Using a Customized DNA Microarray for Expression Profiling of the Estrogen-Responsive Genes to Evaluate Estrogen Activity among Natural Estrogens and Industrial Chemicals

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We developed a DNA microarray to evaluate the estrogen activity of natural estrogens and industrial chemicals. Using MCF-7 cells, we conducted a comprehensive analysis of estrogen-responsive genes among approximately 20,000 human genes. On the basis of reproducible and reliable responses of the genes to estrogen, we selected 172 genes to be used for developing a customized DNA microarray. Using this DNA microarray, we examined estrogen activity among natural estrogens (17β-estradiol, estriol, estrone, genistein), industrial chemicals (diethylstilbestrol, bisphenol A, nonylphenol, methoxychlor), and dioxin. We obtained results identical to those for other bioassays that are used for detecting estrogen activity. On the basis of statistical correlations analysis, these bioassays have shown more sensitivity for dioxin and methoxychlor. *Key words:* **endocrine disruptor, estrogenicity, expression profile, microarray.** *Environ Health Perspect* **112:773–781 (2004). doi:10.1289/txg.6753 available via** *http://dx.doi.org/* **[Online 12 February 2004]**

Endocrine disruptors mimic natural hormones, thereby causing various effects or damage in humans and other animals. Estrogenic compounds are a particularly serious problem because their effects can be transferred to children through damage to the female reproductive organs in the mothers. The effects of estrogenic compounds first appear in the estrogen-responsive genes that include estrogen receptors (ERs), followed by changing expression levels of many other genes and resulting in cellular responses that appear as various symptoms (McDonnell and Norris 2002). As we identify more signaling pathways within the cell, we become aware of more cases in which common pathways are used for different signalings. Dioxin, for example, has an effect through the aryl hydrocarbon receptor, which to some degree may share common cascades with the ER pathway [Carlson and Perdew 2002; reviewed by Safe (2001)]. Therefore, unraveling signaling pathways will provide clues not only to the estrogen signaling pathway alone but also to other pathways.

Estrogenic chemicals can act upon the cell through two major pathways: *a*) direct interaction with the ERs and *b*) interactions with other molecules first. As estrogen binds to ERs more tightly than to other molecules, the major effects originate from the first pathway. However, when the chemical has low estrogen activity and the activity of other interactions is high, estrogen activity can be masked or disguised by the second pathway. Furthermore, the major estrogen activity is not conducted by a unique pathway. First, there are at least two types of ERs, ER-α (Green et al. 1986) and ER-β (Kuiper et al. 1996), which differ in their affinity for ligands and the way in which they transduce signals (Katzenellenbogen and Katzenellenbogen 2000). Differences in affinity between ER-α and ER-β were reported for methoxychlor and its analog DDT (Jacobs et al. 2003). As for the ER-α, tamoxifen is an antagonist against natural estrogen but has agonist activity in the uterus, whereas ICI 182,780, a well-known pure antagonist, does not show such activity (Branham et al. 1996). This difference can be explained by the difference in the ligand-dependent or -independent activation functions assisted by coactivators and has been observed in other chemicals [reviewed by McDonnell et al. (2002); McKenna and O'Malley (2002)]. This indicates that, even for the first pathway, using any one of these signaling pathways as an indicator of estrogen activity would be biased and specific signals could be enhanced, resulting in differences between the expected and real biological outcomes.

The second pathway is more complex. It may include various metabolic and modification pathways for chemicals, and estrogen activity could be higher or lower than the original, depending on the products (Beresford et al. 2000). Methoxychlor, for example, is metabolized to mono- and bisphenolic forms by oxygenase (Bulger et al. 1978) or by cytochrome P450 isoforms (Hu and Kupfer 2002). These metabolites have more estrogen activity than methoxychlor. Such a metabolic activation of estrogenic chemicals was also reported for bisphenol A and bisphenol B (Yoshihara et al. 2001), 2-nitrofluorene (Fujimoto et al. 2003), and styrenes (Kitamura et al. 2003). Metabolic inactivation or inactivation by modification could also occur in many chemicals. As estrogen activity results in growth and proliferation of the cell through the activity of transducing signals by means of hormones, growth factors, cytokines, and others, monitoring estrogen activity at the steps close to such cellular responses rather than at the beginning (receptor binding, for example) is crucial for reliable evaluation of estrogenicity.

Previously we found that a significant number of genes responded to estrogen in a DNA microarray analysis and we characterized some of them, including solute carrier family 7, member 5 (*SLC7A5*), retinoblastoma-binding protein 8 (*RBBP8*), and c-*myc* promoter-binding protein 1 (*IRLB*) (Inoue et al. 2002b). We also found that many of these genes responded to estrogen in a manner similar to that in cancer cells from the breast, ovary, stomach, kidney,

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and other sites. Here, using a customized DNA microarray with newly selected estrogen-responsive genes, we outline an experimental system with more sensitivity for evaluation of estrogen activity in natural and industrial chemicals on the basis of statistical analysis of gene response. Our goal is to establish an experimental system with more sensitivity for the evaluation of estrogen activity in these chemicals, which can be applied even to those having low activity.

Materials and Methods

Cell Culture and Materials

MCF-7 cells were obtained from JCRB Cell Bank (National Institute of Health Sciences, Tokyo, Japan) and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C under 5% carbon dioxide. Cells were cultured in phenol red-free RPMI 1640 medium with 10% FBS treated with dextran-coated charcoal for 3 days and treated with ethanol (vehicle) or a variety of chemicals for 72 hr. 17β-Estradiol (E_2), estriol, estrone, genistein, diethylstilbestrol (DES), bisphenol A, nonylphenol, and methoxychlor were obtained from Sigma–Aldrich (St. Louis, MO, USA) and used at the concentrations of 10 nM (E_2, estriol, estrone, DES) or 10 μM (genistein, bisphenol A, nonylphenol, and methoxychlor). Dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin; purity 99.0%) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA) and used at a concentration of 50 mg/mL in dimethyl sulfoxide.

cDNA Microarray Analysis

GeneChip analysis was conducted using human U95A oligonucleotide probe arrays (Affymetrix, Santa Clara, CA, USA) according to the supplier's protocols, as follows. Total RNA (1 μg) was used to generate a cRNA probe by T7-transcription. The fragmented cRNA (10 μg) was hybridized to the microarrays in 200 μL of a hybridization cocktail by incubation at 45°C for 16 hr in a rotisserie oven set at 60 rpm. The microarrays were then washed with a nonstringent wash buffer [6× $NaCl/NaH_2PO_4$/EDTA (SSPE)] at 25°C, followed by a stringent wash buffer [100 mM MES (pH 6.7), 0.1 M NaCl, and 0.01% Tween 20] at 50°C. The microarrays were stained with streptavidin phycoerythrin (Molecular Probes, Eugene, OR, USA), washed again with 6× SSPE, stained with biotinylated antistreptavidin IgG followed by streptavidin phycoerythrin, and washed a third time with 6× SSPE. The arrays were scanned using a GeneArray scanner (Affymetrix) at a resolution of 3 μm, and the scanned image was quantitatively analyzed with Microarray Suite 4.0 (Affymetrix). For normalizing the data to compare mRNA expression levels among samples, we unified the values to 1,000 as an average of average difference scores corresponding to the signal intensities of all probe sets in each sample.

Microarray analysis using IncyteGenomics (Palo Alto, CA, USA) microarrays was performed as reported previously (Inoue et al. 2002b).

A custom cDNA microarray (EstrArray) was manufactured by InfoGenes Co., Ltd. (Tsukuba, Japan) by mechanical spotting of cDNA (~500 bp to ~1.5 kb) of the genes selected from the above DNA microarray assays [see Inoue et al. (2002b) for details]. The analysis using EstrArrays was performed as follows: After the cells were cultured for 72 hr in the presence of chemicals at indicated concentrations, mRNA was purified using the PolyATract System 1000 (Promega, Madison, WI, USA) according to manufacturer instructions. The quality of mRNA was confirmed by examining the optical density and also by reverse transcription–polymerase chain reaction (RT–PCR) assay for several marker genes (β-actin for all, and pS2 and ER-α for the chemicals with high estrogen activity). Each mRNA was labeled with fluorescent Cyanine 3 (Cy3)-dUTP (for the treatment of chemicals) or Cy5-dUTP (for the control) at 37°C for 1.5 hr using SuperScript II (Invitrogen, Carlsbad, CA, USA) and random primers (a mixture of 6 mers and 9 mers). Both Cy3- and Cy5-labeled probes were mixed and denatured under alkaline conditions for 1 hr. After free fluorescent nucleotides were removed using Microcon-30 columns (Millipore, Bedford, MA, USA), probes were hybridized to EstrArrays for 16 hr in 5× NaCl/Na citrate (SSC) and 0.5% sodium dodecyl sulfate at 65°C. After hybridization, slides were washed twice with 0.05× SSC for 5 min at room temperature. The fluorescent intensities were scanned with a ChipReader (Virtek, Waterloo, Ontario, Canada), and scanned images were analyzed using IPLab (Scanalytics, Fairfax, VA, USA) according to manufacturer instructions. The ratio (Cy3/Cy5) was calculated for each spot, and after transforming the ratio into a logarithmic value ($\log_2$), the value was normalized using internal control genes. Clustering analysis was performed using the Cluster program and the results were displayed with the TreeView program [for both programs see Eisen et al. (1998)]. The genes spotted on EstrArray or GenBank accession numbers (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=nucleotide)] were as follows: *ACO2, ADORA2A, AGTR1, AIM1, AKR1C4, APPL, AR (AREG), ARHGDIA, ARNT2, ASNS, ASS, ATF3, BF, BRCA1, CAMK2A, CAPNS1, CBX1, CCNA1, CCR2, CDC14, CDC6, CDH18, CDIPT, CDKN1A, CDSN, CEBPB, CLIC4, CPT1A, CtIP (RBBP8), CTNND2, CTSD, D53 (TPB52L1), DAZAP2, DDEF, DHCR24, DHX29, EDN2, EFEMP1, EGR3, EIF3 (EIF3S9), ENO2, ENO3, FBP1, FOS, FRA2, FTH1, FUT8, GARS, GFPT1, GOT1, gp96 (TRA1), GRP78 (HSPA5), GUCA2B, H3F3B, HAX1, HDAC6, HMMR, HSP70, IEX-1 (IER3), IFRD1, IGFBP4, IGFBP5, IL1R1, IL-2RB, ILK, IMP4, ISG20, JUN, KRT16, KRT8, LAMP3, LCN2, LGALS3BP, MAL, MAN1A1, MAP1, MATN2, MBP-1 (IRLB), MGP, MIC1, MTHFD2, NCKAP1, NPY1R, PACE4, PCK2, PCYT1A, PDZK1, PEG10, PHGDH, PI3KC3, PIG11, PMAIP1, PMP22, PMPCA, PRKCD, PRKCSH, PSAT1, PTPN18, PVR, QSCN6, RACGAP1, RAP1GAP, RCN1, RDH11, RHOC, RIP140, RSK, RUNX1, S100P, SCD, SECTM1, SELENBP1, SERPINA, SFTPB, SH3BGR, SH3BP5, SHMT2, SLC12A2, SLC1A4, SLC1A5, SLC26A3, SLC7A11, SLC7A5, SORD, STC2, SYNGR2, TACSTD2, TAF9, TCN1, TFII-I (GIF2I), TFIIS (TCEA1), TIEG, TM4SF1, TRB3, TSPAN-1, U5-116KD, ULK1, VAMP5, WARS, XPOT, YARS, ZNF231,* and expressed sequence tags (ESTs) (L05367, NM_052965, AL109840, XM097954, NM_017867, NM_017867, NM_014846, NM_173481, and NM_024092), along with the expression markers *AHR, CCND1, CYP19A1, CYP1A1, ERBB2, ESR1, ESR2, HSD17B2, NCOA1, NCOA3, PGR, STS,* and *TFF1*, and the calibration markers *ACTB, ACTN1, CPEB2, FLJ12748, FUSIP1, G0S2, G6PD, GCLM, GTF2H2, HNRPK, IL6ST, KANK, KIAA0349, KRT6E, LOC129401, NAV1, NMA, NPM1, PAK4, PRKCD, RPL35, SDR1, SLC25A16, SLC29A2, SOCS2, TNFRSF7,* and *ZNF147*. Different parts of the same gene or cDNA were used for some genes (a total of 12 genes), giving multiple plots in the figures. The expression markers are the marker genes for estrogen [all except cytochrome P450 1A1(*CYP1A1*)] or dioxin (*CYP1A1*) responses, and the calibration markers are the genes for adjusting the signals between Cy3 and Cy5 labels (therefore they are not estrogen responsive).

Real-Time Quantitative RT-PCR

mRNA was isolated using a PolyATract System 1000 (Promega) as described previously. The first-strand cDNA was synthesized from 200 ng mRNA using

SuperScript II (Invitrogen). Quantitative PCR was carried out using a LightCycler-FastStart DNA Master SYBR Green I kit (Roche Molecular Biochemicals, Mannheim, Germany). The PCR conditions were as follows: denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 94°C for 10 sec, annealing at 57°C for 5 sec, and extension at 72°C for 20 sec. After PCR a melting curve was constructed by increasing the temperature from 72 to 95°C. The product was resolved in agarose gels to ensure that the correct product was amplified in the reaction. PCR was repeated 3 times for each gene, and the average and standard deviations were calculated. The PCR primers were as follows: *SLC7A11*, 5′-ACAGTG CCAGAGTGAAGAAACTC-3′ and 5′-CCAGCTAAATCCCTAACTT GGAT-3′; *EGR3*, 5′-CCATGATTCCTG ACTACAACCTC-3′ and 5′-GTGGAT CTGCTTGTCTTTGAATG-3′; *PDZK1*, 5′-CCTTTCTCAAGGAATGAG TTGTG-3′ and 5′-CCGCCTGTAAGA CAAATGATAAC-3′; *S100P*, 5′-GTAC TTTGAGAAGGCAGGACTCA-3′ and 5′-GGAATAATTGCCAACAAACACTT-3′; *AR*, 5′-AAACAAGACGGAAAGT GAAA-3′ and 5′-TTACCTTCGTGCAC CTTTAT-3′; *WARS*, 5′- AGGCATCT TCTTCTCACACAGAG-3′and 5′- GAT ACTTCTCGTCATCCGTCATC-3′; *SELENBP1*, 5′-GAAGGTACATGGT CAGTGGAGAA-3′ and 5′-GAGATGTC ATACTGCCTCAGGTC-3′; *ENO2*, 5′-GCACTTTCCACTTCTTCCTTTCT-3′ and 5′-AAGTGACACATGGTCCCT CTCTA-3′; *ARHGDIA*, 5′-CCTCACTA GCCTCTACTCCCTGT-3′ and 5′-ACTGAGGTGACTTGAGTGTTGG-3′; *AGTR1*, 5′-CTGAATAACTCACTG ATGCCATCCCAG-3′ and 5′-GCCAGC AGCCAAATGATGATGCAGGTG-3′; *IGFBP5*, 5′-ATGGATTTGAGAGGAAA GAGAGG-3′ and 5′-AGCACCCTCC TAAGGTTACTCAC-3′; and *SLC12A2*, 5′-GAGGAAATCATTGAGCCAT ACAG-3′ and 5′-GAGCACTAGACACA GCACCTTTT-3′.

Results

We first screened the estrogen-responsive genes in a human mammary tumor cell line, MCF-7, using two different comprehensive DNA microarray systems, UniGem, version 2 (IncyteGenomics) containing 9,182 genes and GeneChip U95A (Affymetrix) containing 12,625 genes (Figure 1). Approximately 300 genes in UniGem, and 850 genes in GeneChip U95A showed a response higher than 2-fold and 3-fold, respectively. To examine the response to estrogen by monitoring transcription of the genes, we selected 172 genes after the reproducibility of their upregulation or downregulation on estrogen treatment (10 nM E_2 for 3 days) was confirmed by repeated DNA microarray and/or RT–PCR analyses (Inoue et al. 2002b; also, data not shown). To confirm that the data obtained were reliable for the genes with various expression levels, we arbitrarily divided the genes into high- and low-expression types. The genes categorized as the high expression type characterized by abundant transcript are summarized in Table 1. These genes had transcripts with expression levels higher than those of the solute carrier family gene 2, member 1 (*SLC2A1*) and the keratin 6B gene in each DNA microarray analysis (both appeared in both DNA microarrays and showed identical expression levels), and included the genes for amino acid transporters, and structural, ion-related, translation-, transcription-, and cell cycle–associated proteins. The expression of most of the tRNA synthetase genes and genes for the TATA-box binding protein–associated factor and histone deacetylase was probably upregulated for enhancing protein synthesis or transcription, respectively. Meanwhile, the genes associated with specific tissues, such as those for the nervous system, showed downregulation. A similar analysis was performed for the genes categorized as the low expression type (Table 2). Upregulation of the genes related to various synthetases, transcription-related, and cell cycle or growth-associated proteins as well as receptors and ion or amino acid transporters was also prominent in this type. Among the tumor-associated genes, oncogenic genes such as for c-*fos*, AML (acute myeloid leukemia) 1b, FOS-like antigen 2, and a v-*jun* homolog were upregulated, whereas tumor suppressor–related genes (absent in melanoma 1) (Ray et al. 1996) were slightly downregulated. Expression of the ER-α gene was downregulated as observed for progressive breast tumors (Lapidus et al. 1998; Yoshida et al. 2000).

On the basis of the information obtained from the estrogen-responsive genes shown above, we constructed a customized DNA microarray, EstrArray, that contains 203 genes, including genes showing either upregulation (108 genes) or downregulation (64 genes) in their expression. EstrArray also contains calibration markers for adjusting the fluorescent levels between Cy3- and Cy5-labeled cDNAs (28 genes) and expression markers such as the genes for trefoil factor, the ER-α and ER-β, steroid sulfatase, and other estrogen-related proteins (14 genes, 11 showing estrogen responsiveness, resulting in a total of 203 genes).

We used this microarray system to analyze natural estrogens and industrial chemicals (Figures 2, 3). First, we examined the reproducibility of the assay by repeating the analysis using E_2 twice (E_2 and E_2-2), which resulted in very similar profiles (Figure 2) and gave a high correlation coefficient (R = 0.928) (Figure 3A). When the reproducibility was examined for the genes of the high and low expression types separately as examined in Tables 1 and 2, the high expression type showed a higher score (R = 0.935) (Figure 3C) than the total gene score. Moreover, the low-expression type also showed a relatively high score (R = 0.910) (Figure 3B), suggesting a high reproducibility even for the low-expression type. Cluster analysis indicated that very similar profiles were obtained among the chemicals already known to have estrogen activity (10 nM E_2, 10 nM DES, 10 μM nonylphenol, 10 nM estriol, 10 μM genistein, and 10 nM estrone) (Figure 2). Other

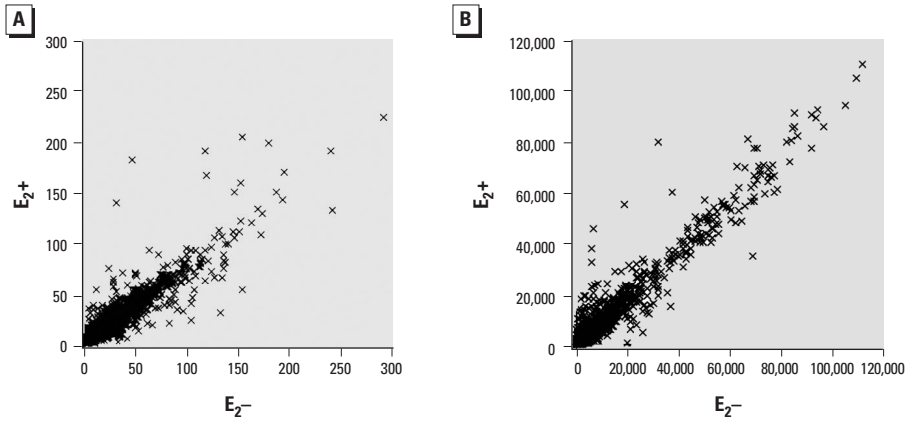

Figure 1. Expression profiling of the human genes using DNA microarrays. The response to 10 nM E_2 was examined with comprehensive sets of human genes on (*A*) UniGem, version 2 (IncyteGenomics) and (*B*) GeneChip U95A (Affymetrix). Each contains a total of 9,182 and 12,625 genes, respectively. The vertical and horizontal axes are indicated by arbitrary units derived from fluorescent intensities.

Table 1. The high expression type in the estrogen-responsive genes.[a,b]

Amino acid transporters	
5.3	Solute carrier family 7 (*SLC7A5*)
2.4	Solute carrier family 1 (*SLC1A5*)
Calcium and other ion related	
2.9	Ferritin (*FTH1*)
2.0	Reticulocalbin 1 (*RCN1*)
–2.8	Calpain, small polypeptide (*CAPN2, CAPNS1*)
Binding proteins	
2.7	SH3-domain binding protein (*SH3BP5*)
2.1	Inhibitor of DNA binding 4 (*ID4*)
2.0	HS1 binding protein (*HAX1*)
Receptors	
2.0	Poliovirus receptor (*PVR*)
Tumor-associated proteins	
3.3	Tumor rejection antigen (*TRA1*)
3.1	FOS-like antigen (*FOSL2*)
2.5	Pituitary tumor-transforming (*PTTG1*)
–2.1	*Ras* homolog gene family, member C (*ARHC*)
–2.6	c-*myc* promoter-binding protein (*IRLB*)
Synthetases, transferases, kinases, or others	
3.9	Methylene tetrahydrofolate dehydrogenase (*MTHFD2*)
2.9	Argininosuccinate synthetase (*ASS*)
2.7	Alpha-1-antichymotrypsin (*SERPINA*)
2.4	Cathepsin D (*CTSD*)
2.2	Serine hydroxymethyltransferase 2 (*SHMT2*)
2.1	Mannosidase alpha (*MAN1A1*)
2.0	Protein geranylgeranyltransferase type I (*PGGT1B*)
–2.0	Phosphatidylinositol synthase (*CDIPT*)
–2.2	Sorbitol dehydrogenase (*SORD*)
–2.3	Aconitase 2, mitochondrial (*ACO2*)
–2.6	Carnitine palmitoyltransferase (*CPT1A*)
–2.6	Fructose-bisphosphatase (*FBP1*)
–3.7	Isocitrate dehydrogenase, mitochondrial (*IDH2*)
Nervous system–related proteins	
–2.0	Protein tyrosine phosphatase (*PTPN18*)
–2.3	Synaptogyrin (*SYNGR2*)
–2.7	Enolase gamma, neuronal (*ENO2*)
Translation-associated proteins	
5.7	Tryptophanyl-tRNA synthetase (*WARS*)
3.5	Alanyl-tRNA synthetase (*AARS*)
3.1	Glycyl-tRNA synthetase (*GARS*)
2.9	Tyrosyl-tRNA synthetase (*YARS*)
2.0	Isoleucine-tRNA synthetase (*IARS*)
–2.2	Eukaryotic translation initiation factor, subunit 9 (*EIF3S9*)
Transcription-associated proteins	
2.1	TATA box binding protein-associated factor (T*AF9*)
2.0	Histone deacetylase (*HDAC6*)
–2.6	GATA-binding protein 3 (*GATA3*)
–2.8	General transcription factor IIi, pseudogene 1(*GTF2I*)
Cell cycle or growth-associated proteins	
2.0	CDC6 homolog (*CDC6*)
–2.0	Mal, T-cell differentiation protein (*MAL*)
–2.2	Protein kinase C substrate 80K-H (*PRKCSH*)
–2.9	Protein kinase C delta (*PRKCD*)
Cellular responsive proteins	
4.9	Heat shock 70kDa protein 5 (*HSPA5*)
–3.6	Heat shock 70kDa protein 1 (*HSP70*)
–3.4	Clusterin (*CLU*)
Structural proteins	
2.8	H3 histone, family 3B (*H3F3B*)
2.2	Keratin 8 (*KRT8*)
–2.0	Membrane component, surface marker 1 (*M1S1*)

[a]Fold increases or decreases (in negative values) are shown. [b]Gene names are either from UniGene (htttp://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene) or they are the conventional names. For the latter, the UniGene names are in parentheses upon first mention.

Table 2. The low-expression type in the estrogen-responsive genes.[a,b]

Receptors	
6.2	Transcobalamin I (*TCN1*)
3.0	Nuclear receptor interacting protein 1 (*RIP140*)
3.0	Exportin, tRNA (*XPOT*)
–3.0	Estrogen receptor 1 (*ESR1*)
Calcium and other ion-associated proteins	
5.5	Stanniocalcin (*STC2*)
5.1	S100 calcium-binding protein A9 (*S100A9*)
4.7	S100 calcium-binding protein P (*S100P*)
–3.7	Cadherin 18 (*CDH18*)
Amino acid transporters	
5.5	Solute carrier family 1, member 4 (*SLC1A4*)
4.3	Solute carrier family 12, member 2 (*SLC12A2*)
Membrane proteins	
3.6	Semaphorin 3B (*SEMA3B*)
3.5	Hyaluronan-mediated motility receptor (*HMMR*)
Binding proteins	
–4.4	Selenium-binding protein 1 (*SELENBP1*)
Estrogen-associated proteins	
11.7	Trefoil factor 1 (*TFF1*)
8.1	Tumor protein D52-like 1 (*TPD52L1*)
Oncogene-associated proteins	
5.1	FOS-like antigen 2 (*FRA2*)
3.0	v-*jun* avian sarcoma virus 17 oncogene homolog (*JUN*)
7.5	c-*fos* (*FOS*)
14.4	AML1b (*RUNX1*)
Tumor-associated proteins	
4.2	Phorbol-12-myristate-13-acetate-induced protein 1 (*PMAIP1*)
4.0	Interferon-stimulated gene (*ISG20*)
4.0	Downregulated in adenoma (*SLC26A3*)
3.9	Retinoblastoma-binding protein 8 (*RBBP8*)
–3.1	Absent in melanoma 1 (*AIM1*)
Nervous system–related proteins	
8.2	Amphiregulin (*AREG*)
3.2	Neuropeptide Y receptor Y1 (*NPY1R*)
–3.0	Bassoon (*BSN*)
–3.2	Catenin (cadherin-associated protein), delta 2 (*CTNND2*)
Synthetases, transferases, kinases, or others	
12.1	Asparagine synthetase (*ASNS*)
7.6	Phosphoenolpyruvate carboxykinase 2 (*PCK2*)
6.3	EST, highly similar to phosphoserine aminotranferase
3.9	Glutamic-oxaloacetic transaminase 1, soluble (*GOT1*)
3.8	Phosphoinositide-3-kinase, class 3 (*PI3KC3*)
3.6	Unc-51 (C. elegans)-like kinase 1 (*ULK1*)
3.6	Aldo-keto reductase family 1, member C4 (*AKR1C4*)
3.2	Glutamine-fructose-6-phosphate transaminase 1 (*GFPT1*)
3.1	Ribosomal protein S6 kinase, 90 kDa (*RSK*)
–3.2	Fucosyltransferase 8 (*FUT8*)
–3.3	Enolase 3 (*ENO3*)
–3.5	Paired basic amino acid cleaving system 4 (*PACE4*)
Transcription related	
5.1	CCAAT/enhancer binding protein (C/EBP), beta (*CEBPB*)
4.9	Activating transcription factor 3 (*ATF3*)
4.3	Motilin (*MLN*)
Cell cycle or growth-associated proteins	
5.0	Prostate differentiation factor (*PLAB, MIC1*)
3.5	Insulin-like growth factor-binding protein 4 (*IGFBP4*)
3.4	TGFβ inducible early growth response (*TIEG*)
3.4	Cyclin A1 (*CCNA1*)
3.2	B-factor, properdin (*BF*)
–6.7	EGF-containing fibulin-like extracellular matrix protein 1 (*EFEMP1*)
–7.0	Insulin-like growth factor binding protein 5 (*IGFBP5*)
–12.8	Insulin-like growth factor binding protein 5 (*IGFBP5*)
Cellular responsive proteins	
8.8	Early growth response 3 (*EGR3*)
Structural proteins	
8.2	Matrix Gla protein (*MGP*)
3.9	Microtubule-associated protein 1B (*MAP1*)

[a]Fold increases or decreases (in negative values) are shown. [b]Gene names are either from UniGene (htttp://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene) or they are the conventional names. For the latter, the UniGene names are in parentheses upon first mention.

chemicals showed relatively high correlation coefficients (0.929 for estriol, 0.847 for estrone, 0.692 for DES, 0.909 for genistein, and 0.862 for nonylphenol) Figure 3D–H). Relatively low scores for estrone and DES can be explained by the low response of the genes when they were assayed at the concentration of 10 nM. Bisphenol A and methoxychlor, on the other hand, showed similar but clear differences (0.651 for bisphenol A and 0.556 for methoxychlor) (Figure 3I–J). Dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin; 10 nM), in contrast, showed a very different profile, partly because most genes did not respond well, and naturally showed a very low score (R = 0.213) (Figure 3K). A dioxin marker, *CYP1A1*, responded well.

The response of a total of 12 genes (6 showing upregulation and 6 showing downregulation) to E_2 and other chemicals is summarized in Figure 4A. For example, amphiregulin (*AR*) showed a relatively high response to E_2 (5.4-fold increase). The response to the other chemicals with relatively high estrogen activity (estriol, estrone, DES) was distinguishable although low (1.6- to 1.9-fold increases). The chemicals with low estrogen activity, however, showed a relatively high response when their concentrations were increased to 10 µM. Expression of the AT1 receptor gene (*AGTR1*) was downregulated by the treatment with E_2 (2.8-fold decrease) and all other chemicals (1.2- to 6.4-fold). We next examined the response of the genes to E_2 by the real-time PCR (Figure 4B). The degrees of response were generally higher for the real-time PCR because of higher backgrounds in DNA microarray assay. However, the response was confirmed by both methods.

Discussion

Customized DNA Microarray

DNA microarray technology is one of the most potentially powerful tools in modern toxicogenomics because it can shorten the time for elucidating toxicological phenotypes and widen the way for drug discovery (Inoue 2003). However, determining the relationship between specific gene expression profiles and toxicological phenotypes will be accelerated by the development of customized DNA microarrays, the accumulation of profiles specific to chemicals, and an increase in the knowledge of gene functions (Adachi et al. 2002; Inoue et al. 2002a; Watanabe et al. 2002; Wong et al. 2003).

Here we developed a customized DNA microarray, EstrArray, for expression profiling of estrogen-responsive genes. EstrArray contains 172 estrogen-responsive genes

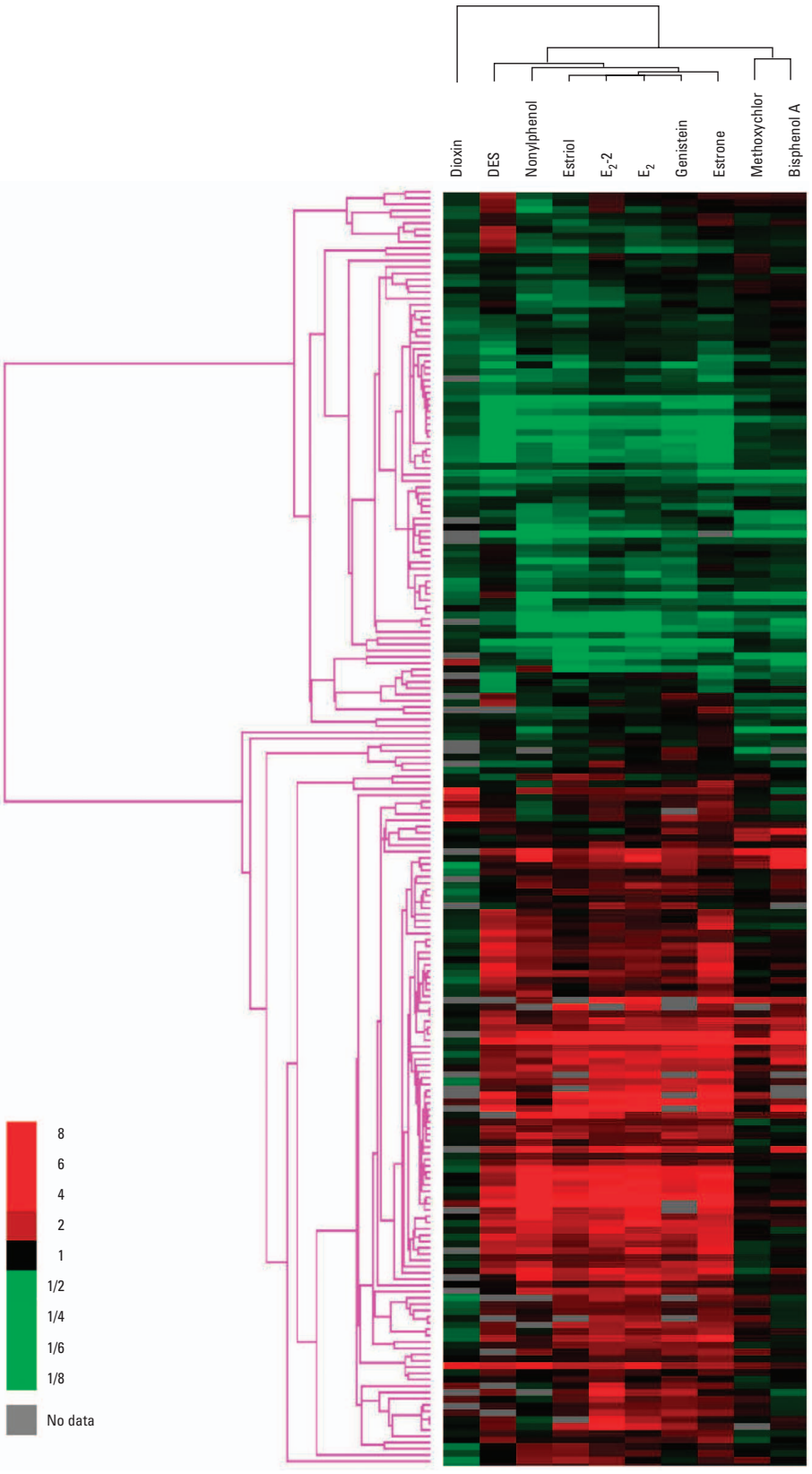

Figure 2. Clustering of gene expression after the treatment of various estrogens and industrial chemicals examined using EstrArrays. E_2-2, E_2 twice. Gene expression profiles were obtained after treatment with 10 nM of E_2, estrone, estriol, and DES, 10 µM nonylphenol, bisphenol A, genistein, and methoxychlor, or 50 µg/mL dioxin. The results of EstrArray analysis are shown as values of $\log_2$ (fluorescent intensity for chemical plus/fluorescent intensity for chemical minus), which were colored according to the color scale.

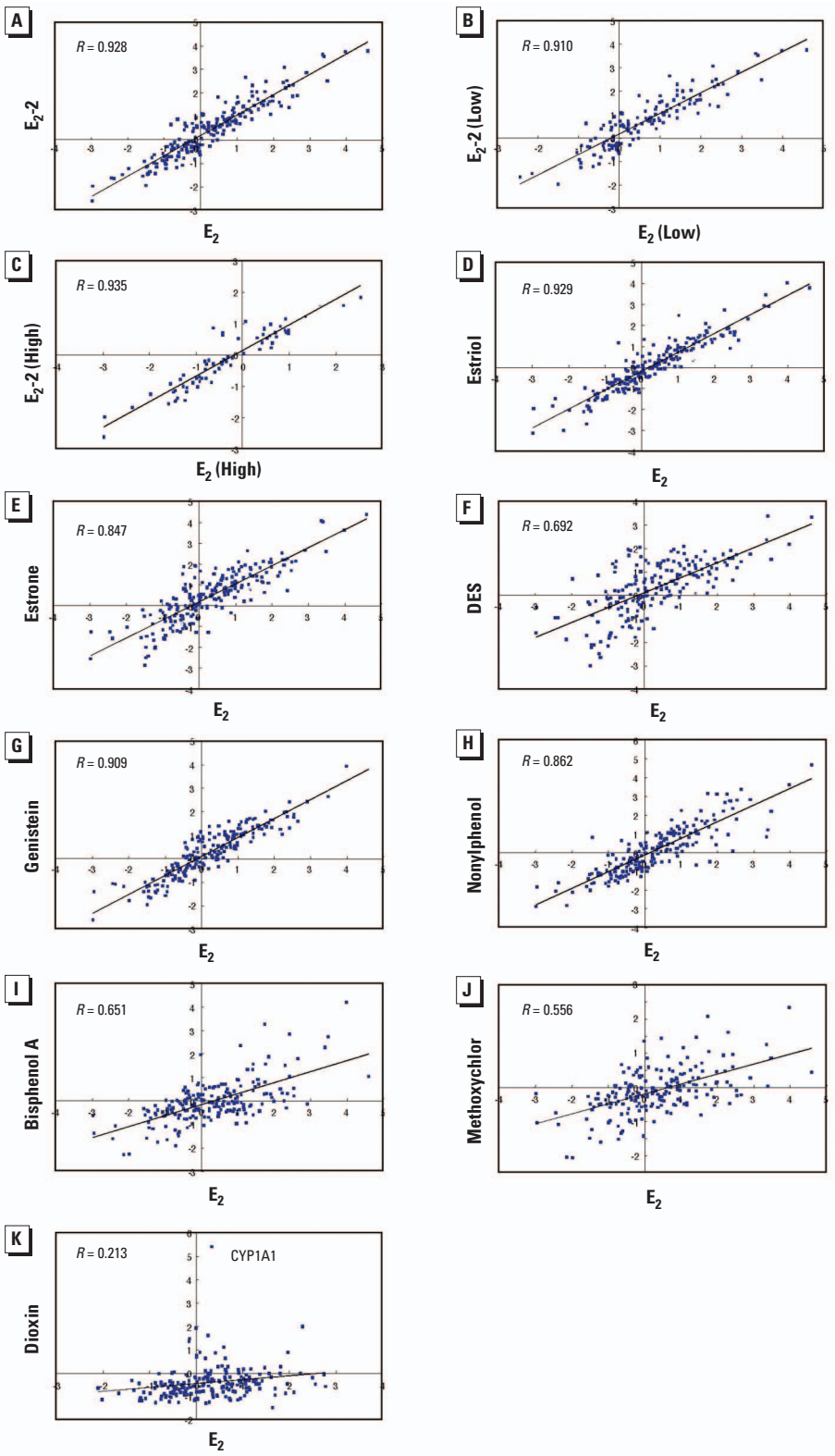

Figure 3. Estrogenicity of chemicals examined using EstrArrays. E_2-2, E_2 twice. Gene expression profiles of estrogen-responsive genes were compared between the independent E_2 treatments and shown in a scatterplot graph (*A*). The same comparison was performed for the genes of the (*B*) low-expression or (*C*) high-expression types. Gene expression profiles were compared between (*D*) E_2 and estriol, (*E*) estrone, (*F*) DES, (*G*) genistein, (*H*) nonylphenol, (*I*) bisphenol A, (*J*) methoxychlor, and (*K*) dioxin. The axes are shown in $\log_2$ (fluorescent intensity for chemical plus/fluorescent intensity for chemical minus) calculated for each chemical. The correlation coefficient (*R*) between two profiles was calculated for each graph on the basis of linear regression between the two profiles. *CYP1A1*, a dioxin marker, is indicated in *K*.

selected from approximately 20,000 genes, almost half the estimated number in the whole human genome. As approximately 95% of the genes examined did not respond to estrogen or were not expressed in MCF-7 cells, the genes used for EstrArray were considered to represent the genes most suitable for monitoring estrogen responsiveness. As we reported previously, some of these genes were characterized extensively to show reproducible estrogen responsiveness by Northern blot analysis (Inoue et al. 2002b) and to examine their potential functions (data not shown). EstrArray also contains marker genes for the calibration of fluorescent levels that cover a wide range of expression levels for normalizing signals between the presence and absence of chemicals. The genes, which show estrogen responsiveness, can be classified into several types according to their function (Tables 1 and 2; summarized in Figure 5). Among the genes related to tumor-associated genes, oncogenes and tumor-promoting genes are generally upregulated, whereas the genes related to tumor suppression and the ER-α gene are downregulated. This is consistent with the effects of estrogen, namely, the promotion of tumorigenesis. For growth- and ion-associated genes and other genes, the expression of various transporters, synthetases, transcription factors, growth response genes, and structural genes was upregulated, indicating enhancement of growth and proliferation of the cell. Meanwhile, the genes related to specific differentiation of the cell, such as those for neuronal proteins, were downregulated.

Genes Responding to Estrogenic Chemicals

Among the estrogen-responsive genes used for EstrArray, the *AR* and *AGTR1* were examined in detail (Figure 4). Both showed a relatively high response to E_2 (5.4-fold increase for AR and 2.8-fold decrease for the *AGTR1*) and a similar tendency of response to the other chemicals examined here. Estrogen responsiveness was low for estriol, estrone, and DES compared with E_2 when they were examined at the concentration of 10 nM, except for the *AGTR1* with estriol. These data and the result of the statistical correlation study (Figure 3) indicate that the genes responded to most chemicals analyzed here in similar ways and suggest that these genes commonly respond to estrogen activity. The difference in the degree of response for each gene, however, might be due to the difference in biological effects originating from structural differences. This difference is particularly important for the evaluation of estrogen activity,

especially when the activity is low, giving an advantage to this assay (discussed below).

The functional relationship of these genes to estrogen signaling is mostly unknown. *AR* is an epidermal growth factor and is expressed in invasive mammary tumors together with its receptor, forming a potential autocrine loop for tumor progression (Ma et al. 2001). *AR* is also a target gene for vitamin D3 (Akutsu et al. 2001) and progesterone (Das et al. 1995) and may go through the ErbB pathway for oncogenic activity by inhibiting apoptosis (Hurbin et al. 2002). Therefore, activation of the *AR* gene may well explain the progression of estrogen-independent breast cancer. The *AGTR1* is a type 1-angiotensin II receptor whose expression is downregulated by estrogen in several tissues. This explains the estrogen deficiency in hypertension and other diseases (Krishnamurthi et al. 1999; Nickenig et al. 1998), although the explanation at the molecular signaling level is not so clear. The pathways common to the epidermal growth factor receptor or the insulin-like growth factor could be potential signaling mechanisms (Touyz and Berry 2002).

Evaluating Estrogenicity with EstrArray

The chemicals used here have estrogen activity in reporter gene assays (Demirpence et al. 1993; Gaido et al. 1999; Inoue et al. 2002a; Pons et al. 1990) and cell proliferation/uterotrophic assays [reviewed by Kanno et al. (2003)] and upregulate estrogen target genes in responsive cells (Nagel et al. 2001; Vivacqua et al. 2003). Dioxin does not have estrogen agonist activity (Astroff and Safe 1988; Spink et al. 1990). Cluster analysis shown in Figure 2 clearly demonstrated similar expression profiles among estrogenic chemicals, E_2, estriol, estrone, genistein, nonylphenol, and DES. Note that the data were obtained for 10 µM in the case of genistein, nonylphenol, and bisphenol A, whereas a concentration of 10 nM was used for the others. Bisphenol A at 10 µM showed less of a tendency to enhance the gene response, although it may show a higher tendency when examined at a higher concentration. Methoxychlor at 10 µM showed an even lower response but showed a meaningful correlation with the profile for E_2. Dioxin, as expected, was classified as the most distant chemical in the clustering here.

The evaluation of the estrogenicity of chemicals used here is unique. First, the estrogenicity of chemicals was compared as expression profiles of estrogen-responsive genes, giving multiple scales provided by the expression of each gene used here

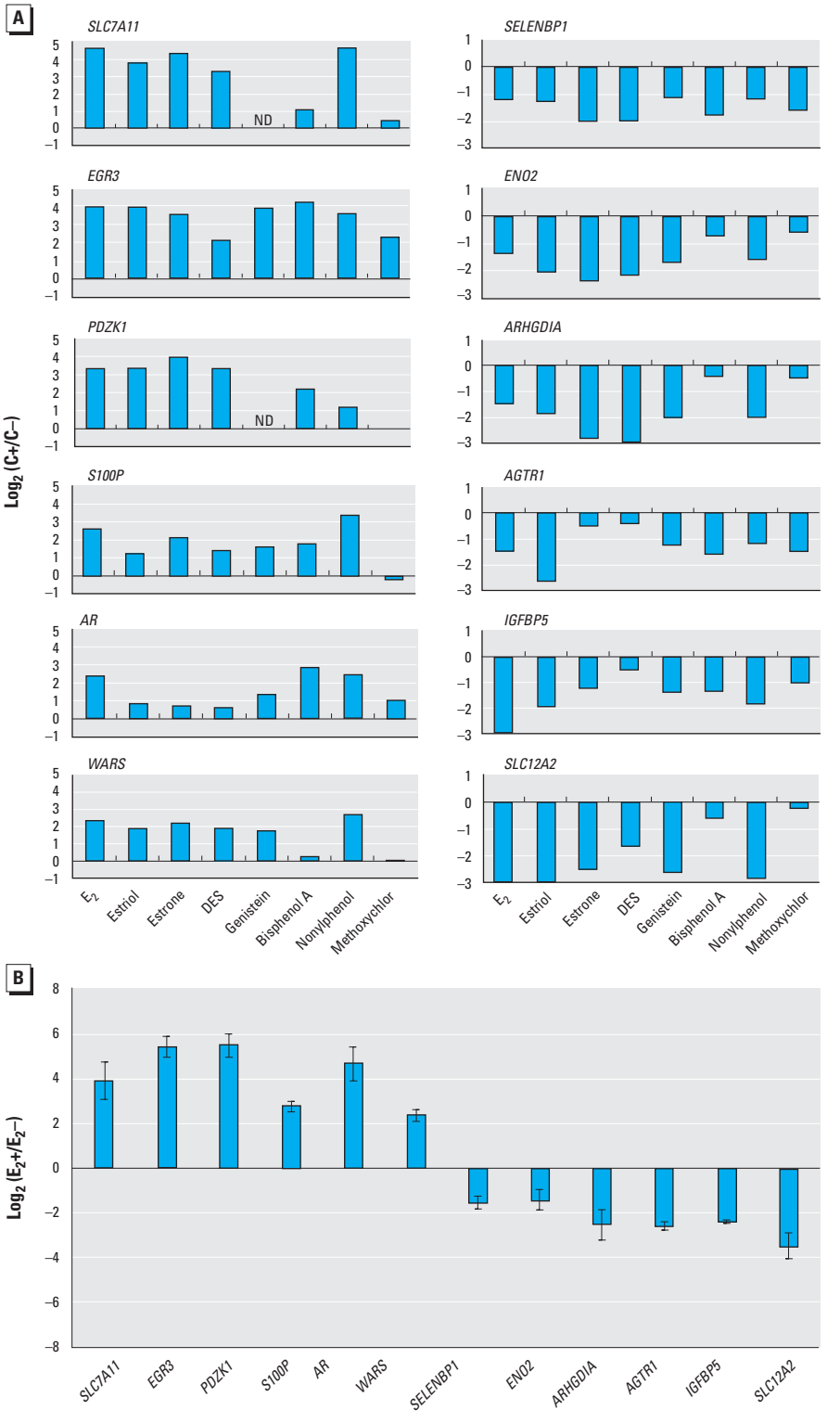

Figure 4. Expression profiles of the genes showing upregulation or downregulation in response to estrogen and estrogenic chemicals. (*A*) Responses to various chemicals analyzed using EstrArrays. The vertical axis marked as $\log_2$ (C+/C–) indicates $\log_2$ (fluorescent intensity for chemical plus/fluorescent intensity for chemical minus) calculated for each chemical. (*B*) The response to E_2 examined by real-time quantitative RT-PCR. The assays were repeated 3 times and the average and the SD (bracketed) in the $\log_2$ values are shown. The genes examined are *SLC7A11* (solute carrier family 7, member 11), *EGR3* (early growth response 3), *PDZK1* (PDZ domain–containing protein), *S100P* (S100 calcium-binding protein P), *AR* (amphiregulin), *WARS* (tryptophanyl-tRNA synthetase), *SELENBP1* (selenium binding protein 1), *ENO2* (enolase 2), *ARHGDIA* (Rho GDP dissociation inhibitor alpha), *AGTR1* (angiotensin II receptor type 1), *IGFBP5* (insulin-like growth factor binding protein 5), and *SLC12A2* (solute carrier family 12, member 2).

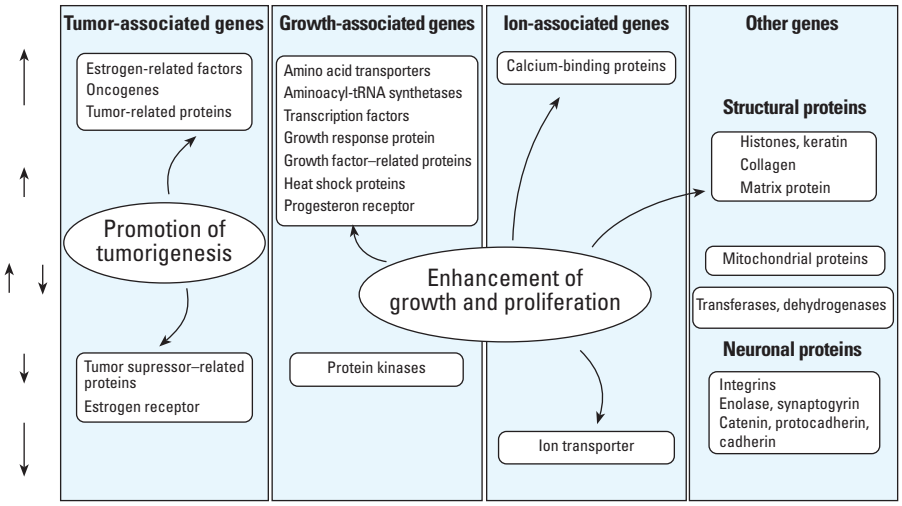

Figure 5. The genes responding to estrogen. The genes were categorized into tumor-, growth- and ion-associated genes and other genes including those for structural and neuronal proteins. Upregulation and downregulation are indicated by arrows on the left side.

compared with the ligand-binding method and reporter gene assays. This is even advantageous when the estrogenicity of chemicals is low, as multiple scales can give statistically significant evaluations. The estrogenicity of methoxychlor was not detected clearly by some assays (Shelby et al. 1996), but here it showed a distinct tendency. Second, the estrogenicity shown here is based on biological effects because not only the target genes of estrogen/estrogen receptor complex but also the genes that are presumably located downstream of the estrogen signaling pathway were included (Inoue et al., in press; Rho et al. in press). Third, with more information, the data can be classified according to the tendency of response among chemicals, specific to steroids, phenol, and phthalate, for example, which are expected to have different effects on the genes. To apply DNA microarray data for the evaluation of estrogen activity among various compounds, we are now constructing a database consisting of DNA microarray data of genes, chemicals, and cells.

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