Table 2
Verification of the microarray results by quantitative real-time RT-PCR

Gene	MCF-7, TCDD			RL95-2, TCDD		
	0.1	1	10	0.1	1	10
NAV1	–	–	–	1.05	1.14	1.61
PCYT1A	–	–	–	1	0.74	0.6*
CDH18	0.42*	0.25*	0.06*	–	–	–
CPEB2	1.05	0.5*	0.22*	0.77	0.46*	0.36*
IGFBP5	0.53*	0.44*	0.51*	0.65	0.59*	0.57
ERBB2	0.84	0.72	0.65	–	–	–
MAN1A1	0.85	0.72	0.66	–	–	–
CTNND2	0.85	0.68*	0.68	0.47	0.68	0.73
APM2	0.71	0.73	0.69	1.23	1.65	1.68
KIAA0373	0.93	0.72	0.72	0.6*	0.74	0.64*
FUT8	1.02	0.81	0.73	–	–	–
ITPK1	0.99	0.93	0.77	–	–	–
CPT1	0.93	0.8	0.77	–	–	–
TFIIH	1.15	0.97	0.77	–	–	–
RCN1	1	0.81	0.8	–	–	–
TGFA	1.08	1.36	0.81	1.21	1.18	1.37
DHCR24	0.98	0.82	0.88	–	–	–
Nup35[b]	1.23	1.15	0.93	0.81	0.71	0.67*
IGF1R	1.25	1.09	1.12	–	–	–
CXCL12	1.51	1.21	1.25	–	–	–
GLCM	1.55	1.53	1.32	1.28	1.47	1.38
NRIP1	1.18	1.24	1.44	1.36	1.75	1.75
MAP6D1	1.56	1.11	1.45	1.22	1.32	1.22
BSN	1.65	1.52	1.47	1.75	2.12*	1.85
ME1	1.85	1.69	1.84	–	–	–
SLC7A5	3.85*	10.62*	19.77*	1.26	1.72	1.8
CYP1A1[a]	119.51*	399.58*	532.86*	18.09	27.48	38.46*

The values given are the means of the ratios of the expression levels to those of the DMSO-treated controls in the triplicate assay.

[a] *CYP1A1* was used as a TCDD-induced positive control.

[b] *Nup35* (AF514993) was used in place of PAC204E5, as they are similar in sequence and AF514993 is a much smaller clone. '–' values which remained undetermined due to expression levels that were below the detection limit. The genes showing responses between vehicle control and each dose were selected by *t*-test in a pairwise comparison for each gene.

* Statistically significant at $P < 0.05$ between the corresponding vehicle control and each dose of TCDD.

functional gene annotation extracted from a public database, and gene expression levels (Ingenuity Systems, Ver. 4). *SLC7A5*, *ME1* and *BSN* in the verified 27 genes were omitted due to lack of information of associations with other genes. Fig. 4A and B shows the predictive pathways including these nine genes, based on pathway analysis. In the MCF-7 cells (Fig. 4A), the pathway showed that TCDD influences *IGF1R* signaling mediated by *AHR* and *MYC*, which in turn are affected by *TGFA* or *NRIP1*.

Table 3
Responsiveness to estrogen treatment determined by quantitative real-time RT-PCR

Gene	MCF-7, E2			RL95-2, E2		
	0.1	1	10	0.1	1	10
CDH18	0.84	0.84	0.75	–	–	–
CPEB2	0.24*	0.19*	0.17*	0.38*	0.62*	0.47*
IGFBP5	0.43*	0.39*	0.78*	0.81	2.02*	1.70
ERBB2	0.33*	0.39*	0.52*	0.71	0.47*	0.71
CTNND2	0.68	0.91	0.50*	0.58*	0.73	0.67*
TGFA	0.82	1.64	1.22	0.85	0.62	0.84
IGF1R	2.04*	1.43	1.49	0.82	0.49*	0.75
CXCL12	2.66*	7.44*	7.01*	–	–	–
NRIP1	0.97	1.10	0.61*	1.34	0.54*	0.82
SLC7A5	3.47*	2.40*	3.65*	1.13	0.76	0.91
CYP1A1	0.18*	0.23*	0.37*	0.80	0.57	0.66

The values given are the means of the ratios of the expression levels to those of the DMSO-treated controls in the triplicate assay. '–' below the detection limit as described in Table 2.

* Statistically significant at $P < 0.05$ between the corresponding vehicle control and each dose of TCDD.

Catenin and cadherin, like *CTNND2* and *CDH18*, also seem to be associated with *IGF1R* signaling. In contrast, the RL95-2 cells did not display the same pathways as the MCF-7 cells due to the lack of change in expression of the genes *ERBB2*, *IGF1R*, *CXCL12* and *CDH18*, suggesting the existence of another pathway under conditions of different ER levels (Fig. 4B). The pathway analysis also suggests this battery of genes has functions associated with cancer, cellular growth and proliferation, and the cell cycle. Therefore, we next focused on the application of *IGFBP5* as an indicator of *IGF1R* signaling in experimental animals.

We analyzed *IGFBP5* gene expression in human and mouse tissues using the public dataset. The data for *IGFBP5* were extracted from the public GEO website in order to obtain information for utilizing *IGFBP5* as a marker in experimental animals exposed to TCDD. The informatic analysis revealed that the expression of *IGFBP5* in the ovary is much higher than in the brain and liver, in both humans and mice (Fig. 5).

3.4. Responsiveness to TCDD in the mouse fetus

IGFBP5 was further investigated in the mouse fetus for responsiveness to TCDD, because cellular growth and proliferation in fetus tissues are very active in comparison to adult tissues. The *IGFBP5* gene expression levels in brain, liver and calvaria of fetuses maternally exposed to TCDD were determined. The brain, liver and calvaria were used since they are known to be ER-rich organs. Ovaries could not be used since in fetuses they are too small to extract RNA for quantitative PCR. The expression of *IGFBP5* was significantly suppressed in the liver of both the male and the female fetuses and in the female fetal calvaria, but was elevated in both the male and the female fetal brain (Fig. 6).

4. Discussion

In our present study, we were successful in identifying novel TCDD-responsive genes using cDNA microarrays of estrogen-

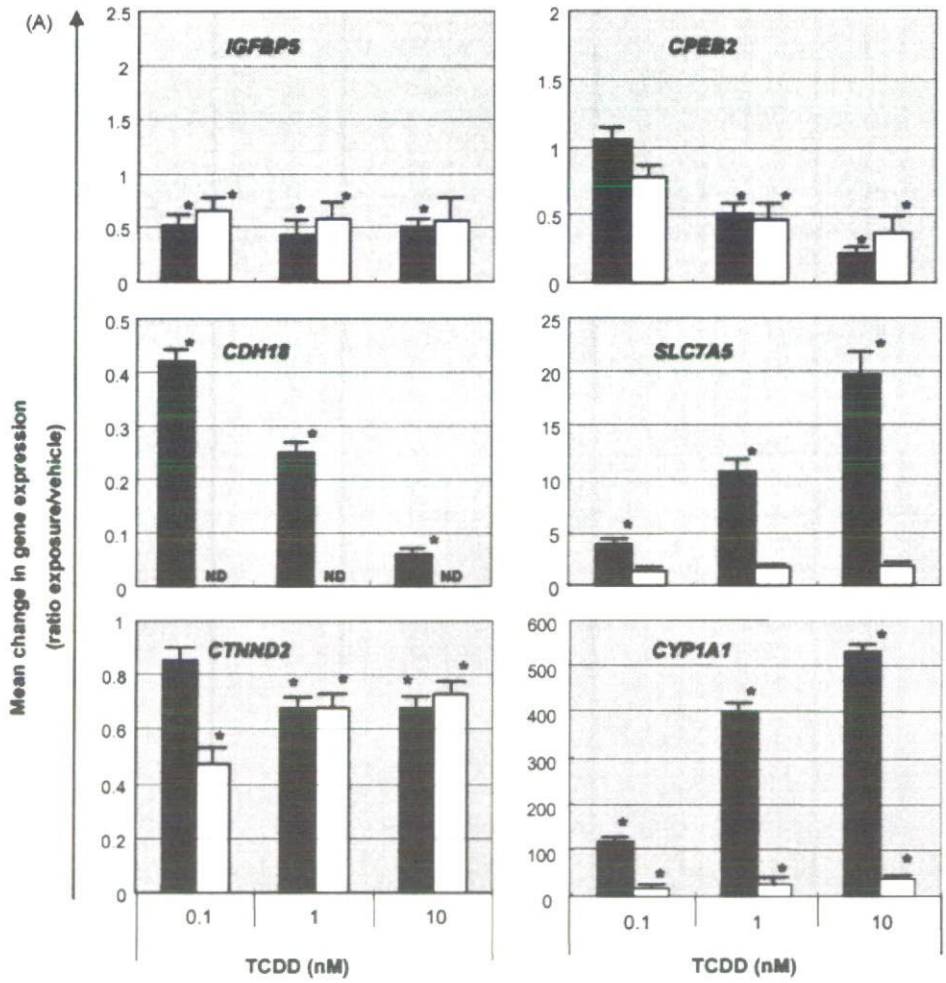

Fig. 3. Representative graphs of the genes altered by TCDD and E2 from Tables 2 and 3. A; TCDD; B, E2. The values given are the means of the ratios of the expression levels to those of the DMSO-treated controls in the duplicate assay. *IGFBP5*, *CPEB2*, *CDH18*, *SLC7A5*, *CTNND2* and *CYP1A1* were selected as representatives. Open bars indicate MCF-7 cells and black bars indicate RL95-2 cells. ND means not detected. The values are given as means ± S.D. ($n = 3$). *The difference from the vehicle control at $P < 0.05$ by *t*-test.

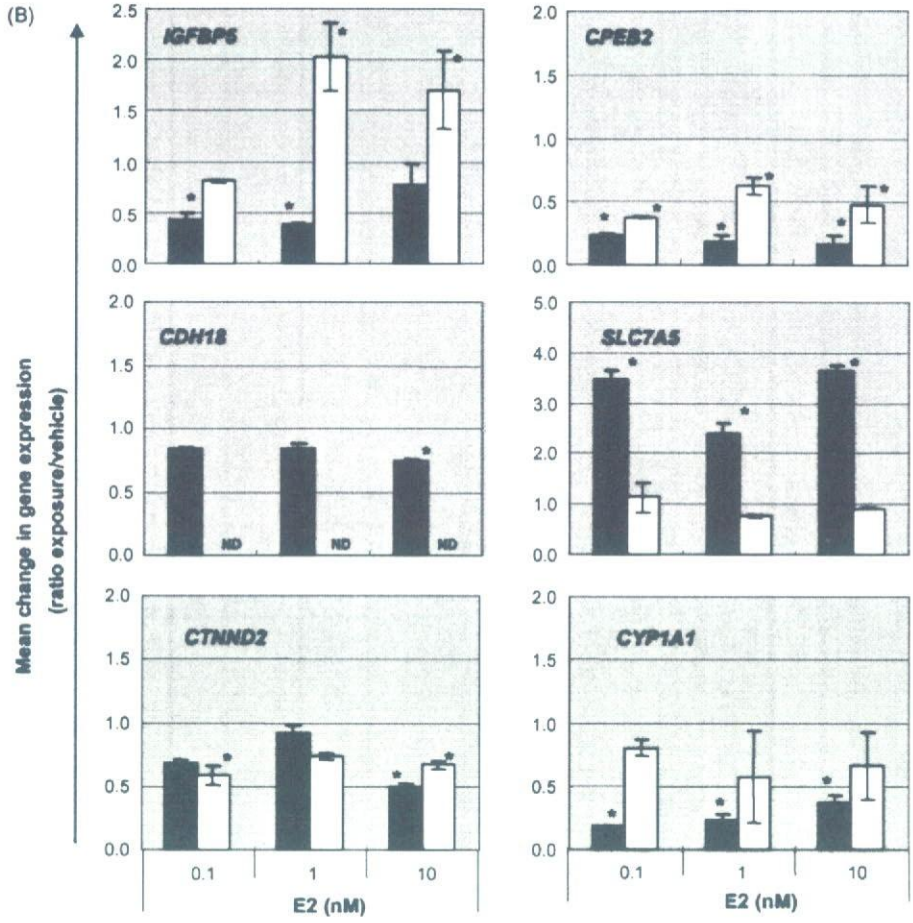

Fig. 3. (*Continued*).

responsive genes. The candidate genes isolated from these screens were also found to have possible applications in model animal systems exposed to TCDD.

The clustering analysis (Fig. 1) and Venn diagrams (Fig. 2B and C) indicate that the individual gene expression profiles were not consistent with the overall dose-dependent response. Only the total number of altered genes showed a dose-dependent increase (Fig. 2A). Although some genes for which expression was altered by TCDD tended to show an overall increase in gene expression with the increase in treatment dosage, genes responsive to TCDD in a dose-specific manner were also identified. Similar results, i.e., a dose-dependent increase in the number of genes responsive to TCDD, were obtained in a study using human lung cell lines (Martinez et al., 2002). Martinez and coworkers observed many genes for which expression was induced by 0.1 nM TCDD, but in our present study only the expression of *PR* was induced in MCF-7 cells by the same dose. This discrepancy might be due to differences between the array materials used in the two studies or the lack of susceptibility of some estrogen-responsive genes to TCDD. In contrast to two other studies in human cell lines, we found no target genes common to all three of the 0.1, 1 and 10 nM TCDD doses except *CYP1A1* (Frueh et al., 2001; Martinez et al., 2002). We propose that the genes responsive to TCDD identified by us in the present study are new, and are associated with the hormonal effects of TCDD action.

The gene profiles for the MCF-7 cells differed from those for the RL95-2 cells at various doses of TCDD, suggesting that each gene has an individual dose response. Along with *CYP1A1* (the positive control), only six genes in both of the cell lines were found to be altered by TCDD (Fig. 2B and C). We expected these differences in the gene expression profiles of the two cell types, considering the differences in transcriptional mechanisms and in the genes involved in cell growth, in particular.

Both MCF-7 and RL95-2 cells express ERα, and, consistent with this, the genes *CYP1A1* and *CYP1B1* were found to be induced by TCDD exposure in both cell lines (Jana et al., 1999, 2000). However, the magnitude of this induction differed between the two cell types, and distinct responses to E2 were observed in the present study (Table 2). Our previous studies have shown that cell proliferation was induced by estrogen exposure in the MCF-7 cells but not the RL95-2 cells (Jana et al., 1999, 2000). This difference in the response to estrogen may therefore partly explain the differing gene expression profiles induced by TCDD in MCF-7 and RL95-2 cells.

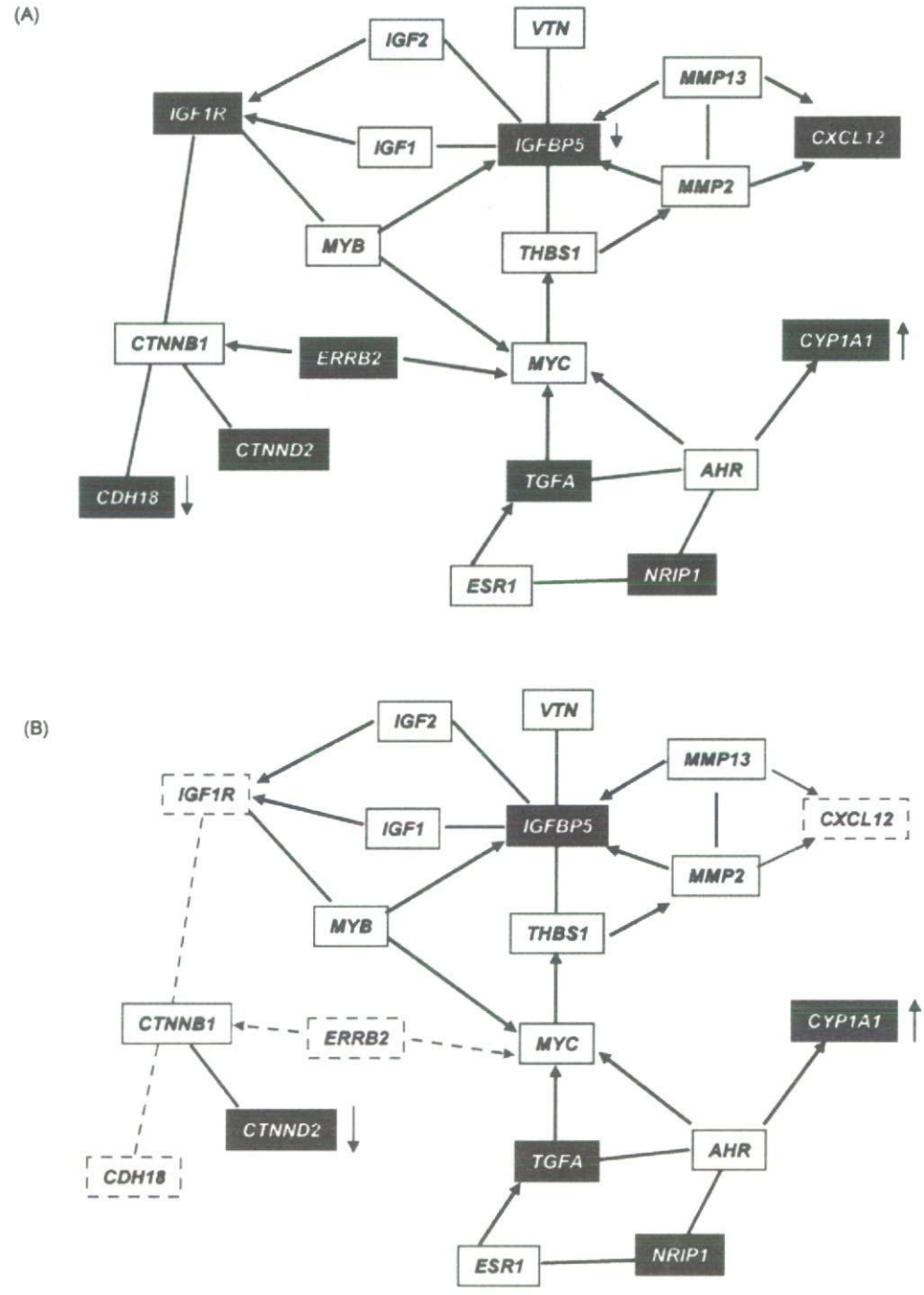

Fig. 4. Illustration of the network of the genes verified to be altered by TCDD exposure in MCF-7 cells (A) and RL95-2 cells (B). These networks display the interaction between the verified genes, showed in Table 2, and associated genes extracted from the knowledge database. The black boxes indicate the verified genes (*CDH18*, *CTNND2*, *ERBB2*, *hIRH*, *IGF1R*, *IGFBP5*, *TGFA*, *NRIP1* and *CYP1A1*). The plain lines and the lines with arrow-heads indicate binding and activating, respectively. The dotted lines and dotted boxes indicate undetected pathways in the RL95-2 cells (B).

Among the TCDD-responsive genes we found in the present study, several genes (*CTNND2*, *IGFBP5* and *CDH18*) were down-regulated by the exposure to TCDD in the MCF-7 cells, as determined by quantitative real-time RT-PCR (Table 2). The frequencies of the estrogen response element (ERE) half-sites and XRE sites in the 5′-upstream region of genes were analyzed in relation to the levels of gene expression induced by E2 or TCDD (Table 4). Interestingly, the correlation coefficient between the frequencies of the EREs plus XREs and the level of gene expression induced by TCDD exposure increased dose-dependently in the MCF-7 cells but not in the RL95-2 cells, suggesting that the increase in gene expression induced by TCDD is associated

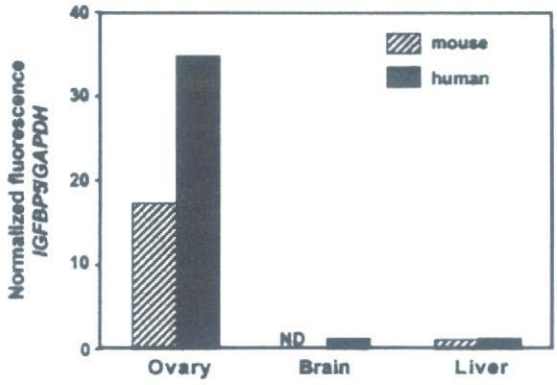

Fig. 5. *IGFBP5* gene expression in human and mouse tissues using the public data collected from GEO. Values were re-calculated as the ratio of target gene against GAPDH value after downloading raw data in microarrays. Each bar represents mean of 2–4 experiments. ND means not detected.

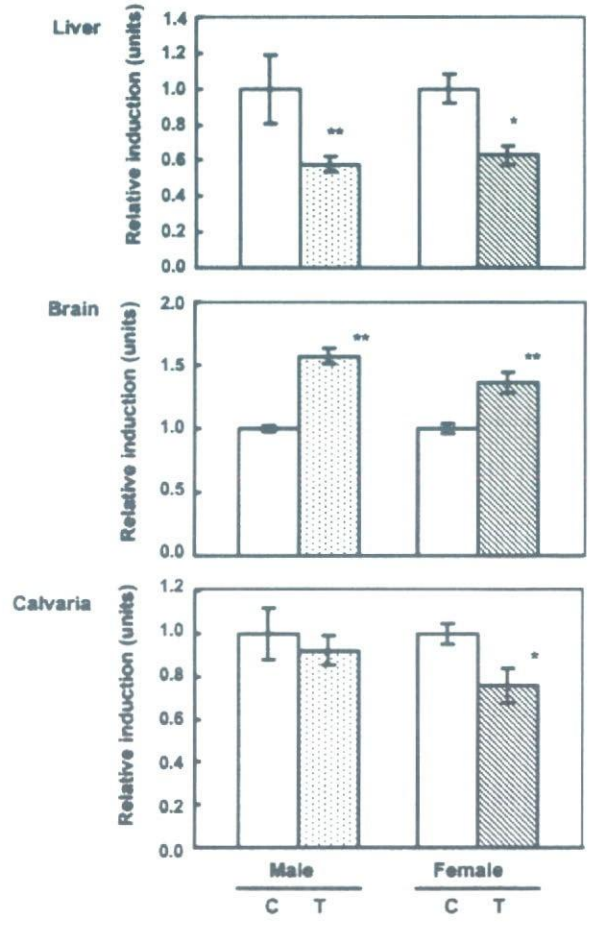

Fig. 6. Application of the identified estrogen-responsive genes induced by TCDD to an animal study model. *IGFBP5* is shown here as a typical example. *, ** at T (TCDD) indicates the difference from the control (C) at $P < 0.05$ and $P < 0.01$, respectively.

Table 4
Number of motifs for ERE, XRE, and both located in the upstream regions of genes

Gene	ERE	XRE	ERE + XRE
NAV1	36	4	40
PCYT1A	51	7	58
CTNND2	39	5	44
IGFBP5	45	2	47
CDH18	18	4	22
ERBB2	58	5	63
APM2	51	10	61
ITPK1	75	10	85
FUT8	37	1	38
CPEB2	54	15	69
KIAA0018	45	6	51
CPTI	64	16	80
KIAA0373	21	2	23
ME1	31	8	39
TFIIH	31	4	35
RCN1	49	3	52
MAN1A1	18	2	20
IGF1R	60	24	84
BSN	48	5	53
GLCM	34	4	38
Nup35	36	4	40
MAP6D1	60	8	68
NRIP1	23	2	25
TGFA	59	11	70
SLC7A5	62	16	78
CXCL12	76	12	88
CYP1A1	68	18	86

ERE (estrogen response element) constitutes half-sites of TGACC, TGAGC, TGACG, TGACA, GCTCA, GGTCA or AGTCA. XREs (xenobiotic response elements) are GGCGT, GTGCG, GCGTG and CACGC. These 5′-upstream sequences were analyzed by an algorithm in TRANSFAC, PATCH.

Table 5
Correlation coefficient between gene expression induced by TCDD and motif numbers in MCF7 and RL95-2 cells

Cell	Element	TCDD		
		0.1	1	10
MCF-7				
	ERE	0.26647	0.26488	0.26685
	XRE	0.35748	0.35592	0.35888
	ERE + XRE	0.30851	0.68819	0.99986
RL95-2				
	ERE	0.44256	0.43150	0.43307
	XRE	0.55015	0.54506	0.54450
	ERE + XRE	0.49333	0.48354	0.48455

with the summation of EREs and XREs. The sum of ERE and XRE and gene expression levels at 10 nM of TCDD showed a strong correlation coefficient of 0.99986 (Table 5). The down-regulation of the genes by TCDD, however, was not clear in this analysis. Judging from the pathway map with the common genes responsive to E2 and TCDD (Fig. 4), it appears that *CTNND2*, *IGFBP5* and *CDH18* are not directly connected to *AHR* signaling, but are mediated by *MYC*, *ERBB2* or *IGF1R* signaling.

IGFBP5 has been suggested to play a significant role in the regulation of organ function, including the development of the

central nervous system (Lee et al., 1995; Ye and D'Ercole, 1998), the involution of the mammary glands (Tonner et al., 1997) and bone physiology (Miyakoshi et al., 2001; Richman et al., 1999). *IGFBP5* has also been known to be a negative regulator of IGF1-induced proliferation of premalignant cells (Lala et al., 2002). Breast cancer tissues showed a positive correlation between ER status and IGF receptor status, and also a negative correlation between ER status and *IGFBP3*, another member of the IGFBP group (Stoll, 1997). These reports suggest that *IGFBP5* could be a good marker for TCDD-induced toxicity in ER-positive tissues.

Our previous studies showed that the effects of TCDD on sexual differentiation and brain development (Ishizuka et al., 2003; Kakeyama et al., 2003) have become one of the important environmental issues, and that TCDD affects the ER status in fetus and neonatal brain. Therefore, we focused on the expression of *IGFBP5* in mouse fetuses exposed to TCDD *in utero*. It is interesting to note that the expression of *IGFBP5* was significantly elevated in the brain but significantly decreased in the liver of fetuses exposed to TCDD *in utero* (Fig. 6). Expression of *IGFBP5* in calvaria was not affected. This variability in responsiveness of *IGFBP5* to TCDD exposure may reflect that ER status varies in different tissues. Further studies will be necessary to clarify the roles of *IGFBP5* in the effects of TCDD, and its roles in TCDD interaction with estrogen in relation to fetal brain development. We believe that the *IGFBP5* gene will provide important clues to the TCDD-estrogen interaction, since estrogen associates either directly or indirectly with the pathways of this gene in order to respond to TCDD.

In summary, we searched for TCDD-responsive genes among the known estrogen-responsive genes in MCF-7 and RL95-2 cells using cDNA microarrays. We successfully identified 32 TCDD-responsive genes out of a total of 165 estrogen-responsive genes. The gene expression profiles induced by TCDD showed significant variance at different doses and between different cell types. Furthermore, the gene *IGFBP5*, which is involved in brain development, was newly identified as a TCDD-responsive gene in the mouse. These results provide important clues as to the mechanisms underlying the effects of TCDD on brain development.

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FULL PAPER *Laboratory Animal Science*

Comparison of Estrogen Responsive Genes in the Mouse Uterus, Vagina and Mammary Gland

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ABSTRACT. Female reproductive organs are mainly regulated by estrogen and progesterone. Specifically, the uterus, vagina and mammary gland show organ-specific mitosis and morphological changes during proliferative events, such as estrous cycle, gestation and lactation. The mechanism underlying these organ-specific estrogen-dependent events is still unknown. We examined, therefore, global gene expression in the mature uterus, vagina and mammary gland of ovariectomized adult mice 6 hr after an injection of 5 μg/kg 17β-estradiol (E_2) using a microarray method in order to identify primary E_2-responsive genes. Half of the E_2 up-regulated genes in the uterus were similar to those in the vagina. E_2 up-regulated the expression of *Insulin-like growth factor 1 (Igf-1)* genes in the uterus and vagina. In the vagina, E_2 up-regulated the expression of IGF binding proteins (*Igfbp2* and *Igfbp5*). In the mammary gland, unlike the uterus and vagina, no gene showed altered expression 6 hr after the E_2 exposure. These results suggest that expression of *Igf-1* and morphogenesis genes is regulated by E_2 in an organ-specific manner, and it is supported by the results of BrdU labeling showing E_2-induced mitosis in the uterus and vagina except the mammary gland. The differences in organ specificity in response to E_2 may be attributed by differences in gene expression regulated by E_2 in female reproductive organs. The candidate estrogen-responsive genes in the uterus and vagina identified by profiling provide an important foundation understanding functional mechanisms of estrogen regulating morphogenesis and maintenance of each reproductive organ.

KEY WORDS: estrogen responsive genes, microarray, tissue specificity.

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Female reproductive organs vary their morphology during reproductive events, such as differentiation, development, estrous cycle, gestation and lactation. Estrogen is known to have differential developmental effects widely on the uterus, vagina, mammary gland, bone, liver, thymus and brain as its target organs. Although the proliferation of uterine and vaginal epithelia, and ductal elongation of mammary gland in mice could be regulated by estrogen alone [20], the mammary gland requires progesterone and prolactin in addition to estrogen to complete the architecture [12, 17, 33]. Ovariectomy and termination of weaning induce apoptosis in epithelial cells in the uterus, vagina and mammary gland [19, 30]. These estrogen target organs are controlled by estrogen receptors (ERα and ERβ) in the epithelial and stromal cells [9, 21, 24]. It is known, moreover, that growth factor(s) from the stroma are involved in epithelial proliferation of these organs [2, 6, 9]. An increase of epithelial and stromal cells in the uterus and vagina is mediated through *Insulin-like growth factor I (IGF-1)* and *Epidermal growth factor (EGF)* [3, 6, 14, 18]. Prolactin also induces expression of *Igf-2* mRNA in the developing mammary gland [13].

Tamoxifen, a selective ER modulator (SERM), acts as an estrogen agonist in the uterus and vagina, but acts as an estrogen antagonist in the mammary gland [22, 32]. The ligand-dependent effect on the mammary gland supports the idea of tissue specificity of gene expression by estrogen. Profiling of estrogen-regulated gene expression is reported recently in the estrogen target cells, tissues and organs [10, 34–36]. However, any comparisons of gene expression in the estrogen target organs have not not been reported. Gene expression reached a maximum 6 hr after E_2 administration in the uterus of ovariectomized adult mice without any histological changes [34]. Thus, we examined global gene expression 6 hr after a single injection of E_2 in order to identify early estrogen-responsive genes in the uterus vagina and mammary gland as the estrogen target organs in ovariectomized adult mice.

MATERIALS AND METHODS

Animals: C57BL/6J mice (CLEA, Tokyo, Japan) at 2 months of age, 20–23 g body weight, were used for mating. Mice were maintained under 12 hr light/12 hr dark at 23–25°C, fed with a commercial diet (CE-2, CLEA, Tokyo, Japan) and provided tap water *ad libitum*. All experiments

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and animal husbandry protocols were approved by the animal care committee of National Institutes of Natural Sciences.

Treatments: 17β-Estradiol (E_2, Sigma, St. Louis, MO) was dissolved in sesame oil. Sixty-day-old mice were ovariectomized and injected with 5 μg E_2/kg body weight after a 10-day recovery period to ensure that endogenous E_2 levels were reduced. Six hr after E_2 injection, 4 mice were killed by decapitation and the uteri, vaginae and mammary glands were collected. Four mice injected with oil vehicle only were used as controls. The tissues were pooled for DNA microarray analysis and the analyses were done on two independent experiments. Two other groups of 4 ovariectomized mice were likewise given E_2 or oil and killed 24 hr after the injection for bromodeoxyuridine (BrdU)-labeling study.

DNA microarray analysis: Total RNA was extracted from tissues (4 mice each) 6 hr after a single injection of 5 μg E_2/kg b.w. or the oil vehicle alone. Ten μg of total RNA were used to synthesize cDNA, which was then used to generate biotinylated cRNA. The cRNA was hybridized to murine U74A version 2 GeneChip expression arrays (Affymetrix, Applied Biosystems (APB), Tokyo, Japan) as described [34]. Total RNA was extracted using TRIzol reagent (Invitrogen, Tokyo, Japan) and purified with an RNeasy total RNA purification kit (Qiagen, Tokyo, Japan). Ten μg of total RNA were converted into double stranded cDNA using the Superscript Choice System (Invitrogen) with a T7-$(dT)_{24}$ primer (APB). Biotin-labeled cRNA was synthesized using the ENZO BioArray High Yield RNA transcript labeling kit (APB). The cRNA was purified by RNeasy (Qiagen). The purified cRNA was fragmented with fragmentation buffer (40 mM Tris, 100 mM K-acetate and 30 mM Mg-acetate) at 94°C for 35 min. Fragmented cRNA was mixed with hybridization buffer containing 100 mM MES [2-(N-morpholino)ethanesulfonic acid], 1 M NaCl, 20 mM EDTA, 0.01% Tween 20 and control oligonucleotides. The quality of cRNA was first assessed by analysis with Test 2 array (Affymetrix). cRNA was hybridized to Murine U74A version 2 GeneChip Expression Arrays (Affymetrix) for 16 hr at 45°C. All preparations were performed following manufacturer's instructions. Arrays were washed and stained with streptavidin-phycoerytherin, and scanned with an Argon-ion Laser Confocal Scanner (APB). Microarray analysis was performed twice on independent samples [34] and these raw data were loaded into NCBIs Gene Expression Omnibus as the dataset GSM159919-GSM159930 (GEO, http://www.ncbi.nlm.nih.gov/geo/). The putative target genes were validated by quantitative RT-PCR (QRT-PCR).

Statistical analysis: Signals in 2 experiments were detected using the robust multichip average (RMA) algorithms, and normalized using Genespring (Silicon Genetics, Redwoods City, CA). Expressed genes more than 40% raw signal of average raw signals in all genes on chip were selected as detected genes for next analysis. Selected genes showing more than 2-fold alterations by E_2 as compared to the tissue-matched oil controls were analyzed further using Genespring software.

Quantitative RT-PCR: One μg total RNA was reverse transcribed using Super Script II reverse transcriptase (Invitrogen) and random primers at 42°C for 50 min. PCR was performed using PE Prism 5,700 Sequence Detection System (PE Biosystems, Tokyo, Japan) with SYBR Green I dye (Molecular Probes, Eugene, OR) and primers selected by Primer Express *ver 1.0* (APB). Primer sets are described in Table 1.

PCR amplification was performed for 2 min at 50°C, for 10 min at 95°C and continued to 40 cycles at 95°C for 15 sec and at 60°C for 1 min. Data were normalized to ribosomal protein 28S RNA using delta Ct method for each primer set. The ratio was calculated as compared with oil controls of uterus.

BrdU-Labeling and Immunostaining: A single injection of 200 mg BrdU (Roche, Grenzacherstrasse, Switzerland)/kg b.w. was given to mice (4 mice each) 1 hr before sacrifice. Uterus, vagina and mammary gland were fixed with neutral-buffered 10% formalin, embedded in paraffin and sectioned at 6 μm. Sections were dipped in PBS and endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 30 min. After washing in 0.5% Tween 20 in PBS twice, sections were dipped in 2N HCl for 20 min, then, neutralized sections borate buffer (0.1 M NaB_4O_7, pH 8.5) twice. After Tween/PBS washing, sections were dipped in 1% BSA/PBS for 20 min. Then, sections were incubated with 1:20 anti-BrdU (Roche). Sections detected with diaminobenzidine staining were analyzed. The BrdU labeling index (%) was estimated by counting BrdU positive cells in 2,000–3,000 epithelial and 10,000–20,000 stromal cells in the uterus and vagina, and in 300–500 epithelial or stromal cells in the mammary glands.

RESULTS

Gene expression in the uterus, vagina and mammary gland exposed to E_2: Approximately 12,400 genes were ana-

Table 1. Primer sets for QRT-PCR

Genebank accession No.	Name	Forward primer	Reverse primer
M13500	Klk 1	ATGGATGGAGGCAAAGACACTT	ACCTTGGAGAACACCATCACAGA
X04480	IGFI	CTACAAAAGCAGCCCGCTCTA	TCCTTCTGAGTCTTGGGCATGT
X81580	IGFBP2	GGAACATCTCTACTCCCTGCACAT	TTGTACCGGCCATGCTTGT
NM_010518	IGFBP5	GGTGTGTGGACAAGTACGGAATGA	ACGTTACTGCTGTCGAAGGCGT
X00525	Ribosomal 28S	AGACCGTCGTGAGACAGGTTAGTT	GCAGGATTACCATGGCAACAA

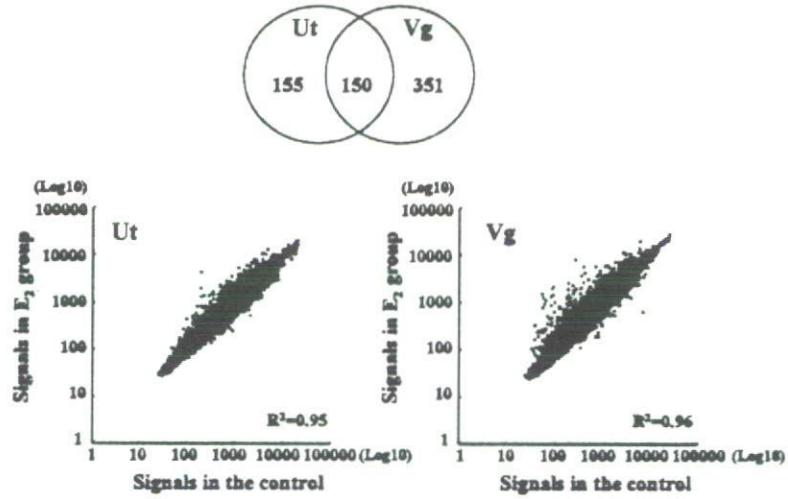

Fig. 1. Gene expression profiles in the uterus and vagina 6 hr after a single injection of 5 μg E_2/kg body weight. The Venn diagram indicatess detected genes in the oil-controls. Scatter plots indicate gene expression levels and the correlations between oil and E_2 groups in all genes. R_2 is correlation between oil and E_2 groups. Ut, uterus; Vg, vagina.

lyzed in the uterus, vagina and mammary gland. The total normalized signals between controls and E_2-exposed mice exhibited high correlations (R^2=0.95–0.96) (Fig. 1). The total number of genes showing at least 2-fold expression change 6 hr after a single injection of E_2 was 656 in all samples (Fig. 1). E_2 did not alter any gene expression more than 2-fold change in the mammary gland in the present study (data not shown). Genes showing organ-specific expression were 155 and 351 in the uterus and vagina, respectively (Fig. 1). Among them, 150 genes were regulated commonly in the uterus and vagina (Fig. 1).

In the uterus, 228 genes were up-regulated and 77 genes were down-regulated by E_2 as compared to the controls (Fig 2). In the vagina, 446 genes were up-regulated and 35 were down-regulated by E_2. In the uterus, 63% of E_2 up-regulated genes were overlapped those in the vagina. E_2 down-regulated common genes in the uterus and vagina were only 6 (Fig. 2). We further analyzed genes related to cell growth and organogenesis to find tissue-specific genes. E_2-responsive genes related to development, cell growth and apoptosis in the uterus and vagina were listed in Table 2.

Expression of Igf-1 family and Kallikrein 1 genes: Since clustering analysis revealed many E_2-regulated genes, we compared expressions of *Kallikrein 1 (Klk1)* genes and *Igf-1* family genes in each tissue using QRT-PCR.

In the controls, expression of *Klk1* mRNA was similar between uterus and vagina, while those of *Igf-1* and *Igfbp5* were lower in the vagina than in the uterus (Fig. 3). In the mammary gland, unlike the uterus and vagina, expressions of all mRNAs examined were very low or undetectable in the ovariectomized mice with or without E_2. In the uterus and vagina, expression of *Igf-1* mRNA was markedly increased by E_2. However, expressions of *Igfbp2* and *Igfbp5*

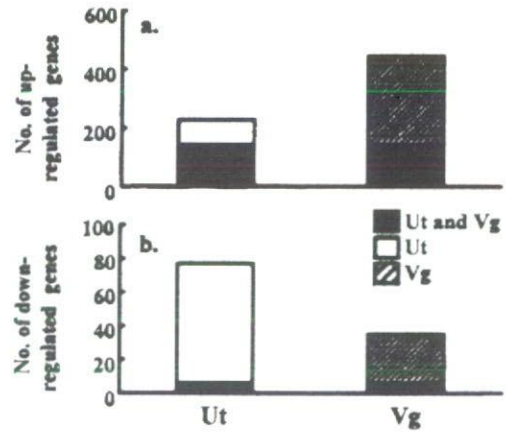

Fig. 2. Number of estrogen up-regulated genes (a) and down-regulated genes (b) in the uterus (Ut) and vagina (Vg). "Ut and Vg" indicates genes showing commonly altered expression by E_2 both in the uterus and vagina, while Ut and Vg indicate organ specific genes, respectively.

mRNAs were increased by E_2 in the vagina (Fig. 3). *Klk1* mRNAs was significantly increased as by E_2 in the vagina.

BrdU incorporation in the uterus, vagina and mammary gland, vagina 24 hr after E_2 injection: BrdU labeled cells were barely detected in epithelial cells of the uterus and mammary gland (0% and 0.05%) in the controls, as compared to those of the vagina (0.24%) in the controls (Fig. 4). The BrdU labeling index in the epithelial cells of the uterus and vagina was significantly increased 24 hr after the E_2 injection as compared with each control. In the mammary gland, however, BrdU-positive cells were not evident in the

Table 2. List of estrogen responsive genes related development, cell growth and apoptosis in the uterus and vagina by microarray analysis

Accession No.	Name	Ratio Ut	Ratio Vg
M60523	Inhibitor of DNA binding 3	**0.42**	0.71
AI840339	Ribonuclease, RNase A family 4	**0.42**	1.32
AA838868	Latent transforming growth factor beta binding protein 4	**0.42**	1.07
L31532	B-cell leukemia/lymphoma 2	**0.43**	0.59
X70298	SRY-box containing gene 4	**0.45**	**0.36**
U88567	Secreted frizzled-related protein 2	**0.45**	0.89
AI843106	Sestrin 1	**0.45**	**0.43**
AW123618	Frizzled homolog 2 (Drosophila)	**0.46**	0.64
AV092014	Peptidoglycan recognition protein 1	1.54	**0.37**
AI834950	Nuclear receptor subfamily 1, group D, member 1	0.58	**0.37**
AF076482	Peptidoglycan recognition protein 1	1.10	**0.40**
AF056187	IGF1 receptor	0.59	**0.41**
AF099973	Schlafen 2	1.02	**0.46**
X07750	Thyroid hormone receptor alpha	0.72	**0.49**
X81580	Insulin-like growth factor binding protein 2 (IGFBP2)	1.00	**2.00**
AW123099	Chromosome segregation 1-like (S. cerevisiae)	1.76	**2.01**
AF003695	Hypoxia inducible factor 1, alpha subunit	1.57	**2.01**
AI747899	Phosphatidylinositol transfer protein, beta	1.24	**2.02**
X03491	Keratin complex 2, basic, gene 4	1.03	**2.08**
X62154	similar to DNA replication licensing factor MCM3 (P1-MCM3)	1.48	**2.09**
AW124529	Tumor necrosis factor superfamily, member 5-induced protein 1	0.97	**2.16**
AF011644	CDK2 (cyclin-dependent kinase 2)-associated protein 1	1.81	**2.19**
AW048763	NMDA receptor-regulated gene 1	1.66	**2.20**
D49382	Septin 2	1.48	**2.20**
AW125478	HtrA serine peptidase 1	1.46	**2.22**
X02452	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	1.95	**2.26**
D10214	Prolactin receptor	1.12	**2.30**
AF014117	Glial cell line derived neurotrophic factor family receptor α 1	1.70	**2.34**
AF041476	Actin-like 6A	1.96	**2.36**
M73329	Protein disulfide isomerase associated 3	1.92	**2.38**
U35846	Apoptosis inhibitor 5	1.76	**2.43**
AF058798	Stratifin	1.32	**2.44**
D12780	S-adenosylmethionine decarboxylase 1	1.89	**2.47**
Z23077	S-adenosylmethionine decarboxylase 1 and 2	1.74	**2.49**
J04766	Plasminogen	1.02	**2.62**
D00613	Matrix Gla protein	0.63	**2.68**
X59846	Growth arrest specific 6	1.14	**2.79**
L12447	Insulin-like growth factor binding protein 5 (IGFBP5)	1.18	**3.09**
AI847054	Phosphatidic acid phosphatase type 2B	1.69	**3.32**
M35523	Cellular retinoic acid binding protein II	1.02	**3.47**
AI837110	Protein arginine N-methyltransferase 1	1.86	**3.49**
M74570	Aldehyde dehydrogenase family 1, subfamily A1	1.31	**4.11**
AV028204	Plasminogen	0.75	**4.38**
AI553024	Zinc finger and BTB domain containing 16	0.52	**4.44**
AW124889	Aldehyde dehydrogenase 18 family, member A1	**2.02**	1.45
AF100777	WNT1 inducible signaling pathway protein 1	**2.03**	1.38
AW260482	NMDA receptor-regulated gene 1	**2.10**	1.50
X13986	Secreted phosphoprotein 1	**2.17**	1.23
U00937	Growth arrest and DNA-damage-inducible 45 alpha	**2.18**	**3.08**
AF079528	Neuropilin 1	**2.20**	1.57
AB003502	G1 to S phase transition 1	**2.21**	1.66
D63784	DnaJ (Hsp40) homolog, subfamily C, member 2	**2.24**	1.96
AI645561	NMDA receptor-regulated gene 1	**2.29**	**2.10**
U53208	DnaJ (Hsp40) homolog, subfamily C, member 2	**2.34**	1.79
AW046181	Serum/glucocorticoid regulated kinase	**2.38**	1.02
AA529583	Mortality factor 4 like 2	**2.39**	**3.56**
V00756	Interferon-related developmental regulator 1	**2.40**	**2.63**
U88327	Suppressor of cytokine signaling 2	**2.42**	1.07
AB012276	Activating transcription factor 5	**2.43**	1.96
M13500	Kallikrein 1	**2.43**	0.92

Table 2. Continued

Accession No.	Name	Ratio Ut	Ratio Vg
U84411	Protein tyrosine phosphatase 4a1	**2.46**	**2.34**
AW048937	Cyclin-dependent kinase inhibitor 1A (P21)	**2.51**	**2.45**
AF055638	Growth arrest and DNA-damage-inducible 45 gamma	**2.53**	**5.03**
AI596034	Receptor tyrosine kinase-like orphan receptor 2	**2.64**	**3.31**
V00727	FBJ osteosarcoma oncogene	**2.66**	**3.22**
L32751	RAN, member RAS oncogene family	**2.77**	**2.36**
D50086	Neuropilin 1	**2.95**	1.27
X99273	Aldehyde dehydrogenase family 1, subfamily A2	**2.99**	1.90
M63801	Gap junction membrane channel protein alpha 1	**3.02**	1.98
AI785289	Guanine nucleotide binding protein-like 3 (nucleolar)	**3.82**	**2.57**
X04480	Insulin-like growth factor 1 (IGF-I)	**4.67**	**4.82**
U83902	MAD2 (mitotic arrest deficient, homolog)-like 1 (yeast)	**4.75**	**4.57**
AF053232	Nucleolar protein 5	**5.09**	**3.32**
X69620	Inhibin beta-B	**10.29**	**7.60**

Bold means more than 2-fold alterations by E_2.

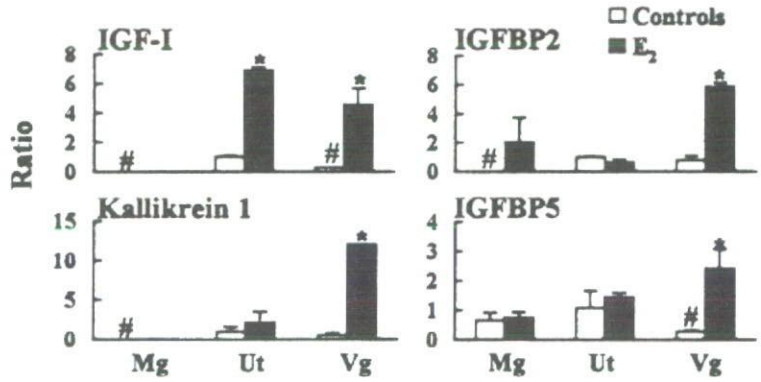

Fig. 3. Ratio of mRNA gene expressions of *Igf-1* family and *Kallikrein 1* in the three organs 6 hr after the E_2 injection using QRT-PCR. *, $P<0.05$ v.s. the control of each tissue; #, $P<0.05$ v.s. the control uterus.

epithelium 24 hr after the E_2 injection. The index was significantly increased by E_2 in the uterine stroma (Fig. 4).

DISCUSSION

Estrogen regulates mitosis and morphological changes in female reproductive organs during proliferative events, such as estrous cycles, gestation and lactation. In order to understand the underlying mechanisms of estrogen functions in reproductive organs, detection of estrogen responsive genes in each reproductive organs are is essential. Effects of estrogen are different in each reproductive organ, therefore we investigated a global gene expression in uterus, vagina and mammary gland after a single injection of E_2.

In the present study, BrdU labeled cells were remarkably increased in the uterine and vaginal epithelia, and in the uterine stroma after the E_2 injection, but not in any parts of the mammary gland. Gene expression in response to estrogen is different among these organs. *Igf-1* is a key epithelial mitogen induced by estrogenic chemicals [28, 31], whereas IGFBP prevents signal pathway by binding to *Igf-1*, and inhibits phosphorylation of Insulin receptor substrate-1 (IRS-1), Phosphatidylinositol 3-kinase (PI3K), Protein kinase B (PKB) and Forkhead transcription factors (FKHRL1) [23]. *Igfbp2* and *Igfbp5* promote apoptosis in the prostate cancer cells and mammary gland cells [23, 26, 27, 29]. Hence, proliferations of uterine and vaginal cells was appear to be regulated by estrogen via *Igf-1* and receptor complex, and its modulator. In the present study, estrogen increased *Igf-1* mRNA and mitosis in the uterus and vagina. However, the *Igf-1* modulators and *Igfbp* mRNAs were also increased in E_2-exposed vagina. This may be accounted for by the suppression of stromal cell proliferation caused by increase of *Igfbp* mRNAs in the stroma rather than the epithelium. Up-regulation of *Klk1* was reported by E_2 in the uterus [34]. *Klk* plays an important role for the release of bradykinin from kininogen, activation of growth factors and alteration of the extracellular matrix in the uterine epithelium [7]. *Klk* is regulated hormonally [7, 8] and the gene expression was found in human ovarian, prostate

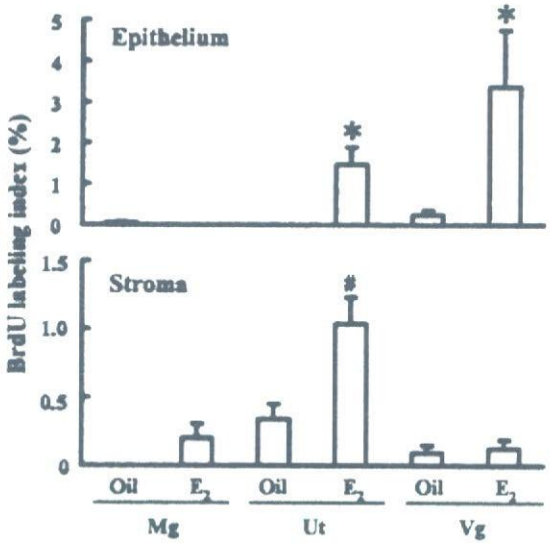

Fig. 4. BrdU labeling index (positive cells/counted cells, %) in epithelium and stroma of the mammary gland, uterus and vagina. Mg, mammary gland; Ut, uterus; Vg, vagina; *, $P<0.05$ v.s. the control of each tissue.

and breast cancer cells and in mouse vagina [15, 25, 37]. We found the up-regulation of *Klk1* gene expression in the vagina by QRT-PCR. Thus, *Klk1* may be related to epidermal proliferation and their expressions can be used for markers of acute response to estrogen in the uterus and vagina.

We found that estrogen regulated genes were markedly limited in the mammary gland as compared to those in the uterus and vagina 6 hr after the E_2 exposure. Global gene expression in E_2-exposed mammary gland has not been reported. Only gene expression in human breast cancer MCF-7 cells treated E_2 *in vitro* was reported [4, 10, 11]. MCF-7 cells treated with E_2 revealed the major down-regulation (70%) of gene expression including transcriptional repressor, antiproliferative and proapoptotic genes, such as *Bcl-2, Cyclin G2* and *TGF-β* family [11]. Moreover, using the serial analysis of gene expression (SAGE) method, 3 up-regulated genes were reported in MCF-7 cells 10 hr after E_2 treatment *in vitro* [4]. The genes reported as E_2 down-regulated genes in MCF-7 cells were not found in the mammary gland in the present study. MCF-7 cells are a single species of mammary cancer cells and show precise response of time- and dose-dependent proliferation to estrogen. Normal mammary gland may need longer than 6 hr to respond to E_2 *in vivo*. Mammary gland has various types of cells, such as epithelial cells stromal cells and adipocytes. Thus, we need further precise experiment to understand estrogen responsive genes in the mammary gland.

Although the mammary gland is known to be one of the target organs of estrogen, ER-α knockout (αERKO) mice showed proliferation and morphogenesis of the mammary gland in adulthood [24]. The mammary gland seems to be regulated by progesterone and prolactin rather than estrogen [5, 12, 15, 17, 24, 33]. The up-regulation of gene expression, such as *IRS-1, Msx-2, C/EBPβ* and *Stat5*, by progesterone was reported in human breast cancer cells [15]. Prolactin induced expression of *Igf-2* mRNA in the developing mammary gland [13]. Thus, mammary gland is possibly regulated largely by progesterone and/or prolactin. This may account for no expression of estrogen responsive genes observed 6 hr after the E_2 exposure and absence of definite mitogenic response in the mammary gland of ovariectomized adult mice 24 hr after the E_2 exposure.

In conclusion, E_2 regulates expression of a number of genes in the vagina and uterus, but not in the mammary gland. Half of E_2-regulated genes in the uterus were in common with the vagina including *Kallikrein* and *Igf* family genes. Differences in expression of these genes in response to E_2 may be leased on the tissue specificity to estrogen exposure. The candidate estrogen responsive genes in the uterus and vagina identified by profiling provide an important foundation to understand functional mechanisms of estrogen regulating morphogenesis and maintenance of the reproductive organ.

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Gene Expression Change in the Müllerian Duct of the Mouse Fetus Exposed to Diethylstilbestrol *In Utero*

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In utero **exposure to diethylstilbestrol (DES) induces various abnormalities in the Müllerian duct of the mouse. In order to understand the underlying molecular mechanisms associated with DES-induced abnormalities of the Müllerian duct, gene expression was examined on Gestation Day (GD) 19 in mouse fetuses exposed to DES (67 μg/kg body weight) from GDs 10 to 18. Microarray analysis revealed that 387, 387, and 225 genes were upregulated and 177, 172, and 75 genes were downregulated by DES in the oviduct, uterus, and vagina, respectively. DES exposure *in utero* commonly upregulated 72 genes and downregulated 15 genes in these three organs. The present study demonstrated that organ-specific gene expression patterns in the mouse Müllerian duct were altered by *in utero* DES exposure. DES-induced changes in expression of genes such as *Dkk2*, *Nkd2*, and *sFRP1* as well as changes in genes of the Hox, Wnt, and Eph families in the female mouse fetal reproductive tract could be the basis for various abnormalities in reproductive tracts following exposure to this estrogenic drug.** Exp Biol Med 232:503–514, 2007

Key words: microarray; gene expression; diethylstilbestrol; oviduct; uterus; vagina; mouse

Introduction

Prenatal diethylstilbestrol (DES) exposure induces persistent malformations of male and female reproductive organs in mice. Female mice exposed perinatally to DES showed noncoiled oviducts, uterine metaplasia, disorganization of the uterine circular muscles, and ovary-independent vaginal epithelial stratification and cornification (1–6).

DNA microarray has been successfully used to analyze estrogen-responsive genes in the mouse uterus and vagina and to begin to identify those genes possibly related to persistent vaginal proliferation induced by neonatal DES exposure (7–12). Therefore, we studied global gene expression, including signal transduction and organogenesis genes in the Müllerian duct, after DES exposure *in utero* using microarray at Gestation Day (GD) 19, and we selected several genes for further study. We focused on the expression of ephrin, Eph family, Wnt, Wnt-antagonists, and Hoxa genes, since they showed altered expression by *in utero* DES exposure in the present study.

Hox genes, expressed in spinal cord, limb, and reproductive tracts, determine anterior to posterior body axis patterning. Hox genes are expressed in the Müllerian duct along its axis, with genes *a-9*, *a-10*, *a-11*, and *a-13* exhibiting anterior to posterior expression pattern at GD

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Table 1. Sequences for Primers Using Quantitative RT-PCR

GenBank accession no.	Name	Sequence of forward primer	Sequence of reverse primer	Length (bp)
AB005457	Hoxa9	CTGACTGACTATGCTTGTGGTTCTC	TCTCGGCATTGTTTTCGGA	84
L08757	Hoxa10	ACAATGTCATGCTCGGAGAGC	TGATGAGCGAGTCGACCAAA	61
U20370	Hoxa11	TTCTGCCACAGGCTTTCGA	TAGTCGGAGGAAGCGAGGTTT	72
U59322	Hoxa13	TGTACAGCATTCGTGGCAAAG	ACAGGCGACAGCTCAATGTG	69
BC019952	Nkd2	ACATTTGATGCAGCTGATGGTT	TGGATGACACAGGAGCACGT	50
NM_020265	Dkk2	TGTCTGAAGCACAGGCTGGAT	CTTCTGGAGCCTCTGATGGC	50
U88566	sFRP1	CCAACAGCCTCACTTTGTAATTCC	CCCTGTCTTATGCTGCTGTTCTTT	60
NM_009523	Wnt4	TGTACCTGGCCAAGCTGTCAT	TTTCTCGCACGTCTCCTCTTC	58
NM_009524	Wnt5a	AGTTTCACTGGTGCTGCTATGTCA	CCACAATCTCCGTGCACTTCT	50
NM_009527	Wnt7a	TTACACAATAACGAGGCGGGT	ACACTCCAGCTTCATGTTCTCCT	56
BC026153	Eph receptor A7	TGTTAAACCAGTGATGTTTTC	CCCATCTGAGGGAAGTCCTTAA	50
BB292785	Eph receptor A3	TTTTGTTACAGCCAAGTGCCAA	TTTCTTACTGCTGACAATTTGCAAT	51
BB706548	Eph receptor A4	AATTTGGGCAGATCGTCAACA	TGTTGGGATTGCGGATGAGT	50
U30244	ephrin B2	CTACAGCTTGTTTAACGGCAGTGT	TTTCCTCATTACAGTGCAAAGGG	50
U67771	Ribosomal protein L8	ACAGAGCCGTTGTTGGTGTTG	CAGCAGTTCCTCTTTGCCTTGT	84

15.5 (13). With the positional expression of Hox genes along the anterior to posterior axis, the Müllerian duct differentiates into three distinct reproductive organs: oviduct, uterus, and upper vagina. Lack of positional Hox gene expression results in reproductive abnormalities due to the loss of organ specificity (14, 15). Lack of *Hoxa-13* expression, for example, resulted in the loss of the caudal Müllerian duct (16). DES repressed the expression of *Hoxa-10* and *a-11* in the mouse uterus at GD 17, which is associated with reduced reproductive performance, including embryo implantation, in adult offspring (17, 18). The lack of Hox gene expression leads to disturbed patterning in the body axis, primarily in limbs, spinal cord, hindbrain, and reproductive tracts (14, 15, 19–22).

Hoxa-13 knockout mice showed the downregulation of the *Eph receptor A7* and inhibition of mesenchymal cell adhesion and apoptosis in limbs (23), suggesting possible functions in reproductive tracts. Eph receptors and ephrin ligands regulate cell–cell communication, cellular movement, and mitogenic responses during development *via* the ERK/MAPK cell signaling pathway (24–26).

Epithelial–mesenchymal differentiation in the Müllerian duct is regulated by Wnt signaling correlated with expression of Hox genes. In female reproductive organs, *Wnt-4*, *–5a*, and *–7a* are expressed during development (27). Lack of *Wnt-7a* expression induced uterine metaplasia, an abnormality similar to that reported in mice exposed to DES *in utero* (28). Wnt-7a maintains the expressions of *Hoxa-10* and *a-11*; thus, lack of Wnt-7a is considered to disrupt segmentation of the reproductive organs. Moreover, Wnt-4 is essential for the early development of female reproductive tracts (29).

Wnt signaling regulates vaginal growth and differentiation through epithelial–mesenchymal interactions. Perinatal DES exposure reduced expression of *Wnt-7a*, which resulted in uterine metaplasia (30), suggesting the presence of suppression factors for Wnt signaling. Secreted frizzled related protein (sFRP) was downregulated by 17β-estradiol treatment in the adult mouse uterus and was shown to compete with Wnt and frizzled (Fz) receptors (31, 32). Dicknock (Dkk; Refs. 33–38) and Naked cuticle (Nkd; Ref. 39) have been reported as Wnt antagonists in various developing organs.

In order to understand the molecular mechanisms underlying the reproductive tract abnormalities reported in female mice exposed prenatally to DES, we analyzed expression changes in Eph family, Wnt, Wnt-antagonists, and Hoxa genes following exposure to this pharmaceutical estrogen.

Materials and Methods

Animals. Mice of the ICR/Jcl strain were kept under a 12:12-hr light:dark cycle at 23°C–25°C, were given a commercial diet (CE-2; CLEA, Tokyo, Japan) and tap water *ad libitum*. All experiments and animal husbandry protocols were approved by the animal care committee of the National Institutes of Natural Sciences. The day on which a vaginal plug was found was considered GD 0. DES (Sigma Chemical Co., St. Louis, MO) was dissolved in sesame oil. Pregnant mice were given daily subcutaneous injections of DES (67 μg/kg maternal body weight) or the oil vehicle alone from GDs 10 to 18 as described previously (40). These experiments were repeated three times.

DNA Microarray Analysis. Total RNA was extracted from the oviduct, uterus, and vagina (7–12 pups/3 litters) at GD 19 using TRIzol (Invitrogen, Tokyo, Japan) and purified with the RNeasy mini kit (Qiagen, Tokyo, Japan). Total RNA quality was examined with a Bioanalyzer 2100 (Agilent Japan, Tokyo, Japan). Purified RNA was processed according to the manufacturer's protocol to prepare the labeled cRNAs, which were hybridized to the mouse expression array 430A (Affymetrix Japan, Tokyo, Japan). Hybridization, washing, and scanning were performed according to the manufacturer's protocol as

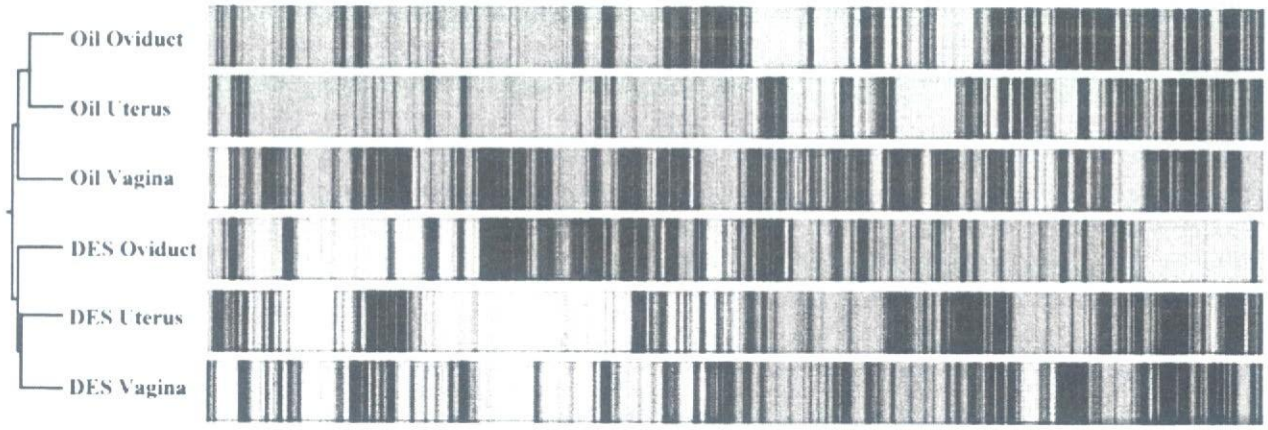


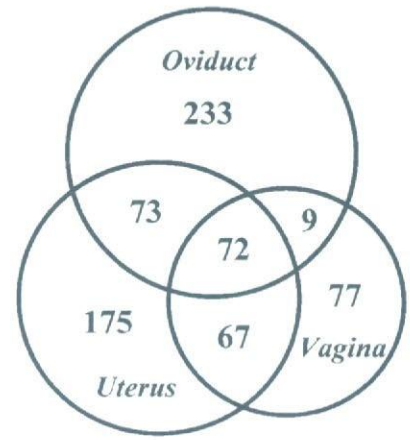
Figure 1. Clustering analysis of DES-responsive genes in the mouse oviduct, uterus, and vagina at GD 19. Each colored bar indicates the expression level of one gene: red, induction; green, repression; yellow, average expression in six groups; gray, not detected. Only genes showing more than a 2-fold change in expression following *in utero* DES exposure are shown here.

described (7). Microarray analysis was performed in triplicate using three different samples.

Data Analysis. Scanned data were analyzed with GeneChip Suit Analysis Software version 5.0 (Affymetrix Japan) to obtain the average intensity of each cell corresponding to each oligonucleotide probe. The averaged fluorescence intensity (2500) of each probe was further analyzed by dChip, a model-based expression analysis program (41), and expression levels were estimated. The PM-only model was used for the analysis, and the estimated values were transferred to the GeneSpring software program (Silicon Genetics, Redwood City, CA) and analyzed. To calculate changes in expression, genes for which average expression levels were more than 1000 fluorescence intensity units under at least one experimental condition were selected, and the average expression values of the treated samples was divided by those from control samples. For the clustering analysis, genes activated more than 2-fold by DES were selected, and similarities between experiments and expression levels were measured by standard correlation using the GeneSpring program as described (7–10). Gene expression changes were estimated by assigning the value of the control uterus as one. These selected genes were listed on http://www.nibb.ac.jp/bioenv1/suzuki/suzukidata004.html. These raw data were loaded into the National Center for Biotechnology Information's Gene Expression Omnibus as the dataset GSE1886 (GEO: http://www.ncbi.nlm.nih.gov/geo/). Categories in DES-regulated genes were determined from the GEO database. Putative target genes were validated by quantitative real-time polymerase chain reaction (Q-PCR).

Quantitative Real-Time PCR. Total RNA was purified as described above. Complementary DNA was synthesized from purified total RNA with Superscript II RT(-) (Invitrogen) and random primers at 42°C for 60 mins. PCR reactions were performed in the PE Prism 5700 Sequence Detection System (PE Biosystems, Tokyo, Japan) with

a. Up-regulated genes



b. Down-regulated genes

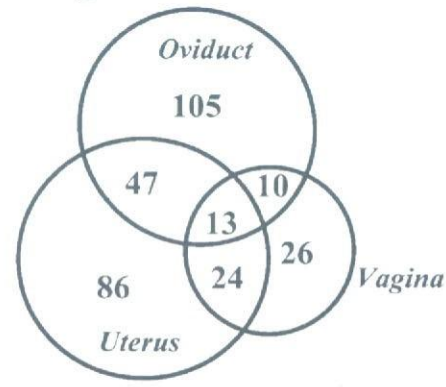


Figure 2. Venn diagrams of the number of upregulated and downregulated genes in the GD 19 oviduct, uterus, and vagina exposed to DES at GDs 10–18. (a) Number of upregulated genes by DES in the Müllerian duct. The number of DES-upregulated genes was the smallest in the vagina. (b) Number of downregulated genes by DES in the Müllerian duct.

Table 2. DES-Regulated Genes Related to Signal Transduction and Organogenesis in the Müllerian Duct at GD 19[a]

Gene accession no.	Fold change			Name
	Ovi	Ut	Vg	
BG066967	10.7	2.7	2.1	RAB20, member RAS oncogene family
BQ174703	2.5	5.1	NC	Double cortin and calcium/calmodulin-dependent protein kinase-like 1
NM_010557	5.4	7.6	NC	Interleukin-4 receptor, alpha
NM_013769	3.2	3.4	NC	Tight junction protein 3
NM_008397	2.6	3.0	NC	Integrin alpha 6
BC027196	2.3	2.9	NC	RIKEN cDNA D530020C15 gene
BC003714	2.6	2.0	NC	Calcium and integrin binding 1 (calmyrin)
NM_080795	NC	2.7	2.3	Ligand of numb-protein X 2
AF039601	NC	2.2	2.2	Transforming growth factor-beta receptor III
AA717838	NC	3.2	2.2	Interleukin-6 signal transducer
NM_021475	7.8	NC	NC	ADAM-like, decysin 1
NM_013602	5.0	NC	NC	Metallothionein 1
U42467	3.7	NC	NC	Leptin receptor
NM_008935	3.1	NC	NC	Prominin 1
M68513	2.3	NC	NC	Eph receptor A3
NM_133485	2.3	NC	NC	Protein phosphatase 1, regulatory (inhibitor) subunit 14c
BC011193	2.0	NC	NC	Prostaglandin E receptor 4 (subtype EP4)
NM_029716	2.0	NC	NC	RIKEN cDNA 0710001E19 gene
AF440694	NC	3.1	NC	Insulin-like growth factor–1 (IGF-I)
BC026642	NC	3.0	NC	Expressed sequence AW049765
NM_016798	NC	2.9	NC	PDZ and LIM domain 3
NM_007429	NC	2.7	NC	Angiotensin II receptor, type 2
NM_009365	NC	2.7	NC	Transforming growth factor-beta 1–induced transcript 1
AF350047	NC	2.6	NC	Regulator of G-protein signaling 3
BE307478	NC	2.5	NC	Ectonucleoside triphosphate diphosphohydrolase 1
AI788797	NC	2.1	NC	Utrophin
NM_019417	NC	2.1	NC	Reversion-induced LIM gene
NM_025278	NC	2.0	NC	Guanine nucleotide (G) binding protein gamma 12
NM_007706	NC	2.0	NC	Suppressor of cytokine signaling 2
BC015254	NC	NC	2.3	Chemokine orphan receptor 1
BB447551	NC	NC	2.3	GATA binding protein 5
BB751088	NC	NC	2.0	G-protein–coupled receptor 49
NM_011196	0.2	0.3	0.2	Prostaglandin E receptor 3 (subtype EP3)
NM_013869	0.3	0.2	0.5	Tumor necrosis factor receptor superfamily, member 19
BC026153	0.3	0.2	0.5	Eph receptor A7
BM946869	0.2	0.2	NC	Stathmin-like 2
BB751088	0.2	0.4	NC	G-protein–coupled receptor 49
NM_019583	0.5	0.3	NC	Interleukin-17 receptor B
BB453355	0.5	0.5	NC	Ephrin B2
AK018789	0.4	NC	NC	Neurotrophic tyrosine kinase, receptor, type 2
AF209905	0.4	NC	NC	Calcitonin receptor-like
NM_133248	0.4	NC	NC	Glomulin, FKBP-associated protein
AW493905	0.5	NC	NC	G-protein–coupled receptor 23
NM_007936	0.5	NC	NC	Eph receptor A4
AK018032	0.5	NC	NC	SH3-domain kinase binding protein 1
AK018504	0.5	NC	NC	Ras association (RalGDS/AF-6) domain family 2
NM_013518	NC	0.4	NC	Fibroblast growth factor 9 (Fgf9)
NM_008016	NC	0.4	NC	Fibroblast growth factor inducible 15
U38501	NC	0.5	NC	Guanine nucleotide–binding protein, alpha inhibiting 1
BC005799	NC	NC	0.5	RIKEN cDNA 5830484J08 gene
BC010581	NC	NC	0.4	Stathmin 1
BC005475	NC	NC	0.4	RIKEN cDNA E430018M08 gene
Organogenesis				
NM_007921	2.7	4.6	2.2	E74-like factor 3
NM_015814	4.2	2.2	NC	Dickkopf homolog 3 (*Xenopus laevis*) (Dkk3)
NM_020265	2.8	4.1	NC	Dickkopf homolog 2 (*Xenopus laevis*) (Dkk2)
L13204	5.8	NC	NC	Forkhead box J1 (Foxj1)
NM_010135	4.4	NC	NC	Enabled homolog (Drosophila)
AK006314	2.4	NC	NC	Spermatid perinuclear RNA binding protein

Table 2. (Continued)

Gene accession no.	Fold change[a] Ovi	Ut	Vg	Name
NM_024226	2.3	NC	NC	Reticulon 4
AW538200	2.1	NC	0.3	Filamin, beta
BM119387	2.1	NC	NC	Villin 2
AI462296	NC	2.5	2.2	Forkhead box O1
BB151515	NC	6.0	2.2	Nerve growth factor receptor
BB759833	NC	NC	3.5	Forkhead box C1
NM_009523	NC	NC	2.4	Wnt-4
BF141691	0.3	0.2	0.3	Naked cuticle 2 (Nkd2) homolog (Drosophila)
L42114	0.1	0.5	NC	Growth differentiation factor 10
NM_009152	0.3	0.3	NC	Semaphorin 3A
BQ176610	0.4	0.3	NC	Semaphorin 5A
NM_009526	0.5	0.4	NC	Wnt-6
BC019150	0.3	0.4	NC	Hoxd-9
BI658627	0.4	0.5	NC	Serected frizzled-related sequence protein 1 (sFRP1)
AK004683	NC	0.1	0.1	Wnt-7a
NM_013601	NC	0.5	0.4	Homeobox, msh-like 2 (Msx2)
BC013463	0.1	NC	NC	Hoxd-10
NM_010450	0.5	NC	NC	Hoxa-11
AK007893	0.2	NC	NC	Sclerostin domain containing 1
D78264	0.3	NC	NC	Olfactomedin 1
NM_010698	0.5	NC	NC	LIM domain binding 2
BC016426	NC	0.1	NC	Homeobox, msh-like 1 (Msx1)
NM_021457	NC	0.4	NC	Frizzled homolog 1 (Drosophila)
AK019458	NC	0.4	NC	Myeloid/lymphoid or mixed lineage-leukemia translocation to 3 homolog (Drosophila)
NM_009519	NC	0.5	NC	Wnt-11
AW107802	NC	0.5	NC	Glypican 3
NM_013598	NC	0.5	NC	Kit ligand
NM_010496	NC	NC	0.4	Inhibitor of DNA binding 2
BG065227	NC	NC	0.5	Tripartite motif protein 37
AF153440	NC	NC	0.5	BMP and activin membrane-bound inhibitor, homolog

[a] Fold change means ratio vs. organ-matched oil controls. NC means no change included with less than 2-fold change and more than 0.5-fold change. Ovi, oviduct; Ut, uterus; Vg, vagina.

SYBR-Green PCR core reagents (Applied Biosystems Japan, Tokyo, Japan) in the presence of the appropriate primers according to the manufacturer's instructions. The primers were chosen to amplify short PCR products of less than 100 base pairs, and their sequences are listed in Table 1.

Each PCR amplification was performed in triplicate using the following conditions: 2 mins at 50°C and 10 mins at 95°C, followed by a total of 40 two-temperature cycles (15 secs at 95°C and 1 min at 60°C). Model 7000 software (Applied Biosystems, Foster City, CA) was used to construct amplification plots from extension-phase fluorescent emission data collected during PCR amplification. Threshold ($C\tau$) values were calculated by determining the point at which fluorescence exceeds a threshold limit.

Gene expression levels were normalized to the expression levels of ribosomal protein L8 mRNA (U67771), and changes in concentration were calculated. Gel electrophoresis and melting curve analyses were performed to confirm correct amplicon size and the absence of nonspecific bands. Quantification of mRNAs was repeated three times with independent mice, and average levels of change were calculated. Statistical analyses of the Q-PCR data were conducted by ANOVA. Q-PCR data were expressed as the relative expression of each gene to that of the control uterus in order to compare differences in gene expression among the three organs derived from the Müllerian duct.

Results

DNA Microarray Analysis. We examined gene expression in the oviduct, uterus, and vagina at GD 19 in DES-exposed mice and oil controls. The correlation coefficients of microarray chips averaged 0.980 (minimum: 0.967; maximum: 0.996). As described above, genes showing at least a 2-fold expression change in DES-exposed mice have been listed at http://www.nibb.ac.jp/bioenv1/suzuki/suzukidata004.html.

To examine the gene expression changes by DES in the three organs of Müllerian duct origin, we analyzed the clustering pattern of DES-regulated genes. Clustering analysis in controls revealed that there was organ specificity in gene expression. Gene expression profiles in the uterus

Table 3. DES-Regulated Common Genes in the Three Organs Derived from Müllerian Duct at GD 19[a]

Gene accession no.	Fold change			Name
	Ovi	Ut	Vg	
Upregulated				
NM_023268	2.0	2.4	2.2	Quiescin Q6
BB284358	2.1	3.4	2.0	EGL nine homolog 3 (*C. elegans*)
BB253720	2.2	2.4	2.1	Procollagen-proline, 2-oxoglutarate 4-dioxygenase, alpha 1 polypeptide
AK009873	2.3	4.8	2.7	RIKEN cDNA 2310047E01 gene
NM_007955	2.4	3.0	2.2	Protein tyrosine phosphatase, receptor type, V
NM_011961	2.4	3.5	2.4	Procollagen lysine, 2-oxoglutarate 5-dioxygenase 2
AV003026	2.4	2.9	2.8	Glutathione *S*-transferase omega 1
AW558468	2.5	2.7	2.4	Natriuretic peptide receptor 2
BG072404	2.5	4.1	2.0	RIKEN cDNA 4631427C17 gene
BC016131	2.5	2.4	2.2	DNA segment, Chr 14, ERATO Doi 813, expressed
NM_008062	2.5	2.6	2.0	Glucose-6-phosphate dehydrogenase X-linked
BB546344	2.5	3.8	3.1	Dehydrogenase/reductase (SDR family) member 8
NM_013650	2.5	4.8	2.9	S100 calcium binding protein A8 (calgranulin A)
NM_013864	2.6	3.5	2.4	*N*-myc downstream regulated 2
BC025020	2.6	2.8	3.1	RIKEN cDNA 2810049G06 gene
BC019434	2.6	3.5	2.1	UDP-glucuronosyltransferase 1 family, member 1
NM_007921	2.7	4.6	2.2	E74-like factor 3
BC026209	2.7	2.8	2.6	Arachidonate 5-lipoxygenase activating protein
NM_009760	2.7	2.8	2.3	BCL2/adenovirus E1B 19-kDa interacting protein 1, NIP3
BC014753	2.7	4.6	2.3	Hydroxysteroid 11-beta dehydrogenase 2
BM209618	2.7	2.1	2.6	Mus musculus cDNA clone C0650E08 3', mRNA sequence.
NM_015786	2.8	3.3	2.3	Histone 1, H1c
NM_013703	2.8	2.7	2.9	Very low-density lipoprotein receptor
BM242294	2.8	2.9	2.4	RIKEN cDNA 6330500D04 gene
NM_019468	2.9	2.7	2.5	Glucose-6-phosphate dehydrogenase X-linked
BB533903	2.9	3.0	2.3	Histone 1, H1c
NM_010050	3.0	12.1	5.9	Deiodinase, iodothyronine, type II
BB114323	3.1	3.3	2.4	3' similar to X85991 M.musculus mRNA for semaphorin B
NM_013655	3.2	2.3	3.7	Chemokine (C-X-C motif) ligand 12
D87867	3.2	4.0	2.1	UDP-glucuronosyltransferase 1 family, member 1
BC021352	3.3	3.8	3.4	Procollagen lysine, 2-oxoglutarate 5-dioxygenase 2
D87867	3.3	4.6	2.2	UDP-glucuronosyltransferase 1 family, member 1
BM209618	3.3	2.1	2.6	Mus musculus cDNA clone C0650E08 3', mRNA sequence
BC013477	3.4	9.7	3.7	Alcohol dehydrogenase 1 (class I)
NM_008735	3.4	3.1	2.4	Mus musculus nuclear receptor interacting protein 1 (Nrip1)
BB353211	3.5	18.6	18.3	Inhibin beta-B
BC005486	3.6	2.9	2.6	E26 avian leukemia oncogene 2, 3' domain
M27695	3.6	3.1	3.1	Urate oxidase
BM239615	3.6	8.5	3.3	Testis-expressed gene 2
NM_011926	3.7	7.2	2.4	CEA-related cell adhesion molecule 1
BM212947	3.7	2.1	2.0	Transmembrane protease, serine 2
AV171622	3.8	3.0	2.3	RIKEN cDNA 3300001H21 gene
NM_011361	4.0	4.1	3.2	Serum/glucocorticoid regulated kinase
BC020531	4.1	2.5	2.1	Spondin 1, (f-spondin) extracellular matrix protein
BC021770	4.2	10.8	4.7	Claudin 10
NM_007799	4.3	3.6	4.7	Cathepsin E
AK012175	4.3	5.1	2.8	RIKEN cDNA 2610528J11 gene
BM207588	4.3	2.2	3.2	Mus musculus transcribed sequences
NM_011488	4.5	2.8	2.6	Signal transducer and activator of transcription 5A
AV171622	4.5	4.3	2.2	RIKEN cDNA 3300001H21 gene
BC024886	4.5	9.0	6.6	Coagulation factor III
NM_053262	4.7	7.5	5.5	Dehydrogenase/reductase (SDR family) member 8
NM_019511	4.8	10.4	12.3	Receptor (calcitonin) activity modifying protein 3
BC022950	4.8	12.1	6.8	RIKEN cDNA 1600029D21 gene
D85596	5.0	6.6	3.4	AMP deaminase 3
BC003705	5.1	8.9	10.3	Surfactant associated protein D
U36502	5.8	4.0	3.2	Signal transducer and activator of transcription 5A
BG069413	6.0	9.3	4.4	Kruppel-like factor 4 (gut)
NM_033648	6.3	5.4	2.9	FXYD domain-containing ion transport regulator 4