RESEARCH COMMUNICATION

Set, a Putative Oncogene, As a Biomarker for Prenatal Exposure to Bisphenol A

Ho-Sun Lee, Myoung-Yun Pyo, Mihi Yang*

Abstract

Background: Bisphenol A (BPA), an endocrine disrupting chemical, has been suspected to pose carcinogenic risks. However, likely mechanisms are obscure and there are difficulties to estimating its real significance for cancer development. Methods: We therefore studied BPA-induced proteomic alterations in immune organs of ICR mice offspring that were prenatally exposed to BPA (15 and 300 mg/L of drinking water). We performed 2D-gel analyses of samples, considering differences in spleen, exposure levels, sex, and ages. Results: From proteomic analyses, we found various proteins were up- or down-regulated by BPA. Among them, SET, a putative oncogene and inhibitor of phosphatase 2A, was significantly down-regulated in a BPA dose-dependent manner. We also confirmed down-regulation of SET in western blot and real time PCR analyses. From gene network analysis, SET is predicted to communicate with other genes including CYP17, which is involved in biosynthesis and metabolism of sex-hormones. Conclusions: This study provided evidence that SET can be applied as a new biomarker for prenatal BPA exposure and suggests a potential new mechanism of action in that BPA may disrupt CYP17 via SET.

Key words: Bisphenol A - SET - prenatal exposure - proteomics - network analysis

Introduction

Most of endocrine disrupting chemicals (EDCs) have been suspected as carcinogens, particularly among second generations. In a case of diethylstylbestrol (DES) of EDCs, DES daughters, who were prenatally exposed to DES by mothers, have an increased risk of developing abnormal cells in the cervix and the vagina that are precursors of cancer, i.e. dysplasia, cervical intraepithelial neoplasia, and squamous intraepithelial lesions (Rubin, 2007).

Bisphenol A [2, 2-bis(4-hydroxyphenyl)propane, BPA] has been considered as a potential EDC. Due to its various usages and increased demands, e.g. substitutes to glass, it has been broadly detected in human urine and blood samples, even the placenta of pregnant women and breast milk (Kuroda et al., 2003; Engel et al., 2006; Ye et al., 2006; Lakind and Naiman, 2008; Yi et al., 2010; Yi et al., 2011). Therefore, exposure to BPA is thought to be quite popular in all populations. Particularly, prenatal exposure to BPA has been emphasized to prevent BPA risks for children's health due to their high susceptibility. BPA has been suspected its carcinogenic risks. However, its carcinogenic end points or mechanisms are obscure and there are even some difficulties to estimate or protect its related cancer risks. Therefore, researchers became to study BPA risks with different ways from those before. Among these studies, omics-approaches and second-hit theories are quite persuasive: For example of omics-approaches, Dolinoy et al. (2007) reported that early developmental exposure to BPA can change offspring phenotype by stably altering the epigenome (2007). In addition, exposure to BPA increased sensitivity of mutagen-induced sister chromatid exchanges in human, although BPA itself is not a mutagen (Yang et al. 2006), and of prostate gland to carcinogens (Prins et al. 2007). Using proteomic approaches, we also reported some BPA-responsive biomarkers, i.e. apolipoprotein A-I precursor (apo-AI), dipeptidylpeptidase III (DPPIII), and vesicle amine transporter 1 (VAT1), particularly in mouse-thymus (Yang et al., 2008).

In the present study, we performed proteomic and systems biological approaches to find out BPA-responsive biomarkers and -carcinogenic mechanisms among prenatally BPA- exposed mice offsprings.

Materials and Methods

Materials

All of the reagents for two dimensional polyacrylamide

*Research Center for Cell Fate Control, College of Pharmacy Sookmyung Women's University, Seoul, Korea *For correspondence: myang@sm.ac.kr*

gel electrophoresis (2D-PAGE) were of analytical grade and obtained from Amersham Pharmacia Biotech Korea Ltd. (Seoul, Korea). Sequencing grade trypsin was purchased from Promega (Madison, WI). Trifluoroacetic acid (TFA) and acetonitrile were from Merck Korea, Ltd. (Seoul, Korea). BPA and alpha-cyano-4-hydroxycinamic acid were from Sigma-Aldrich Co. (St. Louis, MO). Antibodies of SET and Apo A-I were obtained from Abcam (Cambridge, UK).

Animal Care and Treatment

Pregnant ICR mice were arrived at Sookmyung Women's University during the first week of pregnancy from Orient Co. (Seoul, Korea) and maintained under a 12-hr light cycle (lights on 07:00–19:00 h) at 21-24 °C and 40-60 % humidity with free access to pellet food (Samtako, Seoul, Korea) and water continuous access to water.

Pregnant mice group were administered different concentrations of BPA, which was dissolved in 0.1 % ethanol in water into 15 and 300 mg/L through glass bottle for 34-36 days, i.e. from day 7 or 7 days of pregnancy to lactation (days 21 or 21 days from delivery). On the other hand, control pregnant mice drunk 0.1% ethanol in water ad libitum. The 3 and 7 weeks-old offsprings were sacrificed to obtain their immune organ, spleens, which were immediately put into dry ice and kept -80 °C until experiments.

Protein Extraction

Protein extraction of sample spleen was performed as described previously (Yang et al., 2008): In brief, approximately 10 mg of each sample was suspended in 500 μl of lysis buffer containing 7 M urea, 2 M thiourea, 40 mM dithiothreitol (DTT), and 4 % 3-[(3-cholamidopropyl)dimethyl- ammonio] propane-sulfonate and homogenized. After centrifuging the homogenized samples, the protein contents were measured with Bradford assay (Bradford, 1976).

2D Gel Electrophoresis

We performed 2D-PAGE following our method (Yang et al., 2008): In brief, a total of 300 μg of protein was loaded on the IPG (Immobilized pH 4-7 nonlinear gradient) strips using IPGPhor (24 cm, Amersham Pharmacia Biotech Korea Ltd). Isoelectrofocusing was performed at 0-300 V for 3 hr, and 3,500V- 95,000V for 4 hr, and the strips were incubated in equilibration buffer 1 containing 50 mM Tris/HCl, pH8.8, 6 M urea, 30% glycerol, 2% Sodium dodecylsulfate (SDS), bromophenol blue (BPB), and DTT and buffer 2 containing 50 mM Tris/HCl, pH8.8, 6 M urea, 30% glycerol, 2% SDS, BPB, and DTT and 2.5% iodoacetamide for 15 min each. The equilibrated strips were placed on polyacrylamide slab gel and separated at 110 V in running buffer (25 mM Tris/HCL, pH 8.8, 198 mM glycine, and 0.1% SDS). After electrophoresis, proteins were visualized by silver staining. The silver-stained gels were scanned with densitometer 800 (Bio-Rad, Richmond, CA). The digitalized image was analyzed with PDQUEST software (V. 6.1, Bio-Rad), by which all major spots and all changing spots were outlined, quantified, and matched on all gels. We performed the 2 D gel analyses with two different mice for each concentration (control, 15 and 300 mg/L), sex (male and female), and age (3- and 7-week-old): Total number of mice samples was 24.

Mass Spectrometry

Selected proteins from 2D gel were identified with peptide mass finger printing methods (Yang et al. 2008): In brief, the protein spots were cut from gel by spot cutter (Bio-Rad), destained and dried in a speed vacuum concentrator for 5 min and rehydrated with 20 μl of 50 mM NH_4HCO_3 containing 0.2 μg modified trypsin for 30 min on ice. After removal of the solution, 30 μl of 50 mM NH4HCO3 was additionally added and digestion was performed with trypsin overnight at 37 °C. After the removal of residual trypsin, the peptides were desalted using C18 nanoscale column. Custom-made columns were used to desalt and concentrate the peptide mixture prior to mass spectrometric analysis: A column containing 100-300 μL of Poros reverse phase R2 material (20-30 μm bead size, PerSeptive Biosystems, Framingham, MA) was packed into a constricted GELoader tip (Eppendorf, Hamburg, Germany). A 10 mL syringe was used to force the liquid through the column via the application of gentle air pressure. Thirty μL of the peptide mixture from the digestion supernatant was loaded onto the column, and then washed with 30 μL of 5% formic acid. For the MS/MS analysis, the peptides were eluted by the direct application of 2.5 μL of a solution consisting of 60% methanol, 35% H_2O, and 5% formic acid, into a precoated borosilicate nanoelectrospray needle (EconoTipTM, New Objective, Woburn, MA). MS/MS of peptides generated by in-gel digestion was performed by nano-ESI on a Q-TOF mass spectrometer (Micromass, Manchester, UK). The source temperature was 80 °C. A potential of 1 kV was applied to the precoated borosilicate nanoelectrospray needles in the ion source combined with a nitrogen back-pressure of 0–5 psi to produce a stable flow rate (10–30 nL/min). The cone voltage was 40 V. The quadrupole analyzer was used to select precursor ions for fragmentation in the hexapole collision cell. The collision gas was Ar at a pressure of $6–7 \times 10^{-5}$ mbar and the collision energy was 20–30 V. Product ions were analyzed using an orthogonal TOF analyzer, fitted with a reflector, a micro-channel plate detector and a time-to-digital converter. All MS/MS spectra recorded on tryptic peptides derived from spot were searched against protein sequences from NCBInr and EST databases using the MASCOT search program.

Western Blot Analysis

Eighty μg of each sample protein was loaded on 15% (w/v) SDS polyacrylamide gel and separated by electrophoresis. The gels were transferred to

polyvinylidene difluoride membranes (Hybond™ -P, Amersham Biosiences, Burkinghamshire, UK) and blocked with phosphate-buffered saline/5% skim milk/0.05% Tween 20 for 1.5 hours. Primary antibodies, SET and Apo A-I, were diluted to 1:1000 in blocking buffer and incubated for 2 hrs at room temperature. After washing, membranes were incubated for 1.5 hrs with a horse radish peroxidase-conjugated secondary antibody, and developed with ECL Plus western blotting detection kit (GE Healthcare, Bukinghamshire, UK).

Gene Expression Analysis by Real-time PCR

Total RNAs were isolated from homogenized spleen samples by SV Total RNA Isolation System (Promega). One ug of the RNA sample was used for reverse transcription polymerase chain reaction (RT-PCR) and real time PCR. The RNA molecules were subjected to cDNA synthesis using Applied Biosystems High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA) according to manufacturer's suggested protocol. To quantify SET expression, we used TaqMan fluorogenic real time PCR method with an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The primers and probe were 5'-CCGAAGCTGGACAAGTC-3' (sense) and 5'-AGCTACGCGGGAAAACTACAC-3'(antisense), and FAM5'-ACGACTCGA- AGCTCC-TAMRA, respectively. PCR conditions were as followed: 2 min at 50 °C for AmpErase UNG activation and 10 min at 95 °C for UNG inactivation, followed by 40 cycles of 15 sec at °C for denaturing, and 1 min at 60 °C for annealing and extension. After constructing standard curves, mRNA expression levels of SET was quantified and normalized with respect to expression of 18S rRNA (Applied Biosystems), a reference gene. We performed triplicate realtime PCR analyses for each sample.

Global gene network analysis

Using STRING scores (STRING 9.0), we studied global function of SET and its related proteins.

Statistical Analyses

ANOVA was used to analyze differences in body weights, consumption of water and expression of SET due to BPA exposure. Association between BPA exposure levels and mRNA expression of SET was studied with regression analysis. P-values for all tests were computed by JMP version 4 (SAS Institute, Cary, NC), and $p < 0.05$ was used to identify significant associations.

Table 1. Exposure Levels of BPA in Mice

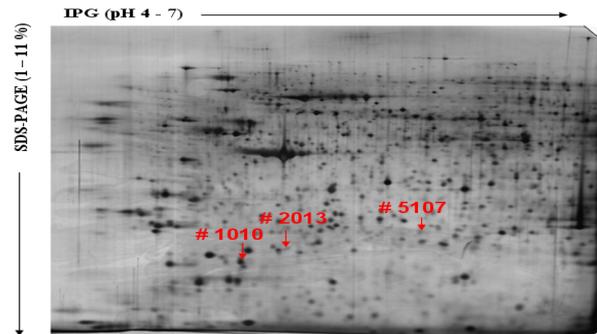| Treatment	N	BPA in drinking water (mg/L)	Body weight water (g)	Consumed drinking (ml/day)	Real exposure to BPA (mg/ kg/ day)
Control	18	0	33.5±2.9	20.6±2.4	0
Low Dose	15	15	35.5±2.9	21.0±3.3	8.9±1.8
High Dose	16	300	34.4±1.3	19.6±1.7	171.1±16.8

Figure 1. Narrow pH Range of Isoelectric Focusing on 2D Gel. Homogenized spleen tissue (350 ug) was loaded onto the gel. Dose-dependent spots (#1010, 2013, and 5107) as well as other features were visible by silver staining
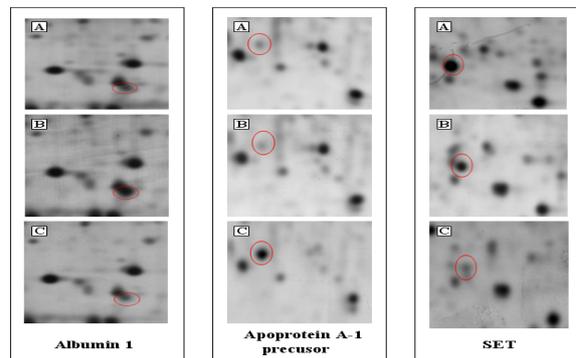

Figure 2. Coordinated Changes of Target Proteins in Spleen Tissue of 3 Weeks-old Offspring. Albumin 1 in male mice; Apo A-I precursor and SET in female mice; A, control; B, 15 mg of BPA/L of water; C, 300 mg of BPA/L of water

Results

BPA Exposure Levels

We calculated the real exposure levels of BPA from different concentrations of BPA in drinking water (Table 1). There were no significant differences in body weight or consumption of drinking water due to BPA exposure (ps> 0.05).

Proteomic analysis

We carried out 2D-PAGE to find responsive proteins for BPA exposure in offspring-spleens. Protein spots were statistically evaluated in order to identify significantly regulated spots by BPA- exposure. Comparison of abundant proteins on 2D gels using PDQUEST program revealed that only the Albumin A1 and SET was significantly down-regulated by BPA; Apo A-I was up-regulated by BPA, compared to controls (Table 2; Figure 1-2). These proteins were identified by peptide mass fingerprinting method using ESI-Q-Tof mass spectrometry and database search.

Validation of the Proteins by Western Blots and Real-time PCR

For the validation of the 2D-gel results, we assessed the protein and gene expression levels by western blots

Table 2. Identification of Responsive Proteins in Mice Spleen

Spot#	Peptide	Sex	Age[a]	expression	NCBI I.D.	Proteins	Description
1010	LVQEVTDFAK	M	3	↓	gi 26341396	Albumin 1 (Unnamed protein product)	Albumin domain, contains five
			7	↓			or six internal disulphide bonds
2103	HSLMPMLETLK	F	3	↑	gi 109571	Apoprotein A-1 precursor	Lipoprotein, HDL component
			7	↑			
5107	IDFYFDENPYFENK	M	3	↓	gi 1711383	SET beta isoform	Phosphatase 2A inhibitor

[a]Age of mice (week); M, male; F, female; ↑, up-regulated; ↓, down-regulated
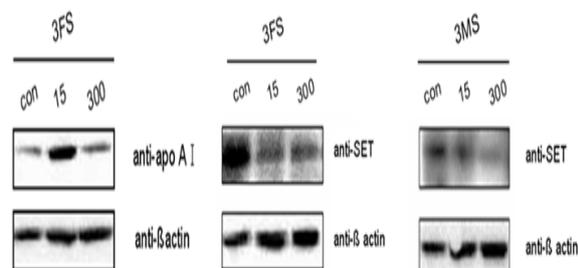

Figure 3. Proteomic Alteration of Apo A-I and SET in BPA-dose Dependent Manner. 3FS, 3-week-old female spleen; 3MS, 3-week-old male spleen; con, control; 15, 15 mg of BPA/L of water; 300, 300 mg of BPA/L of water

and real-time PCR. We confirmed BPA down-regulates SET protein-expression and this result is consistent with the above 2D gel results. However, we could not confirm BPA-modulation in protein-expression of apo A-I through western blots (Figure 3). Therefore, we further studied whether BPA exposure altered mRNA expression of not apo A-I but SET. Performing the real time-PCR, we found that expression of SET was down-regulated in BPA-dose dependent manner, particularly in the spleens of 3 weeks-old female mice (Figure 4).

Global Function of SET

From gene network analysis in Mus musculus, SET is estimated to interact with CYP17A1 (cytochrome P450, family 17, subfamily a, polypeptide 1 Gene), ANAP32A [acidic (leucine-rich) nuclear phosphoprotein 32 family, member A Gene], TREX1 (three prime repair exonuclease 1 Gene), SMYD3 (SET and MYND domain containing 3 Gene, a histone methyltransferase), CDK5 (cyclin-dependent kinase 5 Gene), SMYD1 (SET and MYND domain containing 1 Gene, a transcriptional repressor), DEK [DEK oncogene (DNA binding) Gene], SMYD2 (SET and MYND domain containing 2 Gene), NSD1 (nuclear receptor-binding SET-domain protein 1 Gene, a histone methyltransferase) and HNRN PA2B1 (heterogeneous nuclear ribonucleoprotein A2/B1 Gene) within score 0.924-0.771. Among the above genes, CYP17A1 shows the strongest association with SET (score, 0.924) from supporting papers (Zhang et al., 2001; Belcher et al., 2005; Mellon et al., 2007).

Discussion

The present study was designed as our serial attempts to find BPA-sensitive proteomic biomarkers and to clarify carcinogenic mechanisms of BPA in spleen. In the present study, BPA showed down regulation of SET and it was
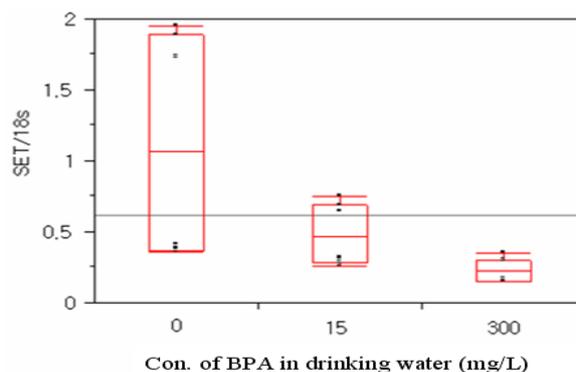

Con. of BPA in drinking water (mg/L)

Figure 4. Effects of BPA Exposure on SET Expression in Spleen of 3-week-old Female Mice (N=9). Expression of SET was decreased in BPA dose-dependent manner (R^2, 0.23; $p=0.04$ by regression analysis)

consistently confirmed by 2D-gel, western blot and real-time PCR analyses. SET was first described as part of the SET-CAN fusion gene, a putative oncogene associated with acute undifferentiated leukaemia (von Lindern et al., 1992): SE in SET refers to the patient with leukemia containing SET translocation and the T in SET refers to translocation (Simon et al., 2012).

In a case of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (TCDD), the most notorious EDC, it has a two-edged blade: It is carcinogenic and triggering lots of endocrine disorders, however, inhibits estrogen-induced responses in the rodent uterus and mammary tumors and in breast and endometrial cancer cell lines through complex inhibitory aryl hydrocarbon receptor (AhR) -estrogen receptor (ER) crosstalk (Safe and McDougal, 2002). Therefore, some of selective AhR modulators, which have structure-similarities with TCDD and are relatively non-toxic, inhibit ER-positive and ER-negative mammary tumor growth, synergize with tamoxifen to inhibit breast cancer growth and block tamoxifen-induced estrogenic activity in the uterus. BPA is also suspected to have two-edged blade, because it is suspected as a carcinogen and EDC, however, down-regulated oncogenic SET in the present study. SET is suggested as a new treatment target in B-cell malignancies and SET antagonists can represent novel agents for treatment of chronic lymphocytic leukemia and non-Hodgkin lymphoma (Christensen et al., 2011). Therefore, some of new chemicals, which have structure-similarity to BPA but are not harmful, can be potential anticancer medicines for SET-mediated cancers.

On the other hand, SET is expected to interact with CYP17A1 (score, 0.924) from the gene network analysis (Zhang et al., 2001; Belcher et al., 2005; Mellon et al., 2007). CYP17 is known to convert pregnenolone and

progesterone to their 17-alpha-hydroxylated products and subsequently to dehydroepiandrosterone and androstenedione. Therefore, CYP17 may be involved in sexual development during fetal life and at puberty. In addition, a recent study showed that BPA inhibits activity of human and rat testicular steroidogenic enzyme including CYP17A1 (Ye et al., 2011). Therefore, the present study provides a potential new mechanism that BPA disrupts CYP17 via SET.

In the present study, the putative BPA-biomarker, SET, was down-regulated in spleens of not 7 weeks- but 3 weeks-old female mice by BPA prenatal exposure. These results suggest that BPA exposure during pregnancy and lactation may induce the proteomic alteration in immune organ, particularly spleen, in young generation. In addition, the spleen undergoes significant molecular remodeling during puberty, resulting in both age and gender-dependent differences in immune system (Lamason et al., 2006). Therefore, SET can be an early and female selective biomarker in immune system from consideration of age and sex.

In conclusion, our present study provides that SET can be a responsive biomarker for BPA-prenatal exposure with proteomic and gene net work approaches. In addition, interaction between BPA and SET should be further studied to clarify BPA- end points.

Acknowledgement

References

Belcher SM, Le HH, Spurling L, et al (2005). Rapid estrogenic regulation of extracellular signal- regulated kinase 1/2 signaling in cerebellar granule cells involves a G protein- and protein kinase A-dependent mechanism and intracellular activation of protein phosphatase 2A. *Endocrinology*, **146**, 5397-406.

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, **72**, 248-54.

Christensen DJ, Chen Y, Oddo J, et al (2011). SET oncoprotein overexpression in B-cell chronic lymphocytic leukemia and non-Hodgkin lymphoma: a predictor of aggressive disease and a new treatment target. *Blood*, **118**, 4150-8.

Dolinoy DC, Huang D, Jirtle RL (2007). Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. *Proc Natl Acad Sci U S A*, **104**, 13056-61.

Engel SM, Levy B, Liu Z, et al (2006). Xenobiotic phenols in early pregnancy amniotic fluid. *Reprod Toxicol*, **21**, 110-2.

Kuroda N, Kinoshita Y, Sun Y, et al (2003). Measurement of bisphenol A levels in human blood serum and ascitic fluid by HPLC using a fluorescent labeling reagent. *J Pharm Biomed Anal*, **30**, 1743-9.

Lakind JS, Naiman DQ (2008). Bisphenol A (BPA) daily intakes in the United States: estimates from the 2003-2004 NHANES urinary BPA data. *J Expo Sci Environ Epidemiol*, **18**, 608-15.

Lamason R, Zhao P, Rawat R, et al (2006). Sexual dimorphism in immune response genes as a function of puberty. *BMC Immunol* , **7**, 2.

Mellon SH, Bair SR, Depoix C, et al (2007). Translin coactivates steroidogenic factor-1-stimulated transcription. *Mol Endocrinol*, **21**, 89-105.

Prins GS, Birch L, Tang WY, et al (2007). Developmental estrogen exposures predispose to prostate carcinogenesis with aging. *Reprod Toxicol*, **23**, 374-82.

Rubin MM (2007). Antenatal exposure to DES: lessons learned future concerns. *Obstet Gynecol Surv*, **62**, 548-55.

Safe S, McDougal A (2002). Mechanism of action and development of selective aryl hydrocarbon receptor modulators for treatment of hormone-dependent cancers. *Int J Oncol* , **20**, 1123-8.

Simon V, Oner SS, Cohen-Tannoudji J, et al (2012). Influence of the Accessory Protein Set on M3 Muscarinic Receptor Phosphorylation and G Protein Coupling. *Mol Pharmacol*, doi:10.1124/mol.111.075523.

von Lindern M, van Baal S, Wiegant J, et al (1992). Can, a putative oncogene associated with myeloid leukemogenesis, may be activated by fusion of its 3' half to different genes: characterization of the set gene. *Mol Cell Biol*, **12**, 3346-55.

Yang M, Kim SY, Chang SS, et al (2006). Urinary concentrations of bisphenol A in relation to biomarkers of sensitivity and effect and endocrine-related health effects. *Environ Mol Mutagen*, **47**, 571-8.

Yang M, Lee HS, Pyo MY (2008). Proteomic biomarkers for prenatal bisphenol A-exposure in mouse immune organs. *Environ Mol Mutagen*, **49**, 368-73.

Ye L, Zhao B, Hu G, et al (2011). Inhibition of human and rat testicular steroidogenic enzyme activities by bisphenol A. *Toxicol Lett*, **207**, 137-42.

Ye X, Kuklenyik Z, Needham LL, et al (2006). Measuring environmental phenols and chlorinated organic chemicals in breast milk using automated on-line column-switching-high performance liquid chromatography-isotope dilution tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*, **831**, 110-5.

Yi B, Kasai H, Lee HS, et al (2011). Inhibition by wheat sprout (Triticum aestivum) juice of bisphenol A-induced oxidative stress in young women. *Mutat Res*, **724**, 64-8.

Yi B, Kim C, Yang M (2010). Biological monitoring of bisphenol A with HLPC/FLD and LC/MS/MS assays. *J Chromatogr B Analyt Technol Biomed Life Sci*, **878**, 2606-10.

Zhang P, Compagnone NA, Fiore C, et al (2001). Developmental gonadal expression of the transcription factor SET and its target gene, P450c17 (17alpha-hydroxylase/c17, 20 lyase). *DNA Cell Biol*, **20**, 613-24.