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Gestational and lactational exposure to bisphenol AF in maternal rats increases testosterone levels in 23-day-old male offspring

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HIGHLIGHTS

- Bisphenol AF bioaccumulated in offspring testes via cord blood and lactation.
- Pups exposed to BPAF showed significant increase in testis testosterone levels.
- 279 differentially expressed genes were identified in pup testes exposed to BFAF.
- Gene expression between two adjacent germ cell types was disrupted by BPAF.

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ABSTRACT

During prenatal and postnatal development, exposure to environmental chemicals with estrogenic activity, such as bisphenol AF (BPAF), may result in reproductive disorders. Currently, the mechanisms behind such disorders in male offspring induced by gestational and lactational exposure to BPAF remain poorly understood. Here, female rats from gestational day (GD) 3–19 were exposed to 100 mg BPAF/kg/day by oral gavage. On the day of birth (postnatal day (PD) 0), cross-fostering took place between treated and control litters, and cross-fostered mother rats were given BPAF 100 mg/kg/day during the postnatal period (PD 3 to PD 19). HPLC-MS/MS analysis showed that BPAF was transferred via cord blood and lactation, finally bio-accumulating in the offspring testes. Pups exposed to BPAF both prenatally and postnatally showed a significant increase in testis testosterone levels compared with that of the control, while all pups exposed to BPAF showed a significant decrease in testis inhibin B (INHB) levels. Compared with the control, RNA-seq revealed that 279 genes were significantly differentially expressed in the testes of pups exposed to BPAF both prenatally and postnatally, including genes involved in cell differentiation and meiosis. These results indicate that gestational and lactational exposure to BPAF in the mother can impair reproductive function in male offspring.

1. Introduction

Human epidemiological and animal study data indicate that reproductive disorders and diseases can begin during prenatal and postnatal development (Kalb et al., 2016). For example, bisphenol A (BPA) exposure is suggested to have endocrine- and neural-disrupting effects in infants, even at very low doses (Kabuto et al., 2004; Doerge et al., 2010; Prins et al., 2011; Kalb et al., 2016). Exposure to endocrine-disrupting compounds (EDCs), a group of biologically active compounds that mimic or antagonize the effects of endogenous hormones, during critical stages of differentiation can interfere with the balance of hormones necessary for normal development, and result in altered gene expression that is not detectable until puberty or much later in life (Zoller et al., 2016).

Bisphenol AF (1,1,1,3,3,3-hexafluoro-2,2-bis(4-hydroxyphenyl) propane, BPAF), an analogue of BPA with a two phenolic ring structure joined by a carbon bridge, is being considered as a replacement for BPA. It has broad applications as a crosslinking

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reagent in the production of fluoroelastomers, and can also function as a monomer in the production of polymides, polyamides, polyesters, polycarbonates, and other polymers (Perez et al., 1998; Akahori et al., 2008; LaFleur and Schug, 2011). Domestic annual production of BPAF in the U.S. ranged from 10,000 to 50,000 pounds between 1986 and 2002 (Stout, 2008), and although the exact annual production is not available for China, more than forty manufacturing sites have been identified (Feng et al., 2012). Studies have reported the occurrence of BPAF in environmental water (river water, sewage) and solid samples (sediment, sludge) (Lu et al., 2014; Song et al., 2014b; Yang et al., 2014b; Ruan et al., 2015; Yu et al., 2015), as well as in indoor dust, foodstuffs, and beverages in the U.S. and seven Asian countries (Liao, 2013). Recent studies on BPAF urinary levels in residents exposed to a BPAF manufacturing plant in southern China (Yang et al., 2014a) showed that children had a higher intake than that of adults through the inhalation of soil particles and dermal exposure via contact with soil and well water (Song et al., 2012).

Several studies have demonstrated that BPAF shows stronger estrogenic activity than that of BPA both *in vivo* and *in vitro* (Matsushima et al., 2010; Feng et al., 2012; Li et al., 2012; Lee et al., 2013; Teng et al., 2013; Li et al., 2014; Song et al., 2014a; Shi et al., 2015; Yang et al., 2016). The higher binding affinity to estrogen receptors (ERs) and estrogenic activity of BPAF has been attributed to its trifluoromethyl (CF_3) group, which is much more electronegative than the methyl (CH_3) group of BPA. BPAF can act as both an agonist for ERα and an antagonist for ERβ (Li et al., 2012). Reduced serum testosterone and increased luteinizing hormone and follicle-stimulating hormone levels have been observed in adult male rats after oral dosing with BPAF (Feng et al., 2012). Investigation into cholesterol biosynthesis, transportation, and steroid biosynthesis indicates that BPAF exposure results in a dramatic decline in the expression levels of both genes and proteins related to these processes (Feng et al., 2012). BPAF also induces apoptosis in neuronal cells and causes damage to the liver and hypothalamic-pituitary-thyroidal axis in zebrafish (Lee et al., 2013; Tang et al., 2015; Yang et al., 2016). Accordingly, the U.S. National Institute of Environment Health Sciences nominated BPAF for further study in 2008 due to its potential risk in comprehensive toxicity (NTP, 2008).

We hypothesized that BPAF can be transferred to offspring through cord blood and breast milk, resulting in BPAF accumulation in the organs and increased offspring vulnerability to the adverse effects of this contaminant. To test this, a cross-fostering model was used to evaluate the effects of gestational and lactational BPAF exposure on testis function in infant male rats. Serum and testis BPAF concentrations were determined, as were specific hormone levels. Transcriptome profiling of infant rat testes was analyzed using RNA-sequences to elucidate the reproductive toxicological mechanism of BPAF, and RNA and protein levels involved in steroidogenesis were investigated to evaluate the adverse effects of BPAF on testicular function.

2. Materials and methods

2.1. Chemicals

Bisphenol AF (BPAF, CAS No. 1478-61-1, purity 97%) and corn oil (CAS No. 8001-30-7) were purchased from Sigma-Aldrich (USA). β-glucuronidase/arylsulfatase (EC 3.2.1.31/EC 3.1.6.1) was obtained from *Helix pomatia* (Roche Diagnostics GmbH, Mannheim, Germany). TRIZOL reagent was purchased from Ambion (Life Technologies, Carlsbad, USA). Oligo-$(dT)_{15}$ primer and ribonuclease inhibitor were procured from TaKaRa Bio (Japan). M-MLV reverse transcriptase was purchased from Promega (Madison, WI, USA). RIPA buffer was obtained from Applygen technologies Inc. (Beijing, China).

2.2. Animals and treatment

We purchased 60 male and 60 female Sprague-Dawley (SD) rats aged 8 weeks old from the Vital River Company, China. Animals were housed singly in cages for acclimation one week before pairing. BPAF dissolved in corn oil was administrated orally via gavage to treatment female rats from gestational day (GD) 3 to GD 19 (positive vaginal smear = GD 0). During the gestational period, 30 females from the control group and 30 females from the treatment group were treated daily at doses of 0 or 100 mg BPAF/kg of body weight, respectively. Doses were selected based on reported values of an oral 50% lethal dose (LD50) in rats of 3400 mg/kg (Halocarbon, 2007). On the day of birth (postnatal day (PD) 0), litters born to treated and control dams were cross-fostered, resulting in the following groups: unexposed control (CC group), pups exposed prenatally (TC group), pups exposed postnatally (CT group), and pups exposed both prenatally and postnatally (TT group). During the postnatal period (PD 3 to PD 19), cross-fostered mothers were given BPAF dissolved in corn oil orally at doses of either 0 (CC and TC) or 100 mg/kg/day BPAF (CT and TT), respectively. During the experimental period, the mothers were weighed every 3 d to administer the dose per kilogram of body weight and the pups were weighed every 6 d. All animals were weighed and euthanized at PD 23. Blood was collected and centrifuged at 3000 rpm at 4 °C. The serum was then collected and stored at −80 °C until further analysis. Testes and epididymides were isolated and weighed. Testes were frozen in liquid nitrogen and stored at −80 °C for RNA isolation and protein extraction.

2.3. Hormone levels

Estradiol, testosterone, luteinizing hormone, and follicle-stimulating hormone were measured by the Beijing North Institute of Biological Technology, China. Inhibin B (INHB) and anti-Müllerian hormone (AMH) were measured using an enzyme-linked immunosorbent assay (ELISA) kit from the Cloud-Clone Corp (Houston, USA) following the manufacturer's instructions. The detecting limits for hormone analysis were as follows: testosterone 0.1–20 ng/mL; inhibin B 1.56–100 pg/mL; estradiol 10–1000 pg/mL; LH 5-200 mIU/mL; FSH 1.0–100 mIU/mL; anti-müllerian hormone 123.5–10,000 pg/mL.

2.4. BPAF concentration in serum and testis

Recent research identified four metabolites of BPAF, including BPAF diglucuronide, BPAF glucuronide (BPAF-G), BPAF glucuronide dehydrated, and BPAF sulfate (Li et al., 2013). In the present study, β-glucuronidase/arylsulfatase was used to transform conjugated BPAF into free BPAF. We transferred 100 mg of homogenized testis or 200 μL of serum samples into 5 mL polypropylene centrifuge tubes containing 500 μL of acetate buffer (pH 5.3). Then, 20 μL of β-glucuronidase/arylsulfatase was added and mixed. The mixture was incubated overnight at 37 °C. After cooling to room temperature, 1.5 mL of acetonitrile was added and the mixture was sonicated for 15 min at room temperature. The extract was then centrifuged at 9000*g* for 10 min at 4 °C. The supernatant was transferred into another tube and dried under a gentle stream of nitrogen gas. The residual was reconstituted in 1 mL of methanol/water (v/v = 50/50) for analysis. BPAF identification and quantification were performed with an Acquity ultra performance liquid chromatograph (UPLC) coupled to a Xevo triple quadrupole mass spectrometer (Waters, Milford, MA, USA). Liquid chromatograph

separation was conducted with an Acquity BEH C18 column (2.1 mm × 50 mm; 1.7 μm; Waters). The mobile phases were A (ultrapure water) and B (methanol). The injection volume was 10 μL. A total flow rate of 0.3 mL/min was used with a gradient elution starting with 30% mobile phase B, followed by a 4 min linear gradient to 100% mobile phase B, which was continued for 2 min. The system was re-equilibrated for 3 min between runs. Samples were ionized in negative electrospray ionization mode. The capillary voltage was 2.5 kV. The source and desolvation temperatures were set at 150 °C and 400 °C, respectively. The nitrogen flow rate was 700 L/h, and ultra-pure argon was used as the collision gas at a flow rate of 0.13 mL/min.

2.5. RNA seq and reverse transcription PCR

Total RNA was isolated from the testis samples using TRIZOL reagent according to the manufacturer's instructions. The concentration of total RNA was measured by absorbance at 260 nm using a NanoDrop2000 spectrophotometer (Thermo Scientific, Waltham, USA). The purity of the RNA was estimated by the 260/280 nm absorbance ratio. Each individual sample of 100 μg RNA from the CC and TT groups was analyzed for RNA-sequencing. The Perl script was used to filter the original data to guarantee data quality for analysis. The clean data were then used for Q30, data quantity, and base content statistics. Reference gene and genome annotation files downloaded from the UCSC genome browser (http://genome.ucsc.edu/) were used for building a reference genome library with Bowtie2 v2.2.3. The clean data were then mapped to the reference genome using TopHat v2.0.12 software. The Integrative Genomics Viewer (IGV) was used to view the mapping results. HTSeqv0.6.0 was run to calculate the counts of each gene, and RPKM (reads per kilobase of exon model per million mapped reads) was used to assess expression quantity. DESeq software was used for analysis of differentially expressed genes. Blast2go was used for gene ontology (GO) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) Automatic Annotation Server (KAAS) was used for enrichment analysis. To better understand the differentially expressed proteins in relation to published literature, interactions among the proteins regarding biological pathways were determined using Pathway Studio software via the ResNet database (version 6.5, Ingenuity Systems, Inc., Redwood City, USA).

Reverse transcription was performed using high purity dNTPs, oligo-$(dT)_{15}$ primer, M-MLV reverse transcriptase, and ribonuclease inhibitor according to the manufacturer's instructions. Reverse transcription was achieved by incubation for 60 min at 42 °C, followed by 5 min at 95 °C. Real-time PCR reactions were performed with the Stratagene Mx3000p qPCR system (Agilent, USA). SuperReal PreMix Plus (SYBR green) kits (Tiangen, Beijing, China) were used for the quantification of gene expression according to the manufacturer's protocols. Rat-specific primers were designed for the genes of interest: CD177 (CD177 antigen), CDC14A (dual specificity protein phosphatase CDC14A), DPYSL3 (dihydropyrimidinase-related protein 3), PTPRJ (receptor-type tyrosine-protein phosphatase eta), RAG1 (V(D)J recombination-activating protein 1), VIT (vitrin), StAR (steroidogenic acute regulatory protein), ERα (estrogen receptor alpha), and AR (androgen receptor) (Supporting Information Table S1). The housekeeping gene β-actin was used as an internal control. Cycling conditions were as follows: 95 °C for 15 min followed by 40 cycles of 95 °C for 10 s, 58 °C for 20 s, and 72 °C for 30 s. After PCR, melting curve analysis was performed to demonstrate PCR product specificity, which was displayed as a single peak. The relative expression ratio (R) of a target gene was expressed as the sample versus the control in comparison to the β-actin gene. R was calculated based on the following equation: $R = 2^{-\Delta\Delta Ct}$, as described in previous studies (Arocho et al., 2006; Huang et al., 2007), where C_t represents the cycle number at which the fluorescence signal is first observed to be significantly different from the background and $\Delta\Delta Ct$ represents $(C_{t,\ target} - C_{t,\ actin})$ treatment $- (C_{t,\ target} - C_{t,\ actin})$ control.

2.6. Western blot analysis

Protein was extracted from testes frozen at −80 °C by homogenizing the sample in RIPA buffer for 5 min, with lysates then clarified by centrifugation at 12,000g for 20 min. The protein content was determined using the BCA Protein Assay Kit (Tiangen, Beijing, China). Each sample was run on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and then transferred to polyvinylidene difluoride (PVDF) transfer membranes (GE Healthcare Bio-Sciences AB, Sweden). The membranes were first blocked with 5% milk in Tris-buffered saline Tween (TBST) for 1 h at room temperature and then incubated with the primary antibodies for anti-AR (Abcam, ab74272), anti-StAR (Santa Cruz, FL-285), anti-P450scc (Millipore, ABS235), anti-CYP17a (Abcam, ab125022), anti-3β-HSD (Abcam, ab150384), anti-CYP19a (Abcam, ab18995), anti-DPYSL3 (Abcam, ab126787) and anti-PTPRJ (Abcam, ab181244) at 4 °C overnight. The membranes were then washed and incubated with an appropriate secondary IgG-HRP antibody. Proteins were visualized using an ImageQuant LAS 4000 imaging system (GE Healthcare Bio-Sciences AB, Sweden) in accordance with the manufacturer's instructions.

2.7. Statistical analyses

SPSS for Windows 19.0 was used for data analysis. The data were presented as means with standard errors (mean ± SEM). The normality of the data was analyzed by means of a Kolmogorov-Smirnov test. The natural logarithm (ln) transformation was done to ensure normality of the distribution for BPAF concentrations in all samples. Differences between four groups were determined by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Litter size, birth weight, sex ratio, and survival rate

Litter size, birth weight, sex ratio, and survival rate of rat pups are shown in the Supporting Information (Table S2). No significant differences between the control and treatment groups were found.

3.2. Effect of BPAF on body and organ weights of litters

The body weights of mother rats from GD 3 to GD 18 are shown in Fig. S1A. Compared with the control group, the body weights of the GD 9 to GD 18 treatment group mothers showed significant decrease ($p < 0.01$).

The body weights of PD 6 to PD 23 male pups and the absolute and relative weights of the testis and epididymis of PD 23 male pups are given in Fig. S1B–D, respectively. Compared with the CC group, the body weights of the TC group pups increased at PD 6 ($p < 0.01$) and PD 18 ($p < 0.05$). However, the body weights of the CT group decreased at PD 12, PD18, and PD 23 ($p < 0.01$). The body weights of the TT group decreased at PD 6, PD12, and PD 16 ($p < 0.05$). During the postnatal period, the body weights of the TC group were significantly higher than those of the CT group ($p < 0.01$). In addition, the absolute and relative weights of the testis and the absolute weights of the epididymis of the CT, TC, and TT groups showed no differences compared with those of the CC

group.

3.3. BPAF concentration in serum and testis

The concentrations of free and total BPAF in serum and testis were detected in all groups via LC-MS/MS. The concentrations of free BPAF in serum were 0.21, 0.34, 9.32, and 29.38 ng/mL, and the concentrations of total BPAF were 0.76, 1.32, 27.60, and 127.00 ng/mL in the CC, TC, CT, and TT groups, respectively (Fig. 1). Free and total BPAF levels in serum were ranked as TT group > CT group > TC group > CC group. The content of free and total BPAF in the TC and CT groups indicated that BPAF was transferred to infants via gestation and lactation, although the concentration of BPAF in cord blood and breast milk was not measured directly. Furthermore, BPAF in the serum was more easily eliminated than that in the testis during exposure.

3.4. Hormone levels in serum and testis

The effects of BPAF on hormone levels in the serum and testis of infant male rats are shown in Fig. 2 and Fig. S2. No significant differences were observed in the levels of serum estradiol, luteinizing hormone, or follicle-stimulating hormone among the four groups, except for a decrease in the TC and CT group compared with the TT group (Fig. S2). Serum anti-Müllerian hormone (AMH) levels in the TT group were lower than those of the CC, TC and CT group, respectively ($p < 0.05$). Only the TT group serum and testis testosterone levels showed significant increase compared with those of the CC group ($p < 0.05$, Fig. 2). The levels of INHB in the serum of the TC and TT groups decreased compared with those of the CC group ($p < 0.05$), while the content of INHB in the testis of all treated groups decreased significantly compared with that of the control group ($p < 0.01$).

3.5. Transcriptome profiling by RNA-sequences and differentially expressed genes in infant rat testis treated with BPAF

RNA-seq was performed to identify the differentially expressed genes in male offspring testis via gestational and lactational exposure to BPAF in maternal rats. RNA transcripts from three samples in both the CC and TT groups were deeply sequenced. A total of 95,140,585 paired-end reads were generated from the six samples. The number of sequences from each sample ranged from 15.8 to 15.9 million. After removal of ambiguous nucleotides, low-quality sequences, and adaptor sequences, a total of 90,000,006 clean reads (94.9%) were harvested for further analysis. Total clean nucleotides of the six samples were 4.5×10^9 nt, showing stability and consistency in sampling, library preparation, and sequencing.

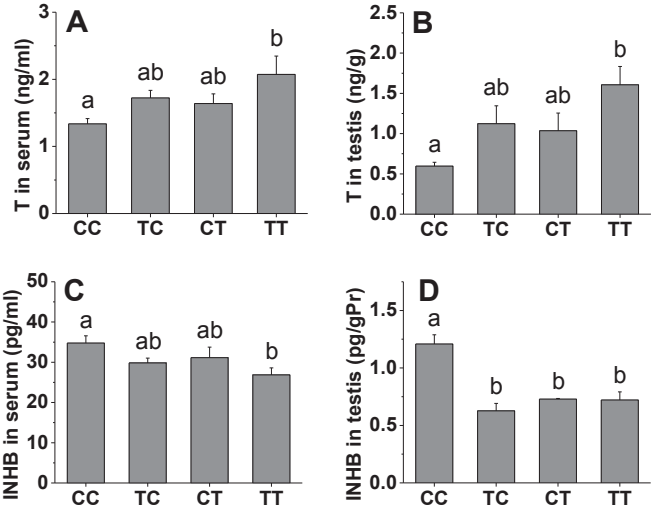

Fig. 2. Testosterone (T) and inhibin B (INHB) levels in the serum and testis of male rat pups. Bars represent the mean ± SEM per group (n = 6). Values not sharing a common superscript differ significantly at $p < 0.05$ by ANOVA and Duncan's multiple range tests.

The clean reads of the six samples were then mapped to the genome sequences, with a mapping rate of 97%–98%. Results showed that 279 genes (236 upregulated with a fold change ≥ 2 and 43 downregulated with a fold change ≤ 0.5) with a false discovery rate (FDR) < 0.05 were significantly differentially expressed in the testis of the TT group compared with the CC group (Table 1). The most over-represented functions were involved in G2/M checkpoint, cell differentiation, meiosis, cell cycle, G2/M transition, and DNA recombination (Fig. 3). We next verified the transcriptional levels of three genes (StAR, ERα, and AR) in the TT group by real-time PCR (Table S3). The mRNA levels of StAR, ERα, and AR in the TT group were also increased compared with that in the CC group, similar to the results from RNA-sequencing. We also identified 11 meiotic-related genes using Pathway Studio software via the ResNet database (version 6.5, Ingenuity Systems, Inc.) (Table 1).

In order to explore whether BPAF exposure interrupted the development of germ cells in 23 day old mice testes, we measured and compared gene expression levels between two adjacent germ cell types (such as preleptotene spermatocytes (plpSC), pachytene spermatocytes (pacSC), and round spermatids (rST)). We referenced the expression levels in three germ cell types (plpSC, pacSC, and rST) from mice in terms of fragments per kilobase of exon model per million mapped reads (FPKM) by RNA-seq and differentially expressed genes since no data were available for rats (Gan

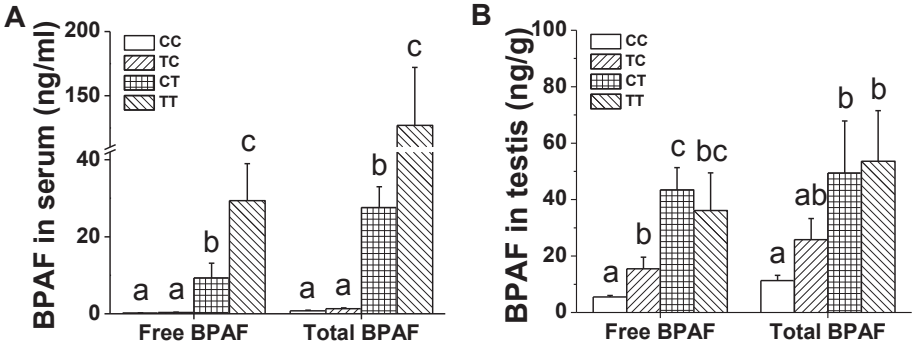

Fig. 1. Concentrations of BPAF in serum and testis from male rat pups. Bars represent the mean ± SEM per group (n = 6). Values not sharing a common superscript differ significantly at $p < 0.05$ by ANOVA and Duncan's multiple range tests.

Table 1
Differentially expressed genes identified by RNA-seq in infant rat testis in the TT group treated with BPAF.

Gene	Full name	Fold change	padj
Cell differentiation			
ABCA1	ATP-binding cassette sub-family A member 1	2.322	0.000
ABCA3	ATP-binding cassette sub-family A member 3	2.441	0.007
ABHD2	Abhydrolase domain-containing protein 2	2.072	0.003
ADAM12	Disintegrin and metalloproteinase domain-containing protein 12	3.232	0.014
ADAM19	Disintegrin and metalloproteinase domain-containing protein 19	2.694	0.000
ADAM23	Disintegrin and metalloproteinase domain-containing protein 23	2.005	0.019
ADCY5	Adenylate cyclase type 5	2.015	0.000
AFF1	AF4/FMR2 family member 1	3.019	0.014
AR	Androgen receptor	3.339	0.000
ARRB1	Beta-arrestin-1	3.662	0.005
ATP2B4	Plasma membrane calcium-transporting ATPase 4	2.369	0.000
CBL	E3 ubiquitin-protein ligase CBL	2.701	0.000
CD79B	B-cell antigen receptor complex-associated protein beta chain	55.815	0.000
CDH5	Cadherin-5	2.250	0.000
CDON	Cell adhesion molecule-related/down-regulated by oncogenes	2.120	0.000
COL4A2	Collagen alpha-2(IV) chain	2.197	0.000
COL5A1	Collagen alpha-1(V) chain	2.747	0.000
CYP1B1	Cytochrome P450 1B1	3.256	0.000
DDR2	Discoidin domain-containing receptor 2	2.058	0.003
DPYSL3	Dihydropyrimidinase-related protein 3	3.471	0.001
EGFR	Epidermal growth factor receptor	2.614	0.029
EGR1	Early growth response protein 1	2.145	0.000
ELF4	ETS-related transcription factor Elf-4	2.003	0.000
ERBB4	Receptor tyrosine-protein kinase erbB-4	2.009	0.020
ESR1	Estrogen receptor	2.872	0.022
FLI1	Friend leukemia integration 1 transcription factor	3.143	0.040
FNDC3B	Fibronectin type III domain-containing protein 3B	2.019	0.000
FSTL1	Follistatin-related protein 1	2.239	0.001
GAN	Gigaxonin	2.109	0.025
GRIK3	Glutamate receptor ionotropic, kainate 3	2.708	0.013
HHIP	Hedgehog-interacting protein	3.944	0.000
HIF1AN	Hypoxia-inducible factor 1-alpha inhibitor	2.082	0.000
IKZF3	Zinc finger protein Aiolos	6.193	0.027
IL17RD	Interleukin-17 receptor D	3.705	0.025
IL2RB	Interleukin-2 receptor subunit beta	2.286	0.019
LDLR	Low-density lipoprotein receptor	2.156	0.000
LIFR	Leukemia inhibitory factor receptor	2.269	0.000
LPAR2	Lysophosphatidic acid receptor 2	2.527	0.000
LPP	Lipoma-preferred partner homolog	3.821	0.001
MAP2	Microtubule-associated protein 2	2.904	0.001
MGAT5	Alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase A	2.372	0.000
MID1	E3 ubiquitin-protein ligase Midline-1	5.114	0.014
MMP19	Matrix metalloproteinase-19	2.051	0.034
NCOA2	Nuclear receptor coactivator 2	2.054	0.000
NDST1	Bifunctional heparan sulfate N-deacetylase/N-sulfotransferase 1	2.298	0.000
NID1	Nidogen-1	2.025	0.000
NLGN2	Neuroligin-2	2.080	0.003
NOTCH3	Neurogenic locus notch homolog protein 3	2.282	0.018
NR4A1	Nuclear receptor subfamily 4 group A member 1	5.001	0.000
NR4A3	Nuclear receptor subfamily 4 group A member 3	2.300	0.008
NRP1	Neuropilin-1	3.107	0.000
NRP2	Neuropilin-2	2.383	0.033
NTN1	Netrin-1	2.704	0.041
PDE4D	cAMP-specific 3′,5′-cyclic phosphodiesterase 4D	2.170	0.002
PDE5A	cGMP-specific 3′,5′-cyclic phosphodiesterase	2.380	0.004
POU2AF1	POU domain class 2-associating factor 1	11.277	0.001
PRKAA2	5′-AMP-activated protein kinase catalytic subunit alpha-2	2.315	0.001
PTCH1	Protein patched homolog 1	2.079	0.000
PTPRJ	Receptor-type tyrosine-protein phosphatase eta	2.305	0.001
RAG1	V(D)J recombination-activating protein 1	Inf	0.001
RGS5	Regulator of G-protein signaling 5	3.390	0.036
SLC16A2	Monocarboxylate transporter 8	2.397	0.032
SLC7A2	Cationic amino acid transporter 2	2.277	0.000
SPDEF	SAM pointed domain-containing Ets transcription factor	12.322	0.021
SPIB	Transcription factor Spi-B	13.206	0.000
SSPN	Sarcospan	3.031	0.000
STAR	Steroidogenic acute regulatory protein, mitochondrial	2.396	0.000
STC1	Stanniocalcin-1	3.274	0.002
TEAD1	Transcriptional enhancer factor TEF-1	2.140	0.000
TGFBR2	TGF-beta receptor type-2	3.933	0.000
TGFBR3	Transforming growth factor beta receptor type 3	2.746	0.000
THBD	Thrombomodulin	2.313	0.001
TNS1	Tensin-1	3.481	0.000

Table 1 (*continued*)

Gene	Full name	Fold change	padj
TRIM25	E3 ubiquitin/ISG15 ligase TRIM25	2.371	0.002
TRIM71	E3 ubiquitin-protein ligase TRIM71	2.380	0.001
UPRT	Uracil phosphoribosyltransferase homolog	7.501	0.023
ZBTB16	Zinc finger and BTB domain-containing protein 16	2.197	0.001
ECEL1	Endothelin-converting enzyme-like 1	0.478	0.000
IL18	Interleukin-18	0.414	0.000
IL24	Interleukin-24	0.458	0.010
MT3	Metallothionein 3	0.451	0.000
NPR2	Atrial natriuretic peptide receptor 2	0.224	0.000
PRTN3	Myeloblastin	0.243	0.023
Meiosis			
AR	Androgen receptor	3.339	0.000
CBL	E3 ubiquitin-protein ligase CBL	2.701	0.000
CDC14A	Dual specificity protein phosphatase CDC14A	2.660	0.042
CDS2	Phosphatidate cytidylyltransferase 2	2.001	0.006
EGFR	Epidermal growth factor receptor	2.614	0.029
ESR1	Estrogen receptor	2.872	0.022
FLI1	Friend leukemia integration 1 transcription factor	3.143	0.040
PDE4D	cAMP-specific 3′,5′-cyclic phosphodiesterase 4D	2.170	0.002
PTCH1	Protein patched homolog 1	2.079	0.000
STAR	Steroidogenic acute regulatory protein, mitochondrial	2.396	0.000
ZYG11A	zyg-11 homolog A (*C. elegans*)	3.334	0.004

Note: Inf: infinity.

et al., 2013). Based on these data, we found that among the 279 differentially expressed genes, six in the TT group BPAF-treated rat testis were classified as differentially expressed genes between two adjacent germ cell types (plpSC and pacSC, pacSC and rST, respectively) (Table 2). The most dramatic changes were observed at the transition from plpSC to pacSC, and from pacSC to rST for RAG1. Other genes also significantly increased from pacSC to rST. Our results confirmed that the data obtained by RNA-seq, except for DPYSL3 and VIT, were not significantly different to that obtained by the RT-PCR method. We also measured the expression patterns of these genes in the TC and CT groups using RT-PCR (Table 2), and found that CD177 in the TC group were upregulated.

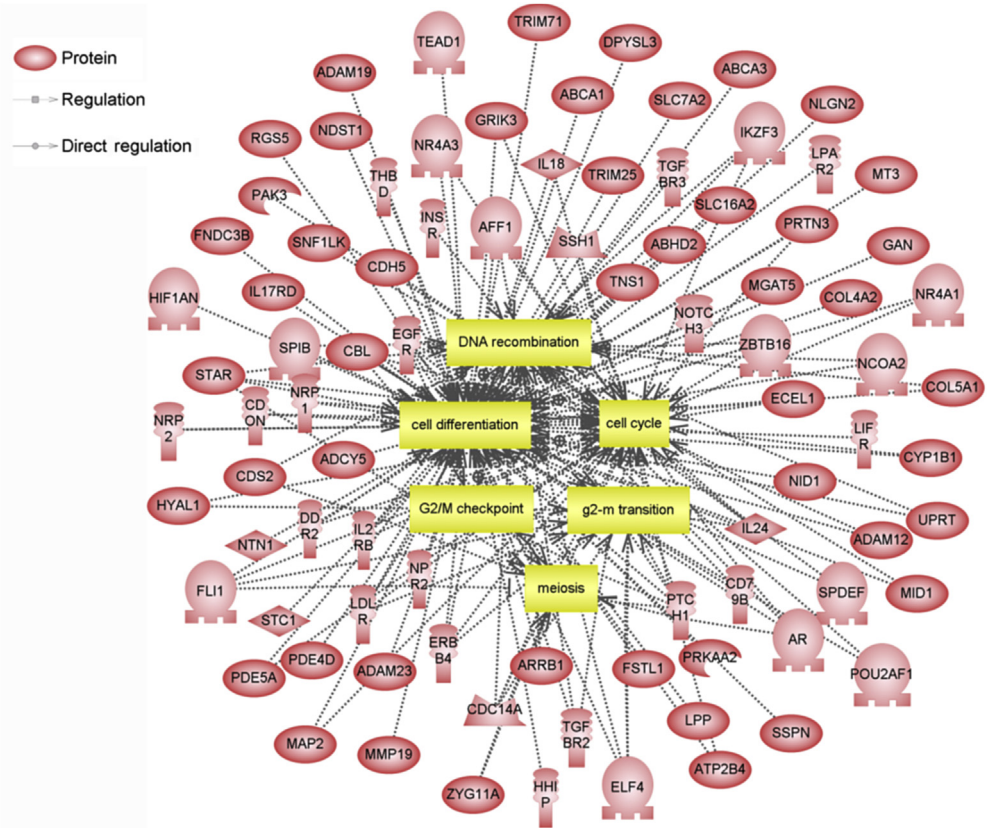

Fig. 3. Pathway studio analysis of differentially expressed genes in infant rat testis in the TT group. Network of genes involved in G2/M checkpoint, cell differentiation, meiosis, cell cycle, G2/M transition, and DNA recombination. Full names of genes are in Table 1.

Table 2
Six differentially expressed genes in infant rat testis treated with BPAF. Differentially expressed genes between two adjacent germ cell types (preleptotene spermatocytes (plpSC), pachytene spermatocytes (pacSC), and round spermatids (rST)) obtained from mice.

Gene symbol	CC group	Fold change				Fold change for *Mus* expression[a] (ratio of FPKM)			
		TC group	CT group	TT group	RNA-seq	pacSC/plpSC	q-value[b]	rST/pacSC	q-value[b]
VIT	1.00[a]	1.40[a]	1.62[a]	2.09[a]	2.09**	5.95	0.005	n.d	
RAG1	1.00[a]	1.68[ab]	1.55[ab]	2.22[b]	Inf**	13.34	0.002	0.22	0.010
PTPRJ	1.00[a]	1.08[a]	1.22[ab]	1.46[b]	2.30**	n.d		26.50	3.79E-10
CD177	1.00[a]	6.04[b]	2.54[ab]	6.64[b]	26.0**	n.d		0.37	0.036
CDC14A	1.00[a]	1.21[a]	1.71[a]	5.56[b]	2.66**	n.d		69.27	2.95E-12
DPYSL3	1.00[a]	0.97[a]	0.86[a]	1.16[a]	3.47**	n.d		44.58	2,28E-13

Values not sharing a common superscript differ significantly at $p < 0.05$ by ANOVA and Duncan's multiple range tests. **: $p < 0.01$, results from RNA-seq. compared with the control group.
[a] Data from Gan et al. (2013).
[b] Corrected *p*-value between plpSC and pacSC.

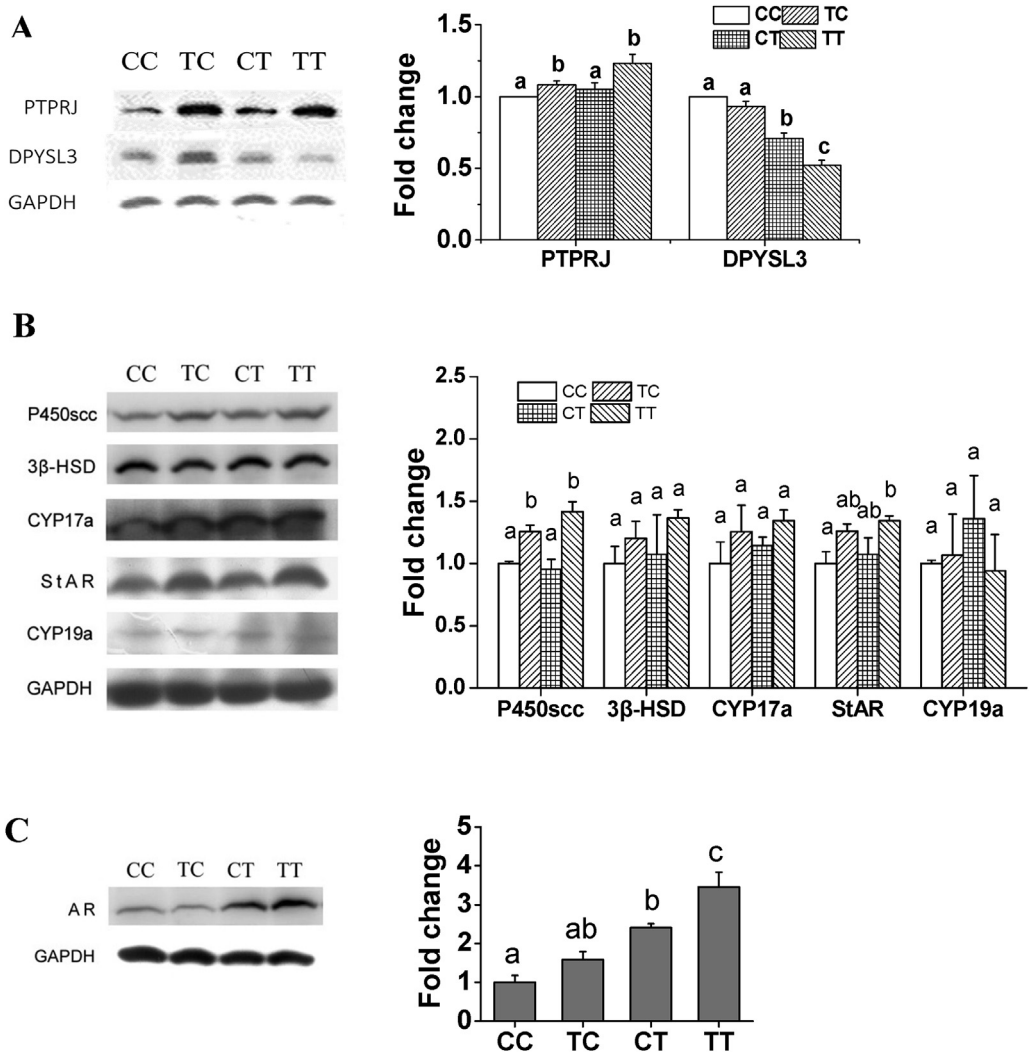

Fig. 4. Western blot analysis of testicular protein expression levels in male rat pups. A) Protein levels of PTPRJ and DPYSL3, B) proteins involved in testosterone synthesis, C) protein levels of AR, representative western blots are shown in the left panels, and results from densitometry analysis are shown in the right panels. Bars represent the mean ± SEM per group (n = 3 or 4). Values not sharing a common superscript differ significantly at $p < 0.05$ by ANOVA and Duncan's multiple range tests.

We evaluated the protein levels of PTPRJ and DPYSL3 in the CC, TC, CT, and TT groups (Fig. 4A). The protein levels of PTPRJ in male rat testis increased in the TC and TT group compared with those in the control group (Fig. 4A). However, the protein levels of DPYSL3 were significantly decreased in the CT and TT groups compared with those in the CC group (Fig. 4A).

3.6. Effect of protein levels of genes involved in steroidogenesis

Seven proteins are involved in testosterone synthesis in the testes. The RNA-sequences obtained in the present study showed that the transcriptional levels of StAR, which is responsible for cholesterol transport to the inner mitochondrial membrane, were

significantly increased in the TT group compared with those in the control group (Table S3). In addition, the mRNA levels of ERα and AR in the TT group were also increased compared with those in the CC group.

We evaluated the protein levels of genes involved in hormone steroidogenesis (P450scc, CYP17a, 3β-HSD, StAR, CYP19a, and AR) in the CC, TC, CT, and TT groups (Fig. 4B). The protein levels of genes involved in steroidogenesis (P450scc and StAR) in the male rat testis increased in the TT group compared with those in the control group (Fig. 4B). In addition, the protein levels of P450scc were increased in the TC group compared with those in the control group ($p < 0.05$). However, no significant differences in protein levels of CYP17a, 3β-HSD, and CYP19a were observed between the control and the BPAF treatment groups. The protein levels of AR were significantly increased in the CT and TT groups compared with those in the CC group (Fig. 4C).

4. Discussion

Recognizing that developing organisms are uniquely sensitive to perturbation by chemicals with estrogenic and/or endocrine disrupting activity, we designed the current study to determine if exposure to BPAF during critical developmental windows could alter hormone programming of the reproductive organs, such that they may respond abnormally to estrogen-like chemicals in later life.

In the present study, the gestational exposure (TC) group showed no significant difference in the content of free BPAF in serum compared with that of the control group, but did show higher free BPAF in the testis. These results indicate that BPAF was eliminated more easily in serum than that in the testis after gestational exposure followed by postnatal 23 d depuration. For lactational exposure, significant differences were found in the concentrations of free and total BPAF in the serum and testis of the CT group compared with those of the control group, respectively. These results imply that BPAF biotransformation or elimination rates in testes were low during the lactational period. Although the concentration of BPAF in milk was not measured directly, the BPAF levels in the serum and testes of lactationally-exposed rats showed that BPAF could be transferred through breast milk. The observed BPAF contamination in the control group infant rats is reasonable, and may have originated from maternal exposure to the plastic material used in their home cages and drinking water bottles. In addition, dietary foods, as well as drinking water and air particles, might also contain trace BPAF and provide a contamination source for maternal rats.

Previous research demonstrated the long-term adverse effects of BPA on reproductive function, including sperm quality, antioxidant capacity, and changes in testicular tissue, in male mice via lactation, even after a long time-depuration from exposure was observed (Kalb et al., 2016). In another study, BPA was detected in breast milk samples from 19 Japanese mothers, suggesting that BPA may affect infants even at very low doses (Nakao et al., 2015). In the present study, gestational and lactational exposure to BPAF in maternal rats increased testosterone levels in the testes, but decreased INHB levels in the serum and testes of infant male offspring.

Testosterone is essential for germ cell survival and development in the testes (Walker, 2009). It is synthesized from cholesterol as a substrate in Leydig cells through a series of reactions catalyzed by four enzymes: P450scc, 3β-HSD, CYP17a1, and 17β-HSD (Payne and Hales, 2004). P450scc converts cholesterol to pregnenolone, which is the first rate-limiting and hormonally-regulated step in the synthesis of all steroid hormones (Hanukoglu, 1992). StAR plays an important role in transferring cholesterol from the outer mitochondrial to the inner mitochondrial membrane in Leydig cells (Manna and Stocco, 2005). In the present study, the protein levels of P450scc only increased significantly in the infant rat testes in the TC and TT group after BPAF exposure. StAR increased at the mRNA and protein levels in the TT group compared with that in the control group. The increased testicular steroid production in the BPAF-treated groups suggests that the steroidogenesis function of Leydig cells in infant male rats was activated by BPAF. These results indicate that BPAF exposure via gestation and lactation would affect cholesterol transport and testosterone biosynthesis in Leydig cells. Previous study showed a decrease in serum testosterone levels, as well as declines in the protein levels of P450scc and StAR, in adult male rats after oral dosing with 200 mg/kg/d BPAF (Feng et al., 2012). Our results indicate that the different effects of BPAF on reproductive functions were dependent on the developmental period of the rat.

Testosterone production is highly regulated via the negative feed-back control of the hypothalamic-pituitary-testicular axis and the primary luteinizing hormone acting on steroidogenic enzymes and steroid transport proteins (Dufau, 1988; Lei et al., 2001). For example, StAR expression can be stimulated via the luteinizing hormone binding to its receptor (LHR) and the cAMP-dependent pathway (Dufau, 1988; Zirkin and Chen, 2000). In this study, no obvious changes in serum luteinizing hormone or follicle-stimulating hormone levels were observed. In addition, mRNA levels of LHR in the testis of the TT group did not change significantly compared with those of the control, as determined by RNA-seq, suggesting that the increase in steroidogenesis in infant male rats by BPAF exposure was independent of the hypothalamic-pituitary-testicular axis.

The main known effects of testosterone in supporting spermatogenesis are to stimulate seminiferous tubule fluid production by the Sertoli cells, regulate the release of mature spermatids from the Sertoli cells, and support the development of pachytene spermatocytes and later germ cell types through stage VII of the spermatogenic cycle. This spermatogenic support appears to be mediated by the secretion of several specific proteins from the Sertoli, peritubular, and germ cells, whose secretions are dependent on both testosterone and a full complement of germ cells. Regulation of spermatogenesis is, therefore, an extremely complex cascade of cell-cell interactions, with the Leydig cells supporting germ cell development through the effects of testosterone on Sertoli and peritubular cell protein secretion, but with germ cells programming the response of these target cells to testosterone.

Inhibin B is a testicular hormone that, in adults, regulates follicle-stimulating hormone secretion by a negative feedback loop. Inhibin B production is increasingly used as a potential biomarker of testicular damage (Pierik et al., 2003). The high levels of inhibin B production during neonatal life and puberty are thought to reflect the proliferation of Sertoli cells. Studies on rats have indicated that a relationship exists between inhibin B and Sertoli cell number (Sharpe et al., 1999). Circulating levels of inhibin B were found to increase rapidly in the rat between PD 3 and PD 10, reaching a peak between 20 and 40 d old and then declining in late puberty by approximately 50% to adult levels (Sharpe et al., 1999). The rapid increase in inhibin B in the postnatal period coincides with the increased number of dividing Sertoli cells (Sharpe et al., 1999). Prior research has indicated that decreased inhibin B levels are related to the impairment of testicular spermatogenesis (Stewart and Turner, 2005). In the present study, although the serum level of follicle-stimulating hormone showed no change, the dramatically reduced inhibin B levels in the serum and testis of infant male rats from the TC, CT and TT groups suggests that BPAF exposure through the uterus and breast milk might impair Sertoli cell function.

Androgen receptor plays an important role in normal

spermatogenesis, and its expression levels rise and fall in adult Sertoli cells in a manner corresponding with the cyclical stages of the seminiferous epithelium. In the rat, the expression of the AR protein is low and difficult to detect, except during stages VI–VIII when AR levels increase dramatically (Bremner et al., 1994). It is during stages VI–IX that the lack of testosterone or AR most strongly affects the processes required for spermatogenesis (De Gendt et al., 2004). Earlier research determined that in the absence of Sertoli cell AR, spermatogenesis in mice does not progress beyond the pachytene or diplotene stages of meiosis (De Gendt et al., 2004; Tsai et al., 2006) and the integrity of junctional complexes making up the blood-testis barrier are not maintained (Meng et al., 2005). In the current study, AR levels were increased following BPAF exposure, suggesting that AR might be involved in the testicular toxicity induced by BPAF exposure.

Estrogen receptors are widely expressed in the testes and play an important role in testicular development and spermatogenesis (Carreau et al., 2011). In adult rat testes, ERα is expressed in Leydig cells, pachytene spermatocytes, and round spermatids in the seminiferous epithelium (Chimento et al., 2010). Although normal estrogen signaling through its receptors is important for maintenance of spermatogenesis and male fertility, over-activation of estrogen signaling through either of its receptors can have detrimental effects on fertility parameters. Estrogen-like xenobiotic chemicals, such as BPA, can alter endocrine function through interaction with ERs (Matthews et al., 2001). In the present study, ERα mRNA was significantly upregulated in infant male rats of mothers given 100 mg BPAF/kg/d. However, Nakamura et al. (2010) reported decreased mRNA levels of ERα in prepubertal rats given 100 and 200 mg/kg/d BPA, which could be related to a reduced number of Leydig cells in the testes. The increased ERα levels in the present study might be partially involved in the increase in testosterone biosynthesis through ERα-independent signaling, though the precise mechanism still remains to be determined. It is likely, however, that both receptor function pathways cross-talk and interact in their target cells.

Earlier research found that exposure to low-dose BPA impairs meiosis in rat seminiferous tubule culture, and that meiotic progression in the presence of BPA is disrupted with increased leptotene and decreased diplotene spermatocyte percentages and partial meiotic arrest at the pachytene checkpoint, resulting in morphological abnormalities at all stages of the meiotic prophase (Ali et al., 2014). In the present study, eleven genes involved directly or indirectly in meiosis were upregulated, indicating that BPAF exposure affected meiosis and DNA recombination (Table 1). These genes often appear to be specific for meiotic and post-meiotic cells. For example, CDC14A is a physiological regulator of the centrosome duplication cycle, and both its overexpression and downregulation can cause aberrant chromosome partitioning into daughter cells (Mailand et al., 2002). In the present study, although we did not observe round spermatids originating from meiotic divisions, we found that CDC14A, CD177, and PTPRJ in the TT group and CD177 in the TC group were upregulated in the testes, especially protein level of PTPRJ in TC and TT groups, suggesting that BPAF exposure might induce the expressions of these genes in pachytene spermatocytes (pacSC).

In summary, BPAF accumulated in the serum and testes of infant male rats via uterine and breast milk exposure. Decreased inhibin B and increased testosterone levels in the testes indicated that Sertoli and Leydig cell functions were both disturbed. Several genes and proteins involved in cholesterol transport and testosterone synthesis were upregulated. Transcriptomic analyses showed that BPAF altered the expression of genes involved in progression G2/M checkpoint, cell differentiation, meiosis, cell cycle, G2/M transition, and DNA recombination. Moreover, several gene expressions between two adjacent germ cell types, such as plpSC and pacSC, and pacSC and rST, were also altered, indicating that BPAF had deleterious effects on meiotic progression and germ cell development during critical stages of differentiation. These results provide additional support for the hypothesis that BPAF can interfere with the hormone balance necessary for normal development. Our transcriptomic data could help to provide candidates for predictive biomarkers of meiotic abnormalities related to the toxic effects of chemicals on spermatogenesis.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.08.059.

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