effect of lead on Sertoli-gonadotropin-culture of rat. Toxicol. Lett., 116, 45-49.

Anastasiadou, M. and Michailidis, G. (2016): Transcriptional changes of cytokines in rooster testis and epididymis during sexual maturation stages and Salmonella infection. Anim. Reprod. Sci., 171, 41-48.

Chao, C., Joyce-Shaikh, B., Grein, J., Moshrefi, M., Raoufi, F., Laface, D.M., McClanahan, T.K., Bourne, P.A., Pierce, R.H., Gorman, D.M. and Pflanz, S. (2011): C17 prevents inflammatory arthritis and associated joint destruction in mice. PLoS One, 6, e22256.

Di-Luoffo, M., Brousseau, C. and Tremblay, J.J. (2016): MEF2 and NR2F2 cooperate to regulate Akr1c14 gene expression in mouse MA-10 Leydig cells. Andrology, 4, 335-344.

Dix, D.J., Allen, J.W., Collins, B.W., Mori, C., Nakamura, N., Poorman-Allen, P., Goulding, E.H. and Eddy, E.M. (1996): Targeted gene disruption of Hsp70-2 results in failed meiosis, germ cell apoptosis, and male infertility. Proc. Natl. Acad. Sci. USA, 93, 3264-3268.

Eveno, C., Broqueres-You, D., Feron, J.G., Rampanou, A., Tijeras-Raballand, A., Ropert, S., Leconte, L., Levy, B.I. and Rocard, M. (2011): Nettin-1 delays colorectal cancer carcinomatosis by inhibiting tumor angiogenesis. Am. J. Pathol., 178, 1861-1869.

Frazee, A.C., Perta, E.A., Jaffe, A.E., Langmead, B., Salzberg, S.L. and Leek, J.T. (2015): Ballgown bridges the gap between transcriptome assembly and expression analysis. Nat. Biotechnol.,
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33, 243-246.

Ge, R.S., Hardy, D.O., Catterall, J.F. and Hardy, M.P. (1999): Opposing changes in alpha-hydroxysteroid dehydrogenase oxidative and reductive activities in rat Leydig cells during pubertal development. Biol. Reprod., 60, 855-860.

Held, T., Paprotta, I., Khulan, J., Hemmerlein, B., Binder, L., Wolf, S., Schubert, S., Meinhardt, A., Engel, W. and Adham, I.M. (2006): Hsp40-deficient mice display increased incidence of male infertility and hydronephrosis development. Mol. Cell. Biol., 26, 8099-8108.

Hessel, E.M., Cruikshank, W.W., Van Ark, I., De Bie, J.J., Van Oosterhout, A.J. (1998): Involvement of IL-16 in the induction of airway hyper-responsiveness and up-regulation of IgE in a murine model of allergic asthma. J. Immunol., 160, 2998-3005.

Hochberg, Y. (1988): A sharper Bonferroni procedure for multiple tests of significance. Biometrika, 75, 800-802.

Hogarth, C.A., Evans, E., Onken, J., Kent, T., Mitchell, D., Petkovich, M. and Griswold, M.D. (2015): CYP26 Enzymes Are Necessary Within the Postnatal Seminiferous Epithelium for Normal Murine Spermatogenesis. Biol. Reprod., 93, 19.

ICH Draft guideline S5(R2). (2005): Detection of Toxicity to Reproduction for Medicinal products & Toxicity to Male Fertility. https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Safety/S5/Step4/S5_R2_Guideline.pdf

Iwase, T., Sano, F., Murakami, T. and Inazawa, K. (1995): Male reproductive toxicity of ethinylestradiol associated with 4 weeks daily dosing prior to mating in rats. J. Toxicol. Sci., 20, 265-279.

Jain, A.K., Singh, D., Dubey, K., Maurya, R., Mittal, S. and Pandey, A.K. (2018): Chapter3 Models and methods for in vitro toxicity. In: In Vitro Toxicology, pp.45-65 Academic Press.

Karlsson, E., Waltersson, M.A., Bostner, J., Perez-Tenorio, G., Olsson, B., Hallbeck, A.L. and Stål, O. (2011): High-resolution genomic analysis of the 11q13 amplicon in breast cancers identifies synergy with 8p12 amplification, involving the mTOR target. Birth Defects Res., 93, 269-280.

Kaneto, M., Kanamori, S., Hishikawa, A. and Kishi, K. (1999): Epididymal sperm motion as a parameter of male reproductive toxicity: sperm motion, fertility, and histopathology in ethinylestradiol-treated rats. Reprod. Toxicol., 13, 279-289.

Kim, D., Langmead, B. and Salzberg, S.L. (2015): HISAT: a fast spliced aligner with low memory requirements. Nat. Methods, 12, 357-360.

Kretzschmar, G., Papke, A., Zierau, O., Möller, F.J., Medjakovic, S., Jungbauer, A. and Vollmer, G. (2010): Estradiol regulates aryl hydrocarbon receptor expression in the rat uterus. Mol. Cell. Endocrinol., 321, 252-257.

Lahousse, S.A., Wallace, D.G., Liu, D., Gaido, K.W. and Johnson, K.J. (2006): Testicular gene expression profiling following prepubertal rat mono-(2-ethylhexyl) phthalate exposure suggests a common initial genetic response at fetal and prepubertal ages. Toxicol. Sci., 93, 369-381.

Li, J., Hou, R., Niu, X., Liu, R., Wang, Q., Wang, C., Li, X., Hao, Z., Yin, G. and Zhang, K. (2016): Comparison of microarray and RNA-Seq analysis of mRNA expression in dermal mesenchymal stem cells. Biotechnol. Lett., 38, 33-41.

Livak, K.J. and Schmittgen, T.D. (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2^(-ΔΔCt) Method. Methods, 25, 402-408.

Martin, M. (2011): Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBNet J., 17, 10-12.

MacLean, G., Li, H., Metzger, D., Chambon, P. and Petkovich, M. (2007): Apoptotic extinction of germ cells in testes of Cyp26b1 knockout mice. Endocrinology, 148, 4560-4567.

Naciff, J.M., Hess, K.A., Overmann, G.J., Torontali, S.M., Carr, G.J., Tiesman, J.P., Foerster, L.M., Richardson, B.D., Martinez, J.E. and Daston, G.P. (2005): Gene expression changes induced in the testis by transplacental exposure to high and low doses of 17alpha-ethynyl estradiol, genistein, or bisphenol A. Toxicol. Sci., 86, 396-416.

Nakamura, N., Merry, G.E., Inselman, A.L., Sloper, D.T., Del Valle, P.L., Sato, T., Ogawa, T. and Hansen, D.K. (2017): Evaluation of culture time and media in an in vitro tests organ culture system. Birth Defects Res., 109, 465-474.

Nakamura, N., Vijay, V., Desai, V.G., Hansen, D.K., Han, T., Chang, C.-W., Chen, Y.-C., Harrouk, W., McIntyre, B., Foster, P.M., Fuscoe, J.C. and Inselman, A.L. (2018): Transcript profiling in the testes and prostates of postnatal day 30 Sprague-Dawley rats exposed prenatally and lactationally to 2-hydroxy-4-methoxybenzophenone. Reprod. Toxicol., 82, 111-123.

Nakamura, N., Sloper, D.T. and Del Valle, P.L. (2019): Evaluation of an in vitro mouse testis organ culture system for assessing male reproductive toxicity. Birth Defects Res., 111, 70-77.

National Research Council. (2011): Guide for the care and use of laboratory animals. National Academy Press, Washington, D.C.

Neenes, J., Koehler, M.R., Kirschner, R., Steinle, C., Kreutzberger, J., Engel, W. and Schmid, M. (1997): Identification of dynin heavy chain genes expressed in human and mouse testis: chromosomal localization of an axonemal dynexin gene. Gene, 200, 193-202.

Pence, L.M., Schmitt, T.C., Beger, R.D., Del Valle, P.L. and Nakamura, N. (2019): Testicular function in cultured postnatal mouse testis fragments is similar to that of animals during the first wave of spermatogenesis. Birth Defects Res., 111, 270-280.

Pertea, M., Pertea, G.M., Antonescu, C.M., Chang, T.C., Mendell, J.T. and Salzberg, S.L. (2015): StringTie enables improved reconstruction of a transcriptome from RNA-seq reads. Nat. Biotechnol., 33, 290-295.

Purzycka-Bohdan, D., Szczerekowska-Dobosz, A., Zablotna, M., Wierzbicka, J., Piotrowska, A., Zmijewski, M.A., Ndoszytko, B. and Nowicki, R. (2016): Assessment of Interleukin 16 Serum Levels and Skin Expression in Psoriasis Patients in Correlation with Clinical Severity of the Disease. PLoS One, 11, e0165577.

Rengaraj, D., Kim, D.K., Zheng, Y.H., Lee, S.I., Kim, H. and Han, J.Y. (2008): Testis-specific novel transcripts in chicken: in situ localization and expression pattern profiling during sexual development. Biol. Reprod., 79, 413-420.

Sasaki, J.C., Chapin, R.E., Hall, D.G., Breslin, W., Moffitt, J., Saldutti, L., Enright, B., Seger, M., Jarvi, K., Hixon, M., Mitchard, T. and Kim, J.H. (2011): Incidence and nature of testicular toxicity findings in pharmaceutical development. Birth Defects Res. B Dev. Reprod. Toxicol., 92, 511-525.

Sato, T., Katagiri, K., Gohbara, A., Inoue, K., Ogonuki, N., Ogura, A., Kubota, Y. and Ogawa, T. (2011): In vitro production of functional sperm in cultured neonatal mouse testes. Nature, 471, 504-507.

Schrimpf, R., Dierks, C., Martinsson, G., Sieme, H. and Distl, O. (2014): Genome-wide association study identifies phospholipase C zeta 1 (PLCz1) as a stallion fertility locus in Hanoverian warmblood horses. PLoS One, 9, e106575.

Shaffer, J.P. (1995): Multiple hypothesis testing. Annu. Rev. psychol., 46, 561-584.

Semet, M., Paci, M., Saïas-Magnan, J., Metzler-Guillemain, C.
RNA sequencing analysis in testis fragments in vitro

Boissier, R., Lejeune, H. and Perrin, J. (2017): The impact of drugs on male fertility: a review. Andrology, 5, 640-663.

Shimomura, K., Shimada, M., Hagiwara, S., Harada, S., Kato, M. and Furuhama K. (2005): Insights into testicular damage induced by ethinylestradiol in rats. Reprod. Biol. Endocrinol., 20, 157-163.

Sironen, A., Hansen, J., Thomsen, B., Andersson, M., Vilki, J., Toppuri, J. and Kotaja, N. (2010): Expression of SPEF2 during mouse spermatogenesis and identification of IFT20 as an interacting protein. Biol. Reprod., 82, 580-590.

Sironen, A., Kotaja, N., Mulhern, H., Wyatt, T.A., Sisson, J.H., Pavlik, J.A., Miiluniemi, M., Fleming, M.D. and Lee, L. (2011): Loss of SPEF2 function in mice results in spermatogenesis defects and primary ciliary dyskinesia. Biol. Reprod., 85, 690-701.

Sousa, M., Ferreira, C., Rabaca, A. and Sa, R. (2017): Assessing male reproductive toxicity during drug development. Andrology (Los Angel.), 6, 185.

Steinberger, A. and Steinberger, E. (1970): Tissue culture of male mammalian gonads. In Vitro, 5, 17-27.

Sun, J., Nawaz, Z. and Slingerland, J.M. (2007): Long-range activation of GREB1 by estrogen receptor via three distal consen-sus estrogen-responsive elements in breast cancer cells. Mol. Endocrinol., 21, 396-405.

Tan, X.J., Xing, X.W., Li, L.Y., Wu, Z.D., Zhong, C.G., Nie, D.S., Fu, J.J., Xiang, Y., Deng, Y. and Lu, G.X. (2005): Molecular cloning of a novel mouse testis-specific spermatogenic cell apoptosis inhibitor gene mTSARG7 as a candidate oncogene. Acta Biochim. Biophys. Sin. (Shanghai), 37, 257-266.

Veughe, K.T., Mikami, A., Paschal, B.M., Holzbaur, E.L., Hughes, S.M., Echeverri, C.J., Moore, K.J., Gilbert, D.J., Copeland, N.G., Jenkins, N.A. and Vallee, R.B. (1996): Multiple mouse chromosomal loci for dynein-based motility. Genomics, 36, 29-38.

Vijay, V., Moland, C.L., Han, T., Fusco, J.C., Lee, T., Herman, E.H., Jenkins, G.R., Lewis, S.M., Cummings, C.A., Gao, Y., Cao, Z., Yu, L.R. and Desai, V.G. (2016): Early transcriptional changes in cardiac mitochondria during chronic doxorubicin exposure and mitigation by dextrazoxane in mice. Toxicol. Appl. Pharmacol., 295, 68-84.

Wang, T. and Hong, W. (2006): RILP interacts with VPS22 and VPS36 of ESCRT-II and regulates their membrane recruitment. Biochem. Biophys. Res. Commun., 350, 413-423.

Wang, X., Wei, Y., Fu, G., Li, H., Satyn, H., Lin, G., Wang, Z., Chen, S. and Yu, L. (2015a): Tssk4 is essential for maintaining the structural integrity of sperm flagellum. Mol. Hum. Reprod., 21, 136-145.

Wang, X.L., Wei, Y.H., Fu, G.L. and Yu, L. (2015b): Testis specific serine/threonine protein kinase 4 (TSSK4) leads to cell apoptosis relying on its kinase activity. J. Huazhong Univ. Sci. Technolog. Med. Sci., 35, 235-240.

Wang, Z., Gerstein, M. and Snyder, M. (2009): RNA-Seq: a revolutionary tool for transcriptomics. Nat. Rev. Genet., 10, 57-63.

Vu, F.J., Lin, T.Y., Sung, L.Y., Chang, W.F., Wu, P.C. and Luo, C.W. (2017): BMP8A sustains spermatogenesis by activating both SMAD1/5/8 and SMAD2/3 in spermatogonia. Sci. Signal., 10, eaal1910.

Yang, B., Yang, E., Liao, H., Wang, Z., Den, Z. and Ren, H. (2015): ARNT2 is downregulated and serves as a potential tumor suppressor gene in non-small cell lung cancer. Tumour Biol., 36, 2111-2119.

Yu, X., Sidhu, J.S., Hong, S. and Faustman, E.M. (2005): Essential role of extracellular matrix (ECM) overlay in establishing the functional integrity of primary neonatal rat Sertoli cell/gonocyte co-cultures: an improved in vitro model for assessment of male reproductive toxicity. Toxicol. Sci., 84, 378-393.

Yu, X., Hong, S., Moreira, E.G. and Faustman, E.M. (2009): Improving in vitro Sertoli cell/gonocyte co-culture model for assessing male reproductive toxicity: lessons learned from comparisons of cytotoxicity versus genomic responses to phthalates. Toxicol. Appl. Pharmacol., 239, 325-336.

Zeleniak, A.E., Huang, W., Brinkman, M.K., Fishel, M.L. and Hill, R. (2017): Loss of MTSS1 results in increased metastatic potential in pancreatic cancer. Oncotarget., 8, 16473-16487.

Zhou, Q., Nie, R., Li, Y., Friel, P., Mitchell, D., Hess, R.A., Small, C. and Griswold, M.D. (2008): Expression of stimulated by retinoic acid gene 8 (Str8) in spermatogenic cells induced by retinoic acid: an in vivo study in vitamin A-sufficient postnatal murine testes. Biol. Reprod., 79, 35-42.