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Toxicogenomic Analysis and Identification of Estrogen Responsive Genes of Di (n-ethylhexyl) Phthalate in MCF-7 Cells

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Abstract

Di (n-ethylhexyl) phthalate (DEHP) is thought to mimic estrogens in their action, and are called endocrine disrupting chemicals. DEHP is used in numerous consumer products, especially those made of flexible polyvinyl chloride and have been reported to be weakly estrogenic. In this study, DEHP were tested for estrogenic properties *in vitro* models and with microarray analysis. First, the E-screen assay was used to measure the proliferation of DEHP in MCF-7 cells, a human breast cancer cell line. DEHP induced an increase in MCF-7 cell proliferation at concentration of 10^{-4} M. Second, we carried out a microarray analysis of MCF-7 cells treated with DEHP using human c-DNA microarray including 401 endocrine system related genes. Of the genes analyzed, 60 genes were identified showing significant changes in gene expression resulting from DEHP. Especially, 4 genes were repressed and 4 genes were induced by DEHP compared to 17β-estradiol. Among these genes, trefoil factor 3 (intestinal), breast cancer 1, early onset and CYP1B1 are involved in estrogen metabolism and regulation. Therefore it suggests that these genes may be associated with estrogenic effect of the DEHP on transcriptional level. The rationale is that, as gene expression is a sensitive endpoint, alterations of these genes may act as useful biomarkers to define more precisely the nature and level of exposure to kinds of phthalates.

Keywords: di (n-ethylhexyl) phthalate (DEHP), gene expression, biomarker, estrogen responsive gene, endocrine disrupting chemicals

There has recently been considerable concern about the potential endocrine-disrupting effects of chemicals released into the environment. Those chemicals that have estrogenic activity are termed xenoestrogens or endocrine disrupting chemicals (EDCs). They are thought to mimic or disturb the function of estrogen and many are known to possess estrogen receptor-binding activity[1].

Many phthalate esters have long been known to be reproductive toxicants when animals are dosed as juveniles or adults, and their teratogenicity is well established[2,3], yet little has been published on the effects of *in utero* and lactational (or continuous multigenerational) exposure to any phthalate ester on postnatal development of the male or female reproductive systems or sexual differentiation of the central nervous system (CNS). Of the approximately 20 phthalate esters in common use, di (n-ethylhexyl) phthalate (DEHP) constitutes approximately half the total; 1-4 million tons are produced per year[2,4]. DEHP is used in numerous consumer products, especially those made of flexible polyvinyl chloride. The use of DEHP in teething rings, pacifiers, and toys for young children has largely been discontinued, but DEHP continues to be used in clothing, toys, food containers, and a variety of building, household, and automotive products[5]. Typical human exposure is estimated to be 4-30 μg DEHP/kg/day, but some individuals have substantially greater exposure resulting from DEHP-plasticized medical devices such as blood bags, hemodialysis tubing and membranes, autophoresis equipment, and nasogastric feeding tubes[6]. The average long-term dialysis patient is reported to receive approximately 12 g of DEHP over the course of a year[7].

DEHP has been known to cause liver tumors in rodents[8] and to enhance tumor promotion in skin[9] and liver[10] of mice. However, in 2000 IARC (the International Agency for Research on Cancer) downgraded DEHP from 2B to 3 group, ie, "not classifiable as to its carcinogenicity to humans"[11]. On the other hand, DEHP caused adverse effects in male reproductive organs in mice and rats, respectively[12].

DEHP at a concentration of 10^{-5} M were weakly estrogenic in the cell proliferation assay with MCF-7 cells[13]. Di-n-alkyl phthalates such as n-butyl benzyl phthalate (BBP), di (n-butyl) phthalate (DBP) and

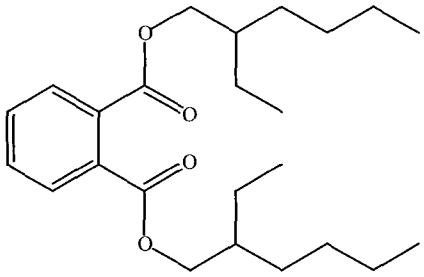

Fig. 1. Structure of di (n-ethylhexyl) phthalate.

DEHP have estrogenic activity that mimics the steroid A ring of receptor binding modes of dialkyl phthalates[14] (Fig. 1.). Experimental results that exposure to DEHP caused prolonged estrous cycles and altered natural ovulation times in female rats[15] might indicate the disruption of the physiological function in female reproductive organs. DEHP showed chronic toxicity in rats through a mechanism thought to involve peroxisome proliferation[16]. On the other hand, DEHP did not show any estrogenic activity in recombinant yeast screen assay[17] and in the binding competition to human uterine estrogen receptor[18].

Currently, a wide variety of testing methods have been developed for EDCs[19]. These include physical and chemical fractionation methods[20-22], the study of biomarkers in sentinel species, and single mode of action (MOA) oriented *in vivo* and *in vitro* assays, and life cycle or multigenerational *in vivo* tests. Development of novel approaches for high-throughput screening for potential EDCs is a major goal in the environmental health research. The need for advancing prediction the adverse biological effects of EDCs in mammalian has made technologies exploiting advances in molecular techniques. Current molecular-level techniques rely on ligand-binding assays[23], enzyme-linked immunosorbent assay (ELISA)[24], and more recently, gene expression profiling[25,26]. In the near future, more reliance will be placed on the development of gene expression assays to determine the intricate interactions between genes that are affected by the exposures. It has the potential to implicate previously unsuspected estrogen, androgen and thyroid hormone-sensitive genes that may later become molecular markers of endocrine disruption[27,28].

In this respect, we designed a high throughput DNA microarray system, namely KISTCHIP-400, which responses to EDCs may be detected by gene expression, to assist in the identification of potential EDCs and to understand molecular toxicological mechanisms of EDCs[29]. In the present study, to improve the controversy on whether DEHP has an estrogenic effect

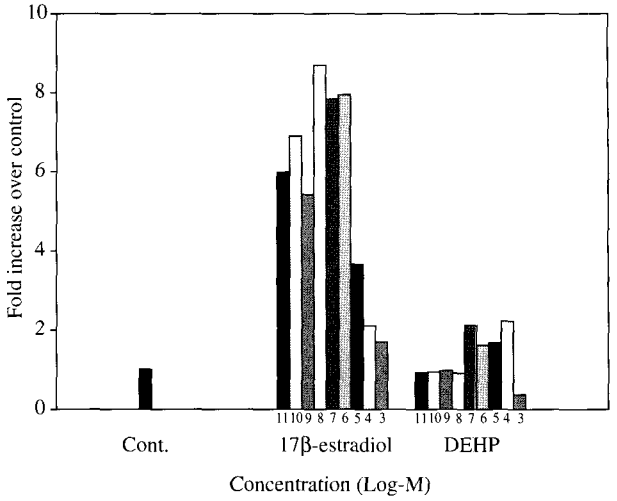

Fig. 2. Estrogenic activity of 17β-estradiol and di (n-ethylhexyl) phthalate by E-screen assay. Cells were exposed to test compounds for 6 days with 5% charchol/dextran-treated serum in the cell culture medium.

or another reproductive effect, we have determined the effect of DEHP and 17β-estradiol on gene expression using KISTCHIP-400, and discussed whether the gene could be used as a biomarker to assess the estrogenic effect of DEHP.

Estrogenic Activity and Dose Selection of DEHP

17β-estradiol at concentrations ranging from 10^{-14} to 10^{-5} M was used as a reference chemical, with known estrogenic effects. The relative cell numbers were measured at day 6 of treatment with DEHP at concentrations from 10^{-11} to 10^{-3} M. The effects of DEHP on the proliferation of MCF-7 cells are shown in Fig. 2. A 1.5-fold increase in cell growth over the control was adopted as a criterion of estrogenicity. Significant proliferation of MCF-7 cells was induced by 17β-estradiol at concentration of 10^{-8} M ($p<0.05$). DEHP significantly stimulated proliferation of MCF-7 cells at a concentration of $10^{-4} \sim 10^{-7}$ M ($p<0.05$). In order to examine microarray for DEHP, we were chosen the 10^{-4} M concentration, the highest estrogenic potency dose.

Analysis of the Gene Expression Patterns Induced by DEHP

To evaluate whether genes spotted upon constructed cDNA microarray responds to DEHP with estrogenic activity, 10^{-4} M DEHP was treated to observe gene expression profiles. Five independent experimental samples for each treatment group were analyzed to

Table 1. List of up- and down-regulated genes by di (n-ethylhexyl) phthalate of 10^{-4} M in MCF-7 cell lines

Accession No.	Gene name	DEHP	17β-estradiol
NM_003226	Trefoil factor 3 (intestinal)	0.892	0.866
NM_007295	Breast cancer 1, early onset	0.811	0.722
U03688	Cytochrome P450, family 1, subfamily B, polypeptide 1	0.654	0.961
AA884709	Cytochrome P450, family 11, subfamily B, polypeptide 1	0.590	0.637
AA130187	Wilms tumor 1	0.701	
Y14039	CASP8_and_FADD-like_apoptosis_regulator	1.241	
R39356	Tumor protein p53 (Li-Fraumeni syndrome)	0.995	
AA485226	Vitamin D (1 25-dihydroxyvitamin D3) re	0.981	
M92424	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)	0.961	
NM_001515	general_transcription_factor_IIH _polype	0.891	
AA453202	Nuclear receptor subfamily 1, group D, member 1	0.838	
X87838	Catenin (cadherin-associated protein), beta 1, 88kDa	0.824	
AA858390	Calcyphosine	0.817	
AA460148	Ataxia telangiectasia mutated (includes complementation groups A, C and D)	0.782	
AA434084	Thyroid hormone receptor associated protein 1	0.757	
R33030	HLA CLASS II HISTOCOMPATIBILITY ANTIGEN	0.727	
AA974848	Dystrophia myotonica-protein kinase	0.709	
NM_004786	Thioredoxin-like 1	0.678	
AA451969	Transcription elongation factor A (SII)-like 1	0.674	
NM_001621	aryl_hydrocarbon_receptor	0.632	
U37230	Ribosomal protein L23a	0.614	
AA486238	Homo sapiens thyroid autoantigen (trunca	0.590	
S79862	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 5	0.589	
AF012108	Nuclear receptor coactivator 3	0.832	−0.664
X58072	GATA binding protein 3	0.816	−0.665
X59408	Membrane cofactor protein (CD46, trophoblast-lymphocyte cross-reactive antigen)	0.747	−1.052
M31470	Ras homolog gene family, member Q	0.721	−0.747
L05779	Epoxide hydrolase 2, cytoplasmic	0.692	−0.796
M30938	X-ray repair complementing defective repair in Chinese hamster cells 5 (double-strand-break rejoining; Ku autoantigen, 80kDa)	−0.748	0.931
AA424833	Bone morphogenetic protein 6	−0.786	0.639
AF043254	TNF receptor-associated protein 1	−0.835	0.619
T87622	Sin3-associated polypeptide, 30kDa	−0.932	0.840
AA487700	Cyclin D1 (PRAD1: parathyroid adenomatosis 1)	−0.960	1.053
NM_001758	Cyclin D1	−1.058	0.863
NM_002014	FK506 binding protein 4, 59kDa	−1.487	0.925
H29521	ATP-binding cassette, sub-family A (ABC1), member 3	−1.494	1.079
AA670429	ad20 g03.s1 Soares_NbHFB Homo sapiens cDNA clone IMAGE: 878836 3' similar to gb: Y00757 NEUROENDOCRINE PROTEIN 7B2 PRECURSOR (HUMAN); mRNA sequence	−1.892	−1.286
Z23115	BCL2-like 1	−0.573	−1.614
AA699864	Coiled-coil domain containing 6	−0.974	−0.659
AA434144	Claudin 3	−1.350	−0.626
H37774	Tuberin TSC2	−0.580	
NM_001985	Electron-transfer-flavoprotein, beta polypeptide	−0.601	
R43309	von Hippel-Lindau syndrome VHL	−0.604	
AA426212	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (protein disulfide isomerase; thyroid hormone binding protein p55)	−0.605	
U42412	protein_kinase _AMP-activated _gamma_1_n	−0.619	
T47483	peptidylprolyl isomerase D (cyclophilin	−0.621	
D13748	Eukaryotic translation initiation factor 4A, isoform 1	−0.627	
AA403083	Presenilin 1 (Alzheimer disease 3)	−0.669	
AA845432	Parathyroid hormone-like hormone	−0.670	
AA455970	Ring finger protein 139	−0.716	
R66447	V-myc myelocytomatosis viral related oncogene, neuroblastoma derived (avian)	−0.787	
D13643	24-dehydrocholesterol reductase	−0.816	
X53587	Integrin, beta 4	−0.826	

Table 1. *Continued*

Accession No.	Gene name	DEHP	17β-estradiol
AA486238	Filamin B, beta (actin binding protein 278)	−0.856	
M11560	Aldolase A, fructose-bisphosphate	−0.906	
AF044773	Barrier to autointegration factor 1	−0.943	
AA725564	Superoxide dismutase 3, extracellular	−0.945	
NM_001273	Chromodomain helicase DNA binding protein 4	−0.953	
AF012108	nuclear_receptor_coactivator_3	−1.271	
R91438	protein phosphatase 1 catalytic subunit	−1.479	

The results of KISTCHIP-400 analysis are shown as values of fold change (fluorescent intensity for chemical plus/fluorescent intensity for chemical minus). Genes presented in box was selected on a common set of genes by 17β-estradiol and DEHP.

Table 2. Estrogen responsive genes of DEHP

Accession NO.	Gene name	Biological function	Fold change vs. Control	
			DEHP	17β-estradiol
NM_003226	Trefoil factor 3 (intestinal)	Defense response	0.892	0.866
NM_007295	Breast cancer 1, early onset	Apoptosis regulation	0.811	0.722
U03688	Cytochrome P450, family 1, subfamily B, polypeptide 1	Electron transport	0.654	0.961
AA884709	Cytochrome P450, family 11, subfamily B, polypeptide 1	Androgen and estrogen metabolism	0.590	0.637
AA670429	ad20 g03.s1 Soares_NbHFB Homo sapiens cDNA clone IMAGE: 878836 3' similar to gb: Y00757 NEUROENDOCRINE PROTEIN 7B2 PRECURSOR (HUMAN); mRNA sequence		−1.892	−1.286
Z23115	BCL2-like 1	Anti-apoptosis	−0.573	−1.614
AA699864	Coiled-coil domain containing 6	Cell growth and/or maintenance	−0.974	−0.659
AA434144	Claudin 3	Transmembrane receptor activity	−1.350	−0.626

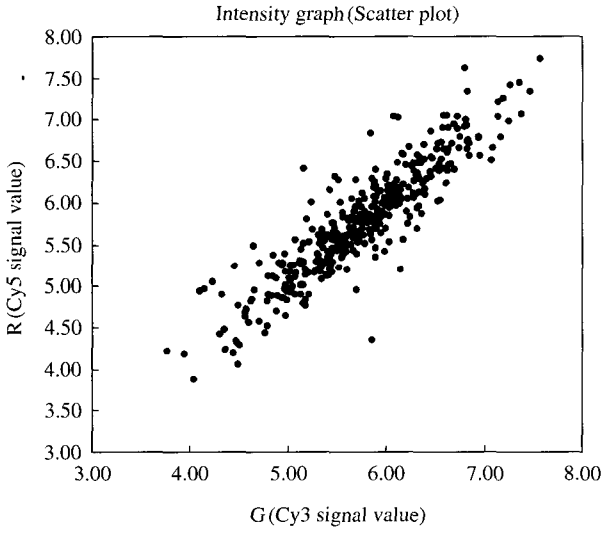

Fig. 3. Scatter plot of fluorescent signal intensity from an hybridization to the KISTCHIP-400 of di (n-ethylhexyl) phthalate treated and untreated MCF-7 cells with Cy3 and Cy5 labeled probes, respectively.

determine RNA transcript levels. Based on the number of genes expressed in control versus treated samples, as well as on the level of expression of individual genes, the overall gene expression pattern was similar between control (vehicle-treated) and estrogenic compound-treated (17β-estradiol and DEHP) (Fig. 3, Table 1). Although the number of genes whose expression is altered by DEHP is not very high, there are many genes whose expression is modified by exposure to DEHP. Of the 416 genes, there were 28 and 32 genes whose expression level showed some evidence of treatment effect (up- or down-regulated) by DEHP, respectively, relative to their respective vehicle control and judged by fold change (at least ± 1.5 fold, up or down) (Table 1). Also, analysis of the data derived from 17β-estradiol and DEHP, indicated that the expression of 8 genes was consistently and significantly regulated in the same direction, although at a different magnitude. These include genes known to be directly regulated by estrogens but also other annotated genes and ESTs that have not been previously identified as estrogen-

responsive one. Table 2 shows the complete list of the 8 genes that showed a statistically significant change in gene expression by DEHP and 17β-estradiol exposure, along with their accession number, fold change (average calculated by comparing treatment versus control) and biological function. Among these, over-expressed genes composed of TFF3 (trefoil factor 3, intestinal), BRCA1 (breast cancer 1, early onset), CYP1B1 (cytochrome p450, family 1, subfamily B, polypeptide 1) and CYP11B1 (cytochrome p450, family 11, subfamily B, polypeptide 1) and down-expressed gene included a human EST sequence, BCL2L1 (BCL2-like1), CCDC6 (coiled-coil domain containing 6) and CLDN3 (claudin 3).

Discussion

Toxicogenomic Approach will be Powerful Tools[30] and May Solve the Biological Relevance of Very Low Quantity With Long Term Exposure of Environmental Hazardous Chemicals at Present

A significant concern has recently been raised about the potential of environmental chemicals that might disrupt endocrine function. Particular attention has been given to chemicals that are able to alter estrogen functions. For this reason, we evaluated whether exposure to estrogenic compounds could be identified by transcripts profiling. In this study we have used this approach to identify the gene expression profile induced by estrogenic effects of DEHP in MCF-7 cells.

An increased awareness of EDCs and their potential to affect wildlife and humans has produced a demand for practical screening methods to identify endocrine activity[31,32]. Despite the exciting prospects of this methodology, a scan of the literature reveals very few toxicogenomic studies. In our previous report, we have constructed a human endocrine related cDNA microarray, called KISTCHIP-400, which contains many of the human genes known or proposed to be involved in endocrine system[29]. We believe that this will serve as a template for future studies in toxicogenomics for performing EDCs monitoring. Through this kind of studies, it is possible to identify the patterns of alteration of gene expression characteristic of exposure to estrogen like EDCs in cultured human cells. And once signatures are identified, the patterns of altered gene expression induced by unknown agents might identify their mechanism of action. This approach could also be applied to identify signatures for various types of tissue-specific EDCs, providing a more rapid test for the possible toxicological effect of drugs or unknown agents and less need to use animals.

The gene expression profile induced by 17β-estradiol exposure identified in the present study by no means should be considered a complete list of genes whose expression can be regulated by estrogenic compounds. Among the genes expressed by DEHP, we pay attention to TFF3, BRCA1 and CYP1B1 because it is first reported that this gene shows the alteration of gene expression followed by not only 17β-estradiol but DEHP treatment in this study. Up-regulation of BRCA1 by 17β-estradiol treatment was previously reported[33]. BRCA1, which functions as a tumor suppressor in human breast cancer cells, is a nuclear phosphoprotein which associates with RNA polymerase II holoenzyme. Mutations in BRCA1 are predicted to be responsible for approximately 45% of inherited breast cancer[34]. Also, other published studies have examined that BRCA1 transcription is induced as a result of not direct responsiveness but the mitogenic activity of 17β-estradiol in estrogen receptor positive cells[33]. CYP1B1 is expressed constitutively in the steroidogenic tissues of mammals and is inducible by peptide hormones, cAMP, and AhR ligands. The mechanism of induction of CYP1B1 is known through the AhR signaling pathway. CYP1B1 is involved in the metabolism of procarcinogens and xenobiotics, such as ethoxyresorufin, theophylline and caffeine[35], and shows overlapping catalytic activities with CYP1A1 (cytochrome p450, family 1 subfamily A polypeptide 1)[35,36]. CYP1B1 mainly catalyzes the conversion of 17β-estradiol to the catechol estrogen metabolite 4OH-17β-estradiol that has been postulated to be involved in mammary carcinogenesis. CYP1A1 encodes EROD and mainly catalyzes 17β-estradiol to its 2OH-17β-estradiol. The CYP1B1/CYP1A1 ratio is a critical determinant of the metabolism and toxicity of estradiol in mammary cells[37]. CYP1B1 activity participates in endocrine regulation and toxicity of estrogens *in vivo*[38]. TFF3 is a member of a family of polypeptides encoded by a cluster of genes on chromosome 21. Through gene expression profiling studies, TFF3 mRNA has been found to be over-expressed in prostate cancer[39].

Thus, the expression of BRCA1, CYP1B1 and TFF3 could possibly serve as a direct marker to assess the DEHP on the reproductive and endocrine system, as the up-regulated genes by DEHP treatment.

In conclusion, we have identified estrogen responsive genes including BRCA1, CYP1B1 and TFF3, which is expressed in MCF-7 cells treated to 17β-estradiol and DEHP. These genes could be a promising biomarker to detect uncertain EDCs including other phthalates. Also, it can be suggested that the KIST-

CHIP-400 is of a highly enough quality so that it can be used for further analysis of the gene expression profiles of safety, MOA and screening of endocrine disrupting related chemicals. We have been proposed that this approach also offers the possibility to identify the molecular marker involved in action of natural and synthetic estrogenic compounds, providing information on interrelationships among the responsive genes. However, the potential use of this estrogenic molecular "fingerprint" to discriminate estrogenic compounds from different classes of chemicals has to be investigated further.

Methods

Cell Culture and Chemical Treatment

MCF-7 cell line originated from human breast cancer cell was a gift from the Prof. Soto (Tufts University School of Medicine, Boston, MA, USA). The cells were cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen Life Technologies, CA) supplemented with 5% fetal bovine serum (FBS) (Invitrogen Life Technologies, CA), penicillin 100 IU/mL and streptomycin 100 μg/mL. Subculturing was conducted every 3 day so as not to exceed 1×10^6 cells/mL. At 80% confluence, cells were treated with 10^{-8} M 17β-estradiol (Sigma, St. Louis MO) and 10^{-4} M DEHP (Wako, Tokyo, Japan) in phenol-red-free DMEM/F12 supplemented with 5% charchol/dextran fetal bovine serum (CDFBS) culture media for 48 h and these chemicals were dissolved in absolute ethanol. Vehicle concentrations were less than 0.1% in all experiments.

Cell Proliferation Experiments (E-screen Assay)

MCF-7 cell line was tested with slight modification described by Soto *et al.*[40]. Cells were trypsinized and plated in 12-well plates at an initial concentration of 3×10^4 cells per well in 5% FBS in DMEM. The cells were allowed to attach for 24 h, then 5% CDFBS supplemented phenol red-free DMEM was substituted for the seeding medium. Appropriated concentrations of the test compounds were added. The assay was stopped after 168 h by mixing with 5 mg/mL of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis MO) and the plate was further incubated for 3 h at 37°C. The medium was removed and the formed formazan crystals were dissolved with 1 mL of dimethylsulfoxide (DMSO; Sigma, St. Louis MO). Finally, the aliquots were transferred to a 98-well plate to read optical density (OD) in a microtiter plate reader at 540 nm[40,41].

RNA Extraction

Total RNA was isolated from MCF-7 cells with 17β-estradiol and DEHP using Trizol reagent (Invitrogen Life Technologies, CA) and purified using RNeasy mini kit (Qiagen, CA) according to the manufacturer's instructions. Genomic DNA was removed using RNase-free DNase set (Qiagen, CA) during RNA purification. The amount of each total RNA sample was measured by a spectrophotometer, and its quality was checked by agarose-gel electrophoresis.

cDNA Microarray Experiments

For cDNA microarray analysis, total RNA was isolated from MCF-7 cells with 17β-estradiol and DEHP treatment. Labeling and hybridization were performed by instruction of MICROMAX direct cDNA microarray system (Perkin Elmer Life Sciences, MA) with minor modification. Briefly, the RNA samples from MCF-7 cells of treated chemicals were labeled with Cy3-dUTP (NEN, MA), and those of non-treated chemicals were labeled with Cy5-dUTP (NEN, MA). The two color probes were then mixed, purified using Microcon YM-100 column (Millipore, MA). Hybridization and washes were performed according to instruction of the Digital Genomics Inc. (Seoul, Korea). Hybridization (hybridization buffer; 25% formamide, 5 × SSC, 0.1% SDS, 0.5 mg/mL polyA, 0.5 mg/mL Cot-1 DNA) was performed in a hybridization oven at 58°C for 16 h. After washing (2 × SSC/0.1% SDS for 5 min at 58°C, 0.1 × SSC/0.1% SDS for 10 min at RT, 0.1 × SSC for 1 min at RT), the slide was dried by centrifugation at 650 rpm for 5 min. Hybridization images on the slides were scanned by ScanArray Lite (Perkin Elmer Life Sciences, MA). Scanned images were analyzed with GenePix 3.0 software (Axon Instruments, CA) to obtain gene expression ratios. The overall intensities were normalized using a correction coefficient obtained from the ratios of housekeeping genes.

Data Analysis

The fluorescent intensity of each spot was calculated by local median background subtraction. We used the robust scatter-plot smoother LOWESS function to perform intensity dependent normalization for the gene expression. Scatter plot analysis was made by Microsoft Excel 2000 (Microsoft). Significance analysis of microarray (SAM) was performed for the selection of the genes with significant gene expression changes[42]. The statistical significance of the differential expression of genes was assessed by computing a q-value for each gene. To determine the q-value we used a permutation procedure, and for each permutation, two-sample t statistics were com-

puted for each gene. Genes were considered differentially expressed when logarithmic gene expression ratios in four independent hybridizations were more than 0.65 or less than −0.65, i.e., 1.5-fold difference in expression level, and when the q-values were < 5.

Reference

1. Danzo, B.J. Environmental xenobiotics may disrupt normal endocrine function by interfering with the binding of physiological ligands to steroid receptors and binding proteins. *Environ. Health Perspect.* **105**, 294-301 (1997).
2. CERHR. NTP-CERHR Expert Panel Report on Di (2-ethylhexyl) Phthalate, http://cerhr.niehs.nih.gov/news/phthalates/DEHP-final.pdf.U.S. Department of Health and Human Services, National Toxicology Program, Center for the Evaluation of Risks to Human Reproduction, Research Triangle Park, NC (2000).
3. Huber, W.W., Grasl-Kraupp, B. & Schulte-Hermann, R. Hepatocarcinogenic potential of di (2-ethyl hexyl) phthalate in rodents and its implications on human risk. *Crit. Rev. Toxicol.* **26**, 365-481 (1996).
4. Blass, C.R. PVC as a biomedical polymer-plasticizer and stabilizer toxicity. *Med. Device Technol.* **3**, 32-40 (1992).
5. NTP. Di (2-ethylhexyl) phthalate (CAS No. 117-81-7). In: Report on Carcinogens, 8th ed, Summary 1998. Research Triangle Park, NC: National Toxicology Program.100-102 (1998).
6. Doull, J. *et al.* A cancer risk assessment of di (2-ethylhexyl)phthalate: application of the new U.S. EPA Risk Assessment Guidelines. *Regul. Toxicol. Pharmacol.* **29**, 327-357 (1999).
7. Faouzi, M.A. *et al.* Exposure of hemodialysis patients to di-2-ethylhexyl phthalate. *Int. J. Pharm.* **180**, 113-121 (1999).
8. Kluwe, W.M. *et al.* Carcinogenicity testing of phthalate esters and related compounds by the National Toxicology Program and the National Cancer Institute. *Environ. Health Perspect.* **45**, 129-133 (1982).
9. Diwan, B.A., Ward, J.M., Rice, J.M., Colburn, N.H. & Spangler, E.F. Tumor-promoting effects of di (2-ethylhexyl)phthalate in JB6 mouse epidermal cells and mouse skin. *Carcinogenesis* **6**, 343-347 (1985).
10. Ward, J.M., Rice, J.M., Creasia, D., Lynch, P. & Riggs, C. Dissimilar patterns of promotion by di (2-ethylhexyl)phthalate and phenobarbital of hepatocellular neoplasia initiated by diethylnitrosamine in B6C3F1 mice. *Carcinogenesis* **4**, 1021-1029 (1983).
11. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. http://www.institutodopvc.org/english/monografia.html March (2003).
12. Poon, R. *et al.* Subchronic oral toxicity of di-n-octyl phthalate and di (2-Ethylhexyl) phthalate in the rat. *Food Chem. Toxicol.* **35**, 225-239 (1997).
13. Blom, A., Ekman, E., Johannisson, A., Norrgren, L. & Pesonen, M. Effects of xenoestrogenic environmental pollutants on the proliferation of a human breast cancer cell line (MCF-7). *Arch. Environ. Contam. Toxicol.* **34**, 306-310 (1998).
14. Asai, D. *et al.* Structural essentials of xenoestrogen dialkyl phthalates to bind to the estrogen receptors. *Toxicol. Lett.* **118**, 1-8 (2000).
15. Davis, B.J., Maronpot, R.R. & Heindel, J.J. Di-(2-ethylhexyl) phthalate suppresses estradiol and ovulation in cycling rats. *Toxicol. Appl. Pharmacol.* **128**(2), 216-223 (1994).
16. Lovekamp-Swan, T. & Davis, B.J. Mechanisms of phthalate ester toxicity in the female reproductive system. *Environ. Health Perspect.* **111**(2), 139-145 (2003).
17. Harris, C.A., Henttu, P., Parker, M.G. & Sumpter, J.P. The estrogenic activity of phthalate esters in vitro. *Environ. Health Perspect.* **105**, 802-811 (1997).
18. Paganetto, G. *et al.* Endocrine-disrupting agents on healthy human tissues. *Pharmacol. Toxicol.* **86**, 24-29 (2000).
19. Jobling, M.A., Bouzekri, N. & Taylor, P.G. Hypervariable digital DNA codes for human paternal lineages: MVR-PCR at the Y-specific minisatellite, MSY1 (DYF155S1). *Human Mol. Genet.* **7**, 643-653 (1998).
20. Huang, C.H. & Sedlak, D.L. Analysis of estrogenic hormones in municipal wastewater effluent and surface water using ELISA and GC/MS/MS. *Environ. Toxicol. Chem.* **20**, 133-139 (2001).
21. Smeds, A. & Saukko, P. Identification and quantification of polychlorinated bisphenols and some endocrine disrupting pesticides in human adipose tissue from Finland. *Chemosphere* **44**, 1463-1471 (2001).
22. Sole, R.V., Bonabeau, E., Delgado, J., Fernandez, P. & Marin, J. Pattern formation and optimization in army ant raids. *Artificial Life* **6**, 219-226 (2000).
23. Takeyoshi, M. *et al.* The efficacy of endocrine disruptor screening tests in detecting anti-estrogenic effects downstream of receptor-ligand interactions. *Toxicol. Lett.* **126**, 91-98 (2002).
24. Nilsen, B.M. *et al.* Development of quantitative vitellogenin-ELISAs for fish test species used in endocrine disruptor screening. *Anal. Bioanal. Chem.* **378**, 621-633 (2004).
25. Imanishi, S. *et al.* Effects of oral exposure of bisphenol A on mRNA expression of nuclear receptors in murine placentae assessed by DNA microarray. *J. Reprod. Dev.* **49**, 329-336 (2003).
26. Nielsen, M., Hoyer, P.E., Lemmen, J.G., van der Burg, B. & Byskov, A.G. Octylphenol does not mimic diethylstilbestrol-induced oestrogen receptor-alpha expression in the newborn mouse uterine epithelium after prenatal exposure. *J. Endocrinol.* **167**, 29-37 (2000).
27. Inoue, T. Toxicogenomics- a new paradigm of toxicology. In: Toxicogenomics (Inoue, T., Pennie, W. D., eds). Tokyo: Springer, 3-11 (2003).

28. Terasaka, S. *et al.* Using a customized DNA microarray for expression profiling of the estrogen-responsive genes to evaluate estrogen activity among natural estrogens and industrial chemicals. *Environ. Health Perspect* **112**, 773-781 (2004).
29. Ryu, J.C. & Kim, Y.J. Construction and validation of human cDNA microarray for estimation of endocrine disrupting chemicals (KISTCHIP-400 ver.1.0). *Mol. Cell. Toxicol.* **1**, 52-61 (2005).
30. Ryu, J.C., Kim, M.K., Choi, M.H. & Chun, T.H. Promising next generation technology in toxicology-toxicogenomics. *Mol. Cell. Toxicol.* **1**, 1-6 (2005).
31. Gaido, K.W. *et al.* Evaluation of chemicals with endocrine modulating activity in a Yeast-based steroid hormone receptor gene transcription assay. *Toxicol. Appl. Pharmacol.* **143**, 205-212 (1997).
32. Skakkebaek, N.E. Endocrine disrupters and testicular dysgenesis syndrome. *Horm. Res.* **57**, 43 (2002).
33. Marks, J.R. *et al.* BRCA1 expression is not directly responsive to estrogen. *Oncogene* **14**, 115-121 (1997).
34. King, M.C., Marks, J.H. & Mandell, J.B. New York Breast Cancer Study Group. Breast and ovarian cancer risks due to inherited mutations in BRCA1 and BRCA2. *Science* **302**, 643-646 (2003).
35. Shimada, T. *et al.* Oxidation of xenobiotics by recombinant human cytochrome P450 1B1. *Drug Metab. Dispos.* **25**, 617-622 (1997).
36. Crespi, C.L. *et al.* Development of a human lymphoblastoid cell line constitutively expressing human CYP1B1 cDNA: substrate specificity with model substrates and promutagens. *Mutagenesis* **12**, 83-89 (1997).
37. Coumoul, X., Diry, M., Robillot, C. & Barouki, R. Differential regulation of cytochrome P450 1A1 and 1B1 by a combination of dioxin and pesticides in the breast tumor cell line MCF-7. *Cancer Res.* **61**, 3942-3948 (2001).
38. Spink, D.C. *et al.* Differential expression of CYP1A1 and CYP1B1 in human breast epithelial cells and breast tumor cells. *Carcinogenesis* **19**, 291-298 (1998).
39. Faith DA. *et al.* Trefoil factor 3 overexpression in prostatic carcinoma: prognostic importance using tissue microarrays. *Prostate* **61**(3), 215-227 (2004).
40. Soto, A.M. *et al.* The E-SCREEN assay as a tool to identify estrogens : An update on estrogenic environmental pollutants. *Environ. Health Perspect.* **103**, 113-122 (1995).
41. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**, 55-63 (1983).
42. Tusher, V., Tribshirani, R. & Chu, C. Significance analysis of microarrays applied to ionizing radiation response. *Proc. Natl. Acad. Sci. USA* **98**, 5116-5121 (2001).