Similarities and differences in uterine gene expression patterns caused by treatment with physiological and non-physiological estrogens

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Abstract

Administration of physiological and non-physiological estrogens during pregnancy or after birth is known to have adverse effects on the development of the reproductive tract and other organs. Although it is believed that both estrogens have similar effects on gene expression, this view has not been tested systematically. To compare the effects of physiological (estradiol; E2) and non-physiological (diethylstilbestrol; DES) estrogens, we used DNA microarray analysis to examine the uterine gene expression patterns induced by the two estrogens. Although E2 and DES induced many genes to respond in the same way, different groups of genes showed varying levels of maximal activities to each estrogen, resulting in different dose–response patterns. Thus, each estrogen has a distinct effect on uterine gene expression. The genes were classified into clusters according to their dose–responses to the two estrogens. Of the eight clusters, only two correlated well with the uterotrophic effect of different doses of E2. One of these clusters contained genes that were upregulated by E2, which included genes encoding several stress proteins and transcription factors. The other cluster contained genes that were downregulated by E2, including genes related to metabolism, transcription and detoxification processes. The expression of these genes in estrogen receptor-deficient mice was not affected by E2 treatment, indicating that these genes are affected by the E2-bound estrogen receptor. Thus, of the many genes that are affected by estrogen, it was suggested that only a small number are directly involved in the uterotrophic effects of estrogen treatment.

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Introduction

Diethylstilbestrol (DES), a non-physiological estrogen, was developed in the late 1930s for clinical use and is thought to mimic the effect of estradiol (E2) (Dodds *et al.* 1938). Subsequently, it was found that treatment of human fetuses and neonatal mice with DES as well as with physiological estrogens causes reproductive tract abnormalities and vaginal adenosis (Kimura *et al.* 1967). It is believed that treatment with physiological and non-physiological estrogens results in these reproductive abnormalities because they both act at the wrong time during development. As DES and E2 have a high affinity for the estrogen receptor and both show

uterotrophic effects, it has been assumed that both estrogens regulate gene expression in the same way. However, this notion has not been extensively tested.

To examine the effect of physiological and non-physiological estrogens on gene expression, we used DNA microarray analysis. DNA microarray technology has been successfully applied to characterize the genome-wide gene expression that results from various stimuli, such as hormone treatment (Feng *et al.* 2000, Flores-Morales *et al.* 2002). High-density oligonucleotide arrays (Lockhart *et al.* 1996) are especially suitable for genome-wide mapping of gene expression because a large number of genes can be analyzed at one time, and the system

can be readily scaled up to allow the simultaneous monitoring of tens of thousands of genes.

In this study, gene expression changes in the uterus following physiological (E2) and non-physiological (DES) estrogen treatment were profiled using DNA microarrays. The uterus has long been used as a measure of estrogenicity because the horn size and uterine weight correlate well with the dose of estrogen administered. Clustering analysis of the gene expression pattern revealed similar gene expression profiles for E2 and DES. However, the expression of some genes were more greatly affected by DES than by E2, and vice versa. The expression of these genes were not altered by estrogen treatment of estrogen receptor (ER) α knockout (α ERKO) mice, which suggests that ER is involved in the estrogenic effects of DES and E2. In addition, by assessing the correlation between the gene expression and the uterotropism caused by various doses of estrogen, we were able to select the genes that appear to be involved in the uterotrophic effects of estrogen treatment.

Materials and methods

Animals

Animals were housed under a 12 h light/12 h darkness cycle. α ERKO mice (Lubahn *et al.* 1993) and their wild-type counterparts (C57/BL6/J background) were ovariectomized at eight weeks of age. Two weeks later, the ovariectomized mice were injected intraperitoneally with estrogen or sesame oil (Nakarai Tesque, Kyoto, Japan) as a vehicle control and whole uteri ($n=4$) were collected 6 h later. Estrogen doses used were 50, 5, 0.5 and 0.05 μ g/kg body weight (b.w.) for 17 β -estradiol (E2) (Sigma-Aldrich Japan, Tokyo, Japan), and 5 and 0.5 μ g/kg b.w. for DES (Sigma-Aldrich Japan). For the uterotrophic assay, the ovariectomized mice were injected intraperitoneally with estrogen or sesame oil every 24 h for 7 days and whole uteri ($n=5$) were collected and weighed. All animal experiments were approved by the institutional Animal Care Committee.

Preparation of labeled cRNA and hybridization

Total uterine RNA was extracted using the TRIZOL reagent (Invitrogen, Tokyo, Japan) and purified using the RNeasy total RNA purification

kit (Qiagen, Tokyo, Japan). Total RNA (10 μ g) was converted into double-stranded cDNA using the SuperScript Choice System (Invitrogen) except that the T7-(dT)₂₄ primer (Amershambiosciences, Tokyo, Japan) was used for first-strand cDNA synthesis. Biotin-labeled cRNA was synthesized using the ENZO BioArray HighYield RNA transcript labeling kit (Amershambiosciences). The cRNA was purified using RNeasy (Qiagen) and partly hydrolyzed in fragmentation buffer (40 mM Tris (pH 8.1), 100 mM potassium acetate, and 30 mM magnesium acetate) by 94 °C heat treatment for 35 min. The fragmented cRNA was mixed with hybridization buffer containing 100 mM 2-(N-Morpholino)ethanesulfonic acid, 1 M NaCl, 20 mM EDTA and 0.01% Tween 20 and control oligonucleotides. The quality of the cRNA was assessed by analysis with the Test 3 array (Affymetrix, Amershambiosciences) containing housekeeping genes. All preparations met Affymetrix's recommended criteria for use on their expression arrays.

After checking the quality of the cRNAs, 15 μ g cRNA were hybridized to the high-density oligonucleotide arrays (Mouse U74 A, Affymetrix, Amershambiosciences) for 16 h at 45 °C. The arrays were then washed, stained with streptavidin-phycoerythrin (Molecular Probes, Funakoshi, Tokyo Japan) and scanned with an argon-ion laser confocal scanner (Amershambiosciences).

Microarray data analysis

Scanned data were analyzed with GeneChip software (Affymetrix, Amershambiosciences). The details of the methods used to analyze the data have been described (Lockhart *et al.* 1996). Briefly, each gene is represented by 20 perfectly matched (PM) and one-base-mismatched (MM) 25-base oligonucleotides. As the MM probes are used to detect background levels and cross-hybridization signals, the relative level of gene expression is represented by the differences between the levels of fluorescent intensity of the PMs and the MMs, which are averaged into the so-called 'average difference'. To normalize the data, the average differences were adjusted to produce an average intensity that equaled 2500.

To be certain of the estrogen-related changes in gene expression revealed by the DNA microarray analysis, we independently repeated the same

experiment at least twice and the differences in intensities were calculated. To maintain reproducibility, we selected for further analysis only those genes whose expression levels at the same condition in two separate experiments did not change by more than twofold. The average values yielded by these independent experiments were then used to denote the gene expression levels. Calculation of Pearson's correlation coefficients and Fisher's z transformation were performed by StatView software (SAS Institute Inc., Cary, NC, USA).

Filtering of genes and clustering analysis were performed by using the GeneCluster program based on self-organizing maps (SOMs) developed by Tamayo *et al.* (1999).

Quantitative-real time-PCR

Total RNA was purified as described above. cDNA was synthesized from purified total RNA by Superscript II RT(-) (Invitrogen) with random primers at 42 °C for 60 min. PCR reactions were performed in the PE Prism 5700 sequence detector (PE Biosystems, Tokyo, Japan) using the SYBR-Green PCR Core Reagents (PE Biosystems) in the presence of appropriate primers according to the manufacturer's instructions. Each PCR amplification was performed in triplicate using the following conditions: 2 min at 50 °C and 10 min at 95 °C, followed by 40 two-temperature cycles (15 s at 95 °C and 1 min at 60 °C). The use of a sequence detector allows continuous measurement of the fluorescent spectra emitted by SYBR-Green intercalated to double-stranded DNA during the PCR amplification. The model 5700 software constructed amplification plots from extension phase fluorescent emission data collected during PCR amplification. Threshold (C_t) values were calculated by determining the point at which fluorescence exceeds a threshold limit (usually 10 times the S.D. of the baseline). The primers were chosen to amplify short PCR products (less than 100 base pairs) and their sequences are as follows: U51196 (APC-binding protein EB1 homolog) CCCATTGCAACACAGAGGACTA, CCAGGATTCTTTTCGCACCAT; AJ005564 (small proline-rich (SPR) protein 2F) TGAGGCTTCAGCAACAATGTCTT, TTGGTGGTGGACACACAGGA; D50086 (neuropilin) CAGCAAGCGCAAGGCTAAG, GAGGTGAAAACGTCCGAAGCT; L40406 (heat shock protein (hsp-E7I)) GGAACGACCGA

AAGTGTTGG, CCTCTGAAGTCCGCTGC AAT; X75285 (fibulin-2) CAGCTCGCATCAC GCACTAC, GGCCGATGCGGAAGATATG; AF087825 (claudin-7) CCCTGGTGTGGGGCT TCTTA, CCACAGCGTGTGCACTTCAT. To obtain standard curves of the genes, serial dilutions of a known amount of a cDNA sample were used. The C_t values of each gene were plotted on these standard curves to obtain the amount of copies present in the initial cDNA sample and fold changes were calculated. Gene expression levels were normalized by the expression levels of 28S (X00525) ribosomal RNA (primer sequences were AGACCGTCGTGAGACAGGTTAGTT and GCAGGATTACCATGGCAACAA). Gel electrophoresis and melting curve analyses were performed to confirm correct amplicon size and the absence of nonspecific bands.

Results

Reproducibility of the DNA microarray experiments

In this study, we used DNA microarray analysis to examine the uterine gene expression in sesame oil-treated mice and in mice treated with four doses of physiological (E2) estrogen or two doses of non-physiological (DES) estrogen. In order to confirm the reproducibility of the DNA microarray data, the intensities of the genes expressed during the same conditions in two independent experiments were compared. As shown in Table 1, the gene expressions at all seven conditions in the two experiments were very similar, as the correlation coefficients were all greater than 0.8 and five of the seven were greater than 0.9. Thus, the microarray data were highly reproducible. In a previous paper, we used Student's t -test with three sets of independent DNA microarray experiments to identify 46 estrogen-induced genes (Watanabe *et al.* 2002). More than 80% of these genes were also found in this study (data not shown), which supports the reproducibility of these microarray experiments.

Different effects of physiological and non-physiological estrogens on gene expression

We first assessed the microarray data obtained with mice treated with 5 µg/kg b.w. of E2 or DES to

Table 1 Correlation between the gene expression values obtained with two identically performed independent DNA microarray experiments. DNA microarray experiments with uterine RNA taken from oil-treated mice or mice treated with four doses of E2 or two doses of DES were repeated twice. The expression levels of the genes in both experiments were measured based on the Affymetrix software and the correlation coefficients between gene expression levels in the two experiments were calculated. The correlation coefficients are shown

	Dose of E2 or DES (µg/kg)				
	0	0.05	0.5	5	50
E2		0.94	0.94	0.87	0.93
DES	0.93	ND	0.83	0.91	ND

Figure 1 Venn diagram of the number of genes induced by E2 and DES. Ovariectomized mice were treated with 5 µg/kg of E2 or DES and total uterine RNAs were purified after 6 h. Expression analysis was performed and the fold changes of gene expression levels were calculated based on the sesame oil control expression levels. The genes that were activated by either E2 or DES or both E2 and DES were counted.

determine whether these estrogens activate the same genes. The number of genes that were activated more than threefold by the estrogens was counted. We found that, in addition to the genes commonly activated by both estrogens (88 genes), some genes were activated by only one of the estrogens (37 genes for E2 and 71 for DES) (Fig. 1). This suggests that E2 and DES are not identical in their gene activation behavior despite a study with yeast reporter assays that revealed the two estrogens had a very similar effect on the genes examined (Korach *et al.* 1985, 1991, Coldham *et al.* 1997).

Dose-dependent activation of genes by physiological and non-physiological estrogens

The identification of genes activated by only one of the two estrogens prompted us to examine the dose-dependent uterine gene-expression patterns resulting from E2 or DES treatment. Thus, we examined the microarray data obtained from oil-treated mice and mice treated with four doses of E2 or two doses of DES. The expression levels recorded for each gene at each estrogen dose in the two experiments were averaged and analyzed by using the GeneCluster program based on self organizing maps (Tamayo *et al.* 1999). This analysis revealed that the expression of estrogen-activated genes as well as estrogen-repressed genes is dependent on the E2/DES dosage used (Fig. 2). Three additional features were also noted from the clustering analysis. First, the *in vivo* activity of DES in altering gene expression is much higher than that of E2. This correlates with the estrogenic activities of E2 and DES estimated by the prepubertal mouse (Coldham *et al.* 1997), although the extent of the difference was greater than the result in prepubertal mouse. The difference between the two systems may be due to the presence *in vivo* of binding proteins such as steroid hormone-binding globulin, which binds to DES more weakly than it binds to E2 (Sheehan & Young 1979), causing more DES to be available for ER binding. Secondly, the genes differed in their dose-dependent activation/repression patterns as we observed that the estrogens induced at least two different dose-response patterns. Some gene clusters, such as classes C, D, G and H, were maximally activated or repressed at the highest estrogen doses used (for example, 50 µg/kg E2, Fig. 2) whereas the genes in classes A, E, and F were maximally activated or repressed at the middle of the dose range (for example, 5 µg/kg E2, Fig. 2). The latter pattern may reflect the well-known phenomenon of antagonism resulting from an overdose of the agonist. These observations suggest that the maximal activity of transcription differs among genes. These observations also suggest that the difference between E2 and DES in the number of the genes they activated by more than threefold at 5 µg/kg doses (Fig. 1) mainly reflects the difference in the relative activity of each estrogen and the difference in the dose-response patterns of the genes. Thirdly, the

Figure 2 Clustering pattern of the genes whose expression is affected by E2 or DES. Based on the DNA microarray data, the dose-dependent gene expression patterns were clustered by a GeneCluster program based on self-organizing maps algorithm (see Materials and methods) and the clusters showing genes that are activated or repressed by both estrogens are indicated. Each graph (A–F) represents the dose-dependent ($\mu\text{g/kg}$; X axis) expression pattern of a cluster represented by normalized values (Y axis). The number of genes in each cluster is also indicated. Solid and dashed lines indicate the E2 and DES dose-responses as means of standardized gene expression levels respectively. The dotted line indicates the gene expression level of αERKO mice. Standard deviations are also indicated.

extent of the gene activation induced by E2 or DES differs depending on the gene. Some genes had a higher maximal activity with DES than E2 whereas other genes showed the opposite pattern. For example, the genes in cluster B were induced by E2 to express much higher levels of message at the optimal E2 concentration compared with DES (Fig. 2). In contrast, in cluster D, DES induced a much higher maximal intensity of gene expression compared with E2 (Fig. 2). Some of the genes are shown with average intensities (Fig. 3). These gene-dependent differences were also confirmed by quantitative-real time-PCR (Q-RT-PCR) on selected genes (Fig. 3). For example, the APC-binding protein EB1 (U51196), the SPR protein 2F (AJ005564) and neuropilin (D50086) were better activated by E2 than by DES, whereas HSP105 (L40406), fibulin-2 (X75285) and claudin-7 (AF087825) were more highly activated by DES compared with the same dose of E2. As the response was not at the maximal effective dose in all cases, the difference can be the result of the difference in the efficacy and potency of the estrogens. The biological significance of these

E2/DES-induced differences and the promoters involved should be examined further.

Relationship between gene expression patterns and uterotrophic activity

As there were at least two types of dose-dependent gene activation patterns, such as those exemplified by clusters A and B, we examined the relationship between these gene activation patterns and their uterotrophic effect. Ovariectomized mice were treated with E2 for one week and the dose-dependent uterotrophic effects were estimated by weighing the uteri. We found that the greatest uterine weights occurred with the highest dose of E2 that was administered (Fig. 4A). This dose-response does not match that of the genes in gene clusters such as A and E (Fig. 2), which are maximally up- or downregulated by the middle dose of E2. This suggests that these genes may not be directly involved in the uterotrophic effect. In contrast, it is likely that the genes that have a dose-response similar to that of the uterotrophic effect are directly involved in the uterotropism of

Figure 3 Quantitative PCR analysis of selected genes that are differently affected by DES and E2. The genes showing big differences in fold changes between E2 and DES were selected and dose curves of the selected genes were plotted from the microarray data. The Y-axis indicates the average gene expression levels calculated from two independent experiments and the X-axis indicates the doses administered (μg/kg). The Genebank accession number is indicated in each panel. For Q-RT-PCR, ovariectomized mice were treated with 5 μg/kg of E2 or DES and uterine RNA was isolated after 6 h. After cDNA synthesis, the fold changes in gene expression levels induced by the estrogens were estimated by quantitative-real time-PCR. After normalizing the expression relative to the expression levels of 28S ribosomal RNA, the fold changes induced by DES were divided by the fold changes induced by E2. These ratios of fold changes are indicated by log2. Each experiment was repeated three times and the errors are indicated. The cluster classes to which the examined genes belong are indicated in parenthesis. U51196, APC-binding protein EB1 homolog; AJ005564, SPR protein 2F; D50086, neuropilin; L40406, Hsp105; X75285, fibulin-2; and AF087825, claudin-7.

E2. This notion is supported by the fact that these genes are not activated by E2 in α ERKO mice (Fig. 2), nor does E2 have any uterotrophic effects in these mice (Lubahn *et al.* 1993).

To select the genes that may be related to the uterotrophic effect of E2, we calculated the

correlation between the gene expression levels and the uterotrophic effects associated with various doses of E2. We selected the genes associated with correlation coefficients that are over 0.85 (positively related genes) or under -0.85 (negatively related genes) (Tables 2 and 3). We used these values for

Figure 4 Correlation between gene expression levels and the uterotrophic effect of varying E2 doses. For the uterotrophic assay, ovariectomized mice were injected intraperitoneally with E2 or sesame oil every 24 h for 7 days and whole uteri ($n=5$) were collected and weighed. (A) The ratio of uterine weight and body weight (mg/g) is indicated in Y-axis, X-axis indicates the doses administered (mg/kg). The correlation between the uterotrophic effect and the expression of each gene at 6 h was calculated. Ten genes with high positive correlation coefficients (>0.85) (B) and ten genes with high negative correlation coefficients (negatively-correlated genes) (<-0.85) (C) were selected and their gene expression levels are indicated. The Y-axis indicates the gene expression levels estimated by DNA microarray and the X-axis indicates the doses administered ($\mu\text{g/kg}$).

the selection because they gave positive values of 98% confidence intervals surrounding r after z transformation.

Included in the positively-correlated genes are stress-related proteins (Stip1, Hspa4, Hspa5 and Hsp105) and insulin-like growth factor I (Igf1). Igf1 has previously been shown to be involved in the regulation of the estrogenic response (Murphy & Ghahary 1990). Of the stress-related proteins, two are known chaperones and two others are related to chaperones. Stip1/mSTI1, conserved from yeast to humans (Blatch *et al.* 1997), is known to interact with Hsp90 and Hsp70 (Lassle *et al.* 1997) and functions as a cochaperone. Hspa5/BiP is also known to have a chaperone-like activity (Hurtley *et al.* 1989, Flynn *et al.* 1991) at the endoplasmic reticulum (Dierks *et al.* 1993). The other stress proteins, Hspa4/apg-2 (Kaneko *et al.* 1997) and Hsp105 (Yasuda *et al.* 1995), are similar

to hsp70RY but their functions are not yet known. Two transcription factors, Atf5 and Egr1, were also activated. These transcription factors may be related to the activation of downstream genes. Also selected were two genes related to sterol synthesis, which we showed in an earlier study to be activated by estrogen (Watanabe *et al.* 2002). When we compared the positively-correlated genes with temporal changes of the expression pattern (Watanabe *et al.* 2003), it was revealed that the expressions of the positively-correlated genes were not temporally correlated. For example, purine-nucleoside phosphorylase was activated as an immediate-early gene, squalene epoxidase was activated as an early-late gene and heat shock protein was gradually activated (Watanabe *et al.* 2003).

With regard to the repressed genes, we found not only genes involved in metabolism but also genes

Table 2 List of selected genes whose expression patterns are upregulated during the uterotrophic effect of E2. The known genes whose E2-induced expression is highly positively correlated with the uterotrophic effect of E2 are indicated. Correlation coefficients (CC), fold changes in E2-induced upregulation of gene expression relative to gene expression levels in oil-treated mice (FC), GenBank Accession numbers (GB#), symbols and gene names are shown

CC	FC	GB#	Symbol	Gene Name
0.87	34.31	X04480	Igf1	insulin-like growth factor 1
0.88	7.01	AB013848	Padi1	peptidyl arginine deiminase, type I
0.90	5.58	D85904	Hspa4	heat shock 70 kDa protein 4
0.86	5.48	X75285	Fbln2	fibulin 2
0.92	5.48	D42048	Sqle	squalene epoxidase
0.90	5.21	AB017189	Slc7a5	solute carrier family 7, member 5
0.86	5.15	U14648	Sfrs10	splicing factor, arginine/serine-rich 10
0.92	5.04	U00937	Gadd45	growth arrest and DNA-damage-inducible 45 alpha
0.86	4.75	M28845	Egr1	early growth response 1
0.92	4.64	AB012276	Atf5	activating transcription factor 5
0.85	4.64	L02526	Map2k1	mitogen activated protein kinase kinase 1
0.91	4.42	AJ002387	Hspa5	heat shock 70 kDa protein 5
0.92	3.91	L32752	Ras12-9	RAS-like, family 2, locus 9
0.93	3.86	L40406	Hsp105	heat shock protein, 105 kDa
0.86	3.81	U59807	Cstb	cystatin B
0.90	3.62	D29016	Fdft1	farnesyl diphosphate farnesyl transferase 1
0.90	3.41	M22679	Adh1	alcohol dehydrogenase 1, complex
0.85	3.25	U27830	Stip1	stress-induced phosphoprotein 1
0.92	3.24	U35374	Pnp	purine-nucleoside phosphorylase
0.85	3.05	D89063	Ddost	dolichyl-di-phosphooligosaccharide-protein glycotransferase

related to detoxification mechanisms such as cytochrome P450 and glutathione S-transferase. Moreover, we selected max dimerization protein 4 (Mad4) (Hurlin *et al.* 1995) and interferon regulatory factor 6 (Irf-6), which are known to be transcription factors. It may be that the downregulation of these transcription factors combined with the upregulation of the transcription factors noted above play an important role in mediating the responsiveness of the uterus to estrogen. Functional analysis of these selected genes should contribute to our understanding of the molecular mechanisms involved in the uterotrophic effects of estrogens, particularly those evoked in the early stages.

Discussion

In this study, we showed that physiological and non-physiological estrogens do not exert identical effects on uterine gene expression. Although both estrogens generally induced similar genes, the expression of some genes was more profoundly affected by E2 than by DES (cluster B in Fig. 2) and vice versa (cluster D in Fig. 2). The fact that E2

and DES induce the expression of genes to different extents suggests that they may exert different physiological effects distinct from estrogen receptor binding activities.

Recently, it was found that the different estrogenic activities of estrogen and tamoxifen are due to their different requirements of a cofactor, steroid receptor coactivator 1 (SRC-1) (Shang & Brown 2002), as the estrogen-like activity of tamoxifen requires high levels of SRC-1 expression whereas estrogen requires lower levels. This study also showed that while tamoxifen can recruit SRC-1 by binding to the ER, it is much less effective in recruiting other cofactors. Interestingly, the efficiency of this ligand-dependent recruitment of cofactors varies depending on the ligand and cofactors involved. For example, while ERs liganded with E2 and DES recruit SRC-3 equally efficiently, E2-liganded ERs can recruit SRC-1 and SRC-2 more efficiently than DES-liganded ERs (Bramlett *et al.* 2001). On the basis of these observations, we could propose that the different gene clusters revealed in this study may relate to the effect of these various coactivators. Although the difference between E2 and DES is not so

Table 3 List of selected genes whose expression patterns are downregulated during the uterotrophic effect of E2. The known genes whose E2-induced expression is highly negatively correlated with the uterotrophic effect of E2 are indicated. Correlation coefficients (CC), fold changes in E2-induced upregulation of gene expression relative to gene expression levels in oil-treated mice (FC), GenBank Accession numbers (GB#), symbols and gene names are shown

CC	FC	GB#	Symbol	Gene Name
-0.91	-6.75	U95826	Ccng2	cyclin G2
-0.86	-5.80	J03953	Gstm3	glutathione S-transferase, mu 3
-0.88	-5.59	AB023957	EIG180	ethanol induced gene product EIG180
-0.93	-5.34	U42467	Lepr	leptin receptor
-0.95	-5.01	D16215	Fmol	flavin containing monooxygenase 1
-0.93	-4.93	M55181	Penk1	preproenkephalin 1
-0.97	-4.71	D83966	Ptpn13	protein tyrosine phosphatase, non-receptor type 13
-0.93	-4.59	AF056187	Igf1r	insulin-like growth factor I receptor
-0.94	-4.51	U88567	Sfrp2	secreted frizzled-related sequence protein 2
-0.89	-4.42	U96724	Pps	putative phosphatase
-0.89	-4.42	AJ132192	Hs1bp3	HS1 binding protein 3
-0.88	-4.38	U32395	Mad4	Max dimerization protein 4
-0.86	-4.34	U09816	Gm2a	GM2 ganglioside activator protein
-0.85	-4.27	M24417	Abcb1a	ATP-binding cassette, sub-family B (MDR/TAP), member 1A
-0.93	-3.90	AF067395	Bnip31	BCL2/adenovirus E1B 19 kDa-interacting protein 3-like
-0.89	-3.83	X98055	Gstt 1	glutathione S-transferase, theta 1
-0.85	-3.80	U90435	Flot1	flotillin 1
-0.85	-3.59	M72394	Pla2g4a	phospholipase A2, group IVA (cytosolic, calcium-dependent)
-0.90	-3.51	L17022	Fcgrt	Fc receptor, IgG, alpha chain transporter
-0.92	-3.36	X15052	Ncam1	neural cell adhesion molecule 1
-0.86	-3.29	AB033921	Ndr2	N-myc downstream regulated 2
-0.91	-3.28	D30779	Pla2g1br	phospholipase A2, group IB, pancreas, receptor
-0.97	-3.26	U73029	Irf6	interferon regulatory factor 6
-0.87	-3.23	U07159	Acadm	acetyl-coenzyme A dehydrogenase, medium chain
-0.93	-3.12	Y08135	Asml3a	acid sphingomyelinase-like phosphodiesterase 3a
-0.87	-3.11	L38444	Tgtp	T-cell specific GTPase
-0.95	-3.08	U69262	Matn2	matrilin 2
-0.92	-3.08	AJ007970	Gbp2	guanylate nucleotide binding protein 2
-0.87	-3.05	D50834	Cyp4b1	cytochrome P450, subfamily IV B, polypeptide 1

drastic as that between E2 and tamoxifen, it can be considered to have weak 'selective estrogen receptor modulator' activity.

Alternatively, the different estrogenic effects of various estrogens may also be due to non-genomic actions of the ER. It is known that signaling cascades such as extracellular signal-regulated kinase (ERK)/mitogen-activated kinase (MAPK) or the modulation of G-protein-coupled receptors are involved in the so-called non-genomic action of estrogen (see references in Levin 2003). Testing these possibilities to determine what is responsible for the different estrogenic effects of various estrogens may facilitate our understanding of the mechanism of estrogenicity.

In our previous study, we examined temporal changes of gene expression after estrogen administration (Watanabe *et al.* 2003). Although we

compared the clustering result of the temporal changes and that of the estrogen response, we could not find a clear correlation between them. This result indicates that sensitivity and efficacy of estrogen are not directly related to temporal changes of the gene expression pattern.

In this study, it was shown that a group of genes showed a dose-response similar to that of the uterotrophic effect. It suggests that we can select the genes that may be directly related to the uterotrophic effect among a large number of estrogen-induced genes (Watanabe *et al.* 2002). Gene expression analysis of the selected genes can be helpful for understanding the inconsistency of estrogenic chemicals in ER binding activity and uterotrophic activity. That the uterotrophic effects of estrogenic chemicals can differ despite equivalent ER-binding activities in the human has long been

known. For example, this phenomenon has been observed in the metabolites of DES, as indenestrol A and B, indanyl-DES and Z-pseudo-DES all have higher ER binding activities but lower uterotrophic effects compared with DES. DES and some of these chemicals show similar temporal nuclear distribution (Korach *et al.* 1985), which suggests that the different uterotrophic effects of these agents may be due to their different gene activation patterns. Transcriptome analyses of the effect of the different DES metabolites will be helpful in testing this notion.

We used quantitative PCR to check the expression of several genes that were revealed by the DNA microarray experiments to be differently expressed in E2-treated uteri compared with DES-treated uteri. However, upon quantitative PCR analysis, some of these genes did not show the same patterns revealed by microarray analysis. We have also observed in our previous study that the fold changes in expression that are estimated by quantitative PCR do not always correlate with those estimated by DNA microarray experiments. These limitations in DNA microarray analysis not only block the accurate characterization of gene expression patterns, they also hinder clustering analysis. Thus, it is necessary to confirm the activation pattern detected by microarray analysis by quantitative PCR. Despite this limitation in DNA microarray assays, this technology still has major advantages in the evaluation of estrogenic activity relative to other methods. Although the yeast system is a conventional means used to assess the effect of estrogen on gene expression, it cannot evaluate the effect of estrogen-binding proteins that exist *in vivo* and it is difficult to evaluate the effect of estrogen on target tissues. Furthermore, while the uterotrophic assay is a useful method for the evaluation of estrogenic activity *in vivo*, it is a time-consuming procedure. Thus, microarray analysis is an ideal method to evaluate the effect of estrogenic activity on gene expression patterns, as it is a quick method that assesses the estrogenic activity *in vivo*.

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