Global Gene Expression in Mouse Vaginae Exposed to Diethylstilbestrol at Different Ages

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Estrogens regulate proliferation and differentiation of cells in target organs such as the female reproductive tract. In mature mice, estrogens stimulate cell proliferation, whereas ovariectomy results in atrophy of the female reproductive tract. In contrast, perinatal exposure to estrogens, including diethylstilbestrol (DES), induces persistent, ovary-independent vaginal stratification and cervico-vaginal tumors later in life. These effects are due to altered cell fate following DES exposure during a critical developmental period. The detailed mechanisms underlying the reversible and irreversible cell proliferation in vaginae induced by DES at different ages has not been clarified. Therefore, we examined differences in gene expression pattern using DNA microarray analysis in mouse vaginae 6 hrs after a single injection of 2 μg DES per gram of body weight, and proliferation of vaginal epithelial and stromal cells 24 hrs after the injection at postnatal days (PNDs) 0, 5, 20, and 70. After DES stimulation, vaginal epithelial and stromal cells showed cell proliferation at PNDs 20 and 70, and at PNDs 0 and 5, respectively. DNA microarray analysis exhibited 54 DES-induced genes and 9 DES-repressed genes in vaginae at PND 0, whereas more than 200 DES-induced genes were found in vaginae at PNDs 5 and 20, and 350 genes at PND 70. Clustering analysis of DES-induced genes in the vaginae at different ages revealed that genes induced by DES at PND 5 were closer to the adult type than that of PND 0. Genes related to keratinocyte differentiation, such as Gadd45α, p21, 14–3–3 sigma, small proline-rich protein 2f (Sprr2f), and Krupple-like factor 4 (Klf4), were induced by DES. The number of DES-induced genes during the critical period, PND 0, was smaller than those found after the critical period. These results give insight toward understanding the molecular mechanisms underlying the critical period in mouse vaginae. Exp Biol Med 231:632–640, 2006

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

Estrogens induce cell proliferation and differentiation, whereas estrogen depletion results in atrophy accompanied by apoptosis in adult female reproductive organs such as the uterus and vagina (1–3). Estrogen exposure during a critical period in early development induces persistent ovary-independent proliferation and keratinization in the vaginal epithelium during adulthood (4, 5). Diethylstilbestrol (DES), a synthetic estrogen used to prevent miscarriage during the 1940s to the early 1970s, induced vaginal clear cell carcinoma and uterine abnormalities in daughters of mothers exposed to DES during pregnancy (6). Similar abnormalities were reported in mice exposed to estrogens during a critical perinatal period (4, 5, 7). In female mice, various abnormalities such as polyovular follicles, oviductal tumors, uterine epithelial metaplasia, persistent vaginal stratification and keratinization, vaginal

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adenosis, and cervico-vaginal carcinomas, were induced by perinatal exposure to estrogens including DES (4, 5, 7–13). Vaginal epithelial proliferation persists even after ovariectomy in adult mice exposed to a sufficient dose of DES during the early neonatal period (4, 5).

During the normal estrous cycle, vaginal epithelial cell proliferation and keratinization occur at the estrous stage (1), whereas keratin 1 (K1) and progesterone receptor expressions were induced at the proestrous stage (14, 15). DES exposure during a critical developmental period results in alteration of the response to estrogen in the vagina, leading to a set of subsequent abnormalities. Epithelial cells failed to undergo apoptosis even after ovariectomy, and persistent expression of various genes was observed in the persistently proliferated vagina (15–17). Reduced expression of estrogen receptor (ER) mRNA and persistent expression of c-fos and c-jun mRNAs were observed in the vaginae of mice exposed to DES at the neonatal stage, even after ovariectomy (15). Persistent phosphorylation of erbB receptors, including epidermal growth factor (EGF) receptor, and sustained expression of EGF-like growth factors were found in the vaginae of mice neonatally exposed to DES (16). Neonatal exposure to a fibroblast growth factor family member, keratinocyte growth factor (KGF), resulted in persistent vaginal epithelial stratification (18). The induction of EGF by estrogens may play important roles in the proliferation of epithelial cells in the uterus and vagina (18–21).

We used DNA microarray to analyze gene expression in neonatally DES-exposed mouse vaginae and observed persistent expression of interleukin-1 (IL-1), IL-1 receptor, insulin-like growth factor-I (IGF-I) mRNAs, and stress-activated protein kinase/c-jun N-terminal kinase (SAPK/JNK), as well as phosphorylation of downstream genes (17).

The critical periods for the induction of abnormalities by estrogenic chemicals during mouse development varies by organ (22). Analyses of the molecular mechanisms underlying the critical sensitive window in each organ is essential for understanding the etiology of the persistent changes induced in the reproductive tracts. Therefore, we examined global expression in vaginae of early genes elicited by DES treatment at different ages in order to understand the differences in estrogen-responsive genes during and after the critical period, and in adulthood.

Materials and Methods

Animals. C57BL/6J mice (CLEA, Tokyo, Japan) were used at postnatal days (PNDs) 0, 5, 20, and 70. Mice were maintained in a 12:12-hr light:dark cycle at 23–25°C. They were fed a commercial diet (CE-2; CLEA), and tap water was provided *ad libitum*. All experiments and animal husbandry protocols were approved by the animal care committee of the National Institutes of Natural Sciences.

Treatments. DES (Sigma Chemical Co., St. Louis, MO) was dissolved in sesame oil. Unless otherwise stated, all materials were obtained from Wako Pure Chemical Industries, Osaka, Japan. The day of birth was designated as Day 0. For microarray experiments, mice at PND 0 (7–12 mice from three litters), PND 5 (7–12 mice from three litters), PND 20 (8 mice), and PND 70 (8 mice) were given a single subcutaneous (sc) injection of 2 μg DES per gram of body weight (bw) or oil vehicle alone. Mice at PND 70 were ovariectomized at 56 days of age. Vaginae from DES-exposed and control mice were collected for DNA microarray analysis and quantitative real time–polymerase chain reaction (QRT-PCR). In order to identify early genes induced by DES, tissues were dissected 6 hrs after the injection as described previously (23–25).

In addition, five mice each were given a single sc injection of 2 μg DES/gram bw or oil vehicle alone for the bromodeoxyuridine (BrdU) experiment.

DNA Microarray Analysis. Total RNA from vaginae was extracted using TRIZOL (Invitrogen, Tokyo, Japan) and purified using an RNeasy mini kit (Qiagen, Tokyo, Japan). Quality and quantity of total RNA were confirmed by the Agilent 2100 bioanalyzer (Agilent, Tokyo, Japan). cRNA probes were prepared from the purified RNA using an Affymetrix cRNA probe kit (Affymetrix, Inc., Santa Clara, CA) according to the manufacturer's protocol. All preparations met the recommended criteria of Affymetrix for use on their expression array. The amplified cRNA was hybridized to high-density oligonucleotide arrays (Mouse U74A; Affymetrix, Inc.) containing approximately 12,500 genes, and the scanned data were analyzed with GeneChip software (Affymetrix, Inc.) and processed as described previously (23). To confirm the estrogen-related changes in gene expression revealed by DNA microarray analysis, we independently repeated the same experiment at least twice. The expression data were analyzed with GeneSpring software (Agilent, Palo Alto, CA) as described previously (26).

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 (23–26). Gene expression change was estimated by the value of control PND 0 as one. Putative target genes were validated by QRT-PCR.

QRT-PCR. Total RNA was purified as described above. cDNA was synthesized from purified total RNA with Superscript II RT (–) (Invitrogen) with random primers at 42°C for 60 mins. PCR reactions were performed in the PE Prism 5700 sequence detector (PE Biosystems, Tokyo, Japan) using SYBR-Green PCR core reagents (PE Biosystems) in the presence of appropriate primers according to the manufacturer's instructions. PCR amplification was performed in triplicate under the following conditions: 2 mins at 50°C, 10 mins at 95°C, followed by a total of 40 cycles of 15 secs at 95°C and 1 min at 60°C.

Gene expression levels were normalized to the expression levels of ribosomal protein L8 mRNA

Table 1. Sequences for Primers Used for QRT-PCR

Genbank accession No.	Name	Forward primer	Reverse primer
U20344	Klf4	ACACAGGCGAGAAACCTTACCA	AATTTCCACCCACAGCCGT
AF058798	14-3-3 sigma	ACAAGGACAGCACCCTCATCA	ACAGCGTCAGGTTGTCTCTCAG
AJ005559	Sprr2a	TCCTGTAGTGTGCTATGAGCAATG	TTGCACAGGAGGGCATGTT
AJ005564	Sprr2f	TGAGGCTTCAGCAACAATGTCTT	TTGGTGGTGGACACACAGGA
AB003502	Gspt1[a]	CAAGTATGCATTGCGCGTTTA	CCCATCTGAGGGAAGTCCTTAA
AI121305	EST	TTATGTCCTCAGTCCGCAGCT	TAGTGTTGCAGGTCTGTGGTCC
AW048937	p21	TGAGACGCTTACAATCTGAGTGG	AACATGTATTGTGGCTCCCTCC
U00937	Gadd45α	GAAGAAGGAAGCTGCGAGAAAA	CCTGGCCATCCTAAATTAGCAGT
U67771	Ribosomal protein L8	ACAGAGCCGTTGTTGGTGTTG	CAGCAGTTCCTCTTTGCCTTGT

[a] Gspt1, G1 to S phase transcript 1.

(U67771), and gel electrophoresis and melting curve analyses were performed to confirm correct amplicon size and the absence of nonspecific bands. The primers were chosen to amplify short PCR products of less than 100 base pairs and their sequences are listed in Table 1.

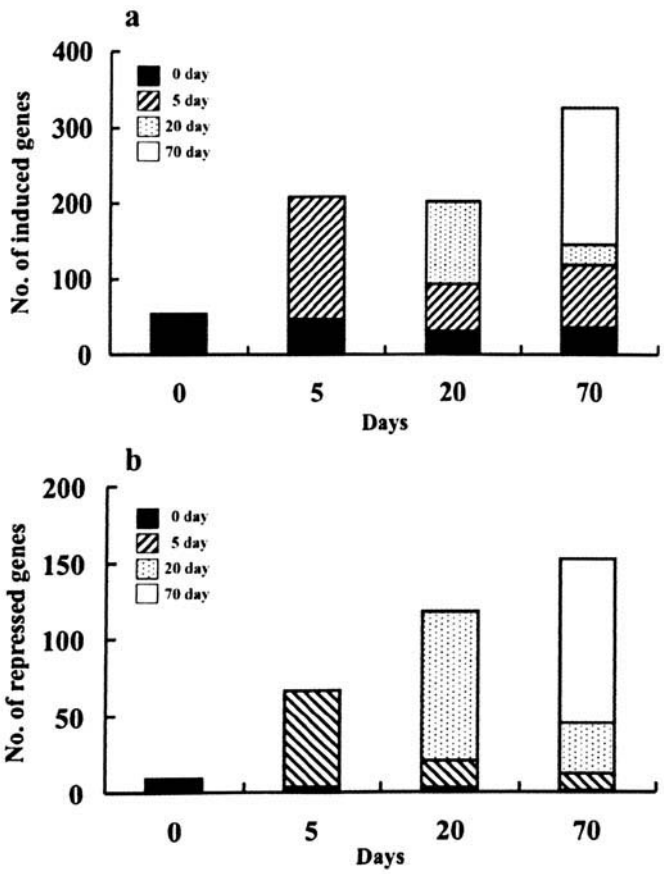

Figure 1. (a) The number of induced genes in vaginae 6 hrs after a single injection of 2 μg DES/g bw at PNDs 0, 5, 20, and 70. Mice at PND 70 were ovariectomized 2 weeks before. The number of DES-induced genes was small at PND 0, but it increased drastically at PND 5. (b) The number of repressed genes in vaginae 6 hrs after a single injection of 2 μg DES/g bw at PNDs 0, 5, 20, and 70. The numbers of DES-repressed genes increased linearly with age. Each pattern in the bar indicates genes commonly induced (a) or repressed (b) genes in respective ages. Days indicate ages given a single injection of DES.

Gene expression levels in DES-treated groups were normalized using control PND 0 as one. Parametric variables were analyzed by one-way analysis of variance (ANOVA) with post-hoc Student's *t* test or Welch's *t* test.

Immunostaining of BrdU. Five mice each given a single sc injection of 2 μg DES were killed 24 hrs after the injection. Two hours before dissection, 0.05 mg/g BrdU (Sigma, Tokyo, Japan) was injected intraperitoneally. Tissues fixed with neutral-buffered 10% formalin were embedded in paraffin. Sections cut at 8 μm were incubated with 0.3% H_2O_2 in methanol for 30 mins at room temperature (RT) to block endogenous peroxidase activity. They were washed with 0.5% Tween-20 in phosphate-buffered saline (PBS), incubated with 2 *N* HCl in water for 20 mins at RT. They were washed with 0.5% Tween-20 in PBS, incubated with 1% bovine serum (BSA) for 20 mins at RT, and with anti-BrdU antibody (Roche, Mannheim, Germany) at a dilution of 1:15 in 1% BSA at 4°C overnight. Washing with 0.5% Tween-20 in PBS, sections were incubated with mixture of 3,3′-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2. Counterstain was conducted with methyl green. The number of BrdU-positive cells in 300 epithelial cells in the middle part of vagina and that in 500 stromal cells were recorded. Proliferation rate (%) was estimated as a percentage of BrdU-positive cells in epithelial cells and stromal cells, separately.

Results

Gene Expression in the Vaginae of Mice Treated with DES at Different Ages. The number of detected genes in the mouse vagina from oil-injected controls was not very different among animals examined at different ages: newborn (PND 0) = 4988 genes, PND 5 = 4937 genes, PND 20 = 4881 genes, and PND 70 = 4903 genes. We selected genes showing at least a 2-fold change in expression in response to DES treatment for further analysis (listed at http://www.nibb.ac.jp/bioenv1/suzuki/). The number of genes induced or repressed by DES was modest at PND 0, but showed a sharp increase with age (Fig. 1). DES exposure induced 54, 208, 202, and 326 genes and repressed 9, 66, 117, and 152 genes in vaginae at PNDs

Table 2. Induced or Repressed Genes in Vaginae 6 Hrs After a Single Injection of DES at Only PND 0

Genbank accession No.	Fold change	Name
AV170770	2.0	EST
AI837116	2.4	Solute carrier family 41, member 1
AI851565	2.5	RIKEN cDNA 1500034J01 gene
U58887	3.3	SH3-domain GRB2-like 3
D50646	0.4	Stromal cell–derived factor 2
AI425990	0.4	RIKEN cDNA C530046L02 gene
AI646638	0.5	Frequently rearranged in advanced T-cell lymphomas 2
M12347	0.5	Actin, alpha 1, skeletal muscle
AI841689	0.5	Chemokine-like factor superfamily 3

0, 5, 20, and 70, respectively (Fig. 1). The number of genes (208) induced by DES at PND 5 was similar to that of PND 20 (202). Ninety-two of 208 (44%) genes induced by DES at PND 5 were also induced at PND 20 by DES. Fifty-eight (28%) PND 5–specific genes were induced by DES (Fig. 1). Four genes (including two enhanced sequence tags [ESTs]) were specific to PND 0 (Table 2 and Fig. 1).

The total number of DES-regulated genes was 781. Some genes showing drastic expression change by DES were selected for further study (Table 3). Many of these genes showed upregulation by a single injection of DES from PND 0 to PND 70. Twenty-five genes, including MAD2, G1 to phase transition 1, c-fos, early growth response 1 (Egr-1), and Kruppel-like factor 4 (Klf4) were induced by DES exposure at all ages examined. MAD2 is an estrogen-responsive gene and assembles the mitotic spindle at the G2/M checkpoint (27). c-fos and Egr-1 were reported to be estrogen-responsive genes in the mouse uterus or mammary gland (or both) (15, 23, 28). Klf4, an inhibitor of the G1/S phase, plays a role in keratinocyte differentiation (29, 30), and is identified as an estrogen-responsive gene in the present study (Table 3).

The number of DES-repressed genes showed an age-dependent increase (Fig. 1). Twenty of 66 (30%) DES-repressed genes at PND 5 were also found at PND 20. Thirty-nine of 117 (33%) DES-repressed genes at PND 20 were also found at PND 70, although the number of age-specific DES-repressed genes was 5 (56%), 39 (59%), 64 (55%), and 107 (70%) at PNDs 0, 5, 20, and 70, respectively (Table 2 and Fig. 1). One of the common DES-repressed genes at all ages was flavin-containing monooxygenase 1 (Fmo 1; Table 3), which regulates metabolism of chemicals (31).

Clustering Patterns and Category of DES-Regulated Genes in the Vaginae of Mice at Different Ages. The 729 genes showing more than a 2-fold change following a single injection of DES at PNDs 0, 5, 20, and 70 were used for clustering analysis. These genes can be grouped as PND 0, PND 5, and PND 70 in controls, and as PND 0 and PND 5–70 in DES-exposed vaginae (Fig. 2) because the clustering patterns of genes in DES-exposed vaginae at PND 5 was more similar to those of PND 20 and PND 70 than that of PND 0 (Fig. 2).

These genes could be categorized into several groups. Genes involved in cell proliferation (15%) and protein modification (17%) were found in the DES-induced genes at PND 0 (Table 4). DES-repressed genes at PND 0 included those involved in cell tissue structure (11%), defense response (11%), transcription (11%), and transport (22%) compared with other groups. DES-regulated genes categorized in organogenesis were repressed by DES at PNDs 5, 20, and 70.

Confirmation of Gene Expression by QRT-PCR. Expression of several genes showing upregulation by DES in mouse vaginae at different ages was confirmed using QRT-PCR. The fold change in gene expression was estimated based on the value of each gene in control PND 0 as one (Fig. 3). Expressions of 14–3–3 sigma , Klf4, Sprr2f, EST (AI121305), and Gadd45α, which promotes the G1 phase and acts at the G2/M checkpoint (32, 33), were increased with age in control mice. Expression of p21 and G1 to phase transition 1 mRNAs were increased at PND 5 in control mice, whereas expression of Sprr2a was decreased at PND 5 in control mice. Expression of these genes, except for Klf4 and EST (AI121305), were induced by DES at PNDs 5, 20, and 70, but not at PND 0. Expression of Klf4 and EST (AI121305) was upregulated by DES at PND 0.

Immunostaining of BrdU. In oil-treated controls, ratios of BrdU-positive cells were not different among postnatal ages either in epithelial cells or in stromal cells. BrdU-positive cells in the vaginal epithelium were increased at PNDs 5, 20, and 70 in DES-treated vaginae as compared with the oil controls. In contrast, a higher number of BrdU-positive cells were found in the stroma in PND 0 and PND 5 mice (Fig. 4).

Discussion

Estrogen, androgen, and KGF exposure for 5 days from the day of birth induces persistent vaginal epithelial stratification in mice (4, 7, 11, 16–18). The persistent vaginal epithelial stratification with superficial keratinization induced by perinatal estrogen exposure was reported to be accompanied by persistent expression of several growth

Table 3. Induced or Repressed Genes in Vaginae 6 Hrs After a Single Injection of DES at PNDs 0, 5, 20, and 70 Using DNA Microarray and QRT-PCR[a]

Genbank accession No.	Gene[b]	Age (days) 0	5	20	70
Induced genes		Ratio by microarray (by QRT-PCR)			
Cell proliferation					
AB003502	G1 to S phase transition 1 (Gspt1)	2.2 (1.4)	2.8 (1.6)	2.1 (2.4)	3.3 (1.2)
V00727	c-fos	4.0	14.5	6.4	9.4
U83902	MAD2	2.1	2.7	3.7	13.8
X59846	Gas6	4.0	3.9	2.1	3.7
AF058798	14-3-3 sigma	–(1.1)	3.4 (3.6)	3.4 (5.3)	2.7 (5.1)
AW048937	p21	–(1.5)	2.4 (1.6)	3.2 (3.9)	3.7 (2.5)
U00937	GADD45α	–(1.8)	6.0 (3.9)	4.5 (4.1)	4.5 (3.6)
Protein modification					
AB013848	Peptidyl arginine deiminase type I	4.1	6.8	3.3	4.5
L02526	Map2k1	2.1	2.1	2.2	2.0
X04591	Creatine kinase, brain	2.1	2.5	2.1	4.3
X59274	Protein kinase C, beta	2.6	2.7	2.1	2.2
Transcription					
M28845	Early growth response 1 (Egr-1)	2.8	4.7	2.9	3.5
U20344	Kruppel-like factor 4 (Klf4)	3.8 (3.5)	4.2 (5.0)	2.1 (4.2)	2.7 (2.0)
Signal cascade					
M63801	Gap junction membrane channel protein alpha 1	2.4	3.5	2.1	2.1
AI596360	RIKEN cDNA 4930422J18 gene	3.1	7.2	2.6	2.8
Unknown					
X67644	Immediate early response 3	2.7	5.3	3.7	2.7
AI121305	RIKEN cDNA 1600029D21 gene	3.8 (2.4)	15.1(14.9)	3.7 (4.3)	3.6 (2.4)
Cell structure					
K02108	K 2, basic, gene 6a	—	6.3	3.0	5.2
AB012042	K 2, basic, gene 6g	—	2.9	7.7	4.1
M36120	K 1, acidic, gene 19	—	—	—	2.6
AJ005559	Sprr2a	–(0.4)	2.2 (4.5)	–(1.3)	4.9 (4.0)
AJ005560	Sprr2b	—	—	—	4.4
AJ005564	Sprr2f	–(1.7)	15.0(14.2)	4.0 (8.0)	7.5 (3.9)
Repressed genes					
Transport					
D16215	Flavin-containing monooxygenase 1 (Fmo1)	0.5	0.3	0.3	0.5

[a] Gene expression change was calculated based on the value of PND 0 as one. QRT-PCR data appear in parentheses; — indicates no change.
[b] Gas6, growth arrest–specific 6; Map2k1, mitogen-activated protein kinase kinase 1; p21, cyclin-inhibitor 21; GADD45α, growth and DNA damage 45α; MAD2, mitotic arrest–deficient; Sprr, small proline-rich protein; K, keratin complex.

factors (16–19, 34, 35). However, the precise mechanism of estrogen effects on the vaginal epithelial proliferation at different ages has not been clearly demonstrated, although several studies demonstrated that neonatal DES exposure induced persistent expression of EGF and EGF-like growth factors (35, 36) and phosphorylation of ERα, EGF receptor, and erbB in mouse vaginae (16, 17).

In the present study, global gene expression in vaginae was analyzed at different ages using DNA microarray analysis. We demonstrated age differences in vaginal responses to estrogen in the induction of gene expression from PND 0 to PND 70. ER staining was found in vaginal stromal cells in CD-1 mice at PND 0 (37), and in both vaginal epithelial and stromal cells, but in only stromal cells in uterine epithelial cells in C57BL/Tw mice at PND 0 (38). Epithelial expression of ER in the reproductive tracts occurs later in C57BL/6 mice than in CD-1 mice (39). Developmental effects of estrogens, including DES on neonatal mouse vaginae, are mediated through stromal ER (40). In the present study, we used C57BL/6 mice. Thus, neonatal mouse vaginae seem to be responsive to estrogen at PND 0. However, in the present study, the number of DES-induced genes in vaginae at PND 0 was smaller compared with those in PNDs 5, 20, and 70 mice. Moreover, vaginal epithelia in mice at PNDs 5, 20, and 70 were proliferated by DES, but not at PND 0 in the present study (Fig. 4). The PND 0 mouse vagina is still under development even without estrogenic stimulation (4, 41). Thus, in terms of gene expression, the vagina at PND 0 is less sensitive to estrogenic stimulation than it is at later stages. It has been shown that the response to DES is different between the Müllerian and urogenital sinus vaginal epithelium; proliferation in the vaginal fornix (Müllerian vagina) is inhibited by neonatal DES and the cell morphology is altered (7). Further

Table 4. Functional Categories of DES-Responsive Genes in Mouse Vaginae Selected in the Present Study[a]

	Age (in days)							
	0		5		20		70	
Category	Induced	Repressed	Induced	Repressed	Induced	Repressed	Induced	Repressed
Biosynthesis	6	0	11	3	13	5	11	6
Cell proliferation	15	0	10	10	10	8	9	6
Cell tissue structure	2	11	9	10	9	5	9	3
Defense response	7	11	5	6	3	4	4	6
Metabolism	7	0	7	3	5	8	6	5
Mitochondrion	0	0	0	0	0	0	1	1
Organogenesis	0	0	0	4	0	3	1	3
Protein modification	17	0	12	7	12	7	9	5
Ribosome	2	0	1	1	0	1	1	0
Signal transduction	7	0	6	6	6	6	6	7
Transcription	7	11	11	10	10	14	12	16
Transport	7	22	7	9	13	13	11	7
Unclassified	23	45	21	31	19	26	20	35
Total (%)	100	100	100	100	100	100	100	100

[a] Total number of clustered genes in each category was 100%. Induced, % of categorized genes in DES-induced gene at each age; Repressed, % of categorized genes in DES-repressed gene at each age.

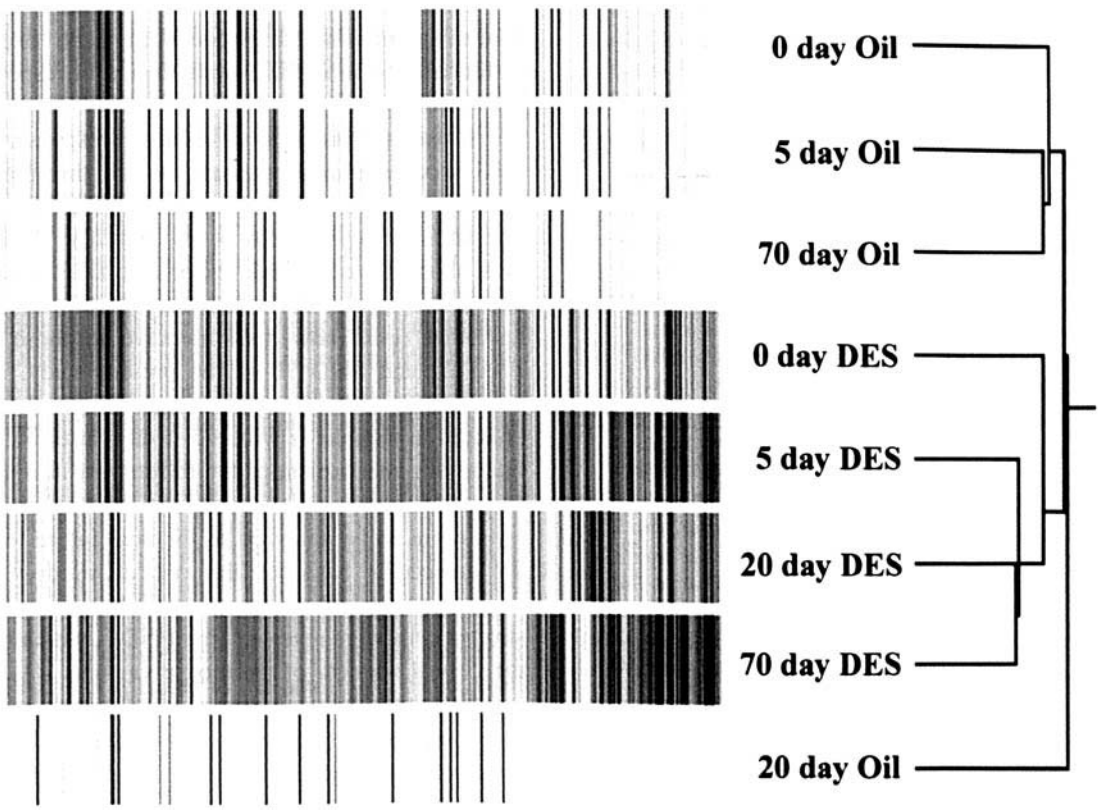

Figure 2. Clustering analysis of DES-responsive genes in mouse vaginae selected in the present study. Mice at PNDs 0, 5, 20, and 70 were stimulated by a single injection of DES. Each color bar indicates the expression level of one gene: red, induction; green, repression; yellow, average expression in eight groups; gray, not detected. Genes showing more than a 2-fold change in expression 6 hrs after a single injection of 2 μg DES/g bw at all ages were used for clustering analysis. Seven-hundred seventy-nine genes were selected. Control mice exhibited separated trees between the neonatal period (PNDs 0 and 5) and adulthood (PNDs 20 and 70). Branching of clustered genes in DES-exposed vaginae at PND 5 was closer to that of PND 0.

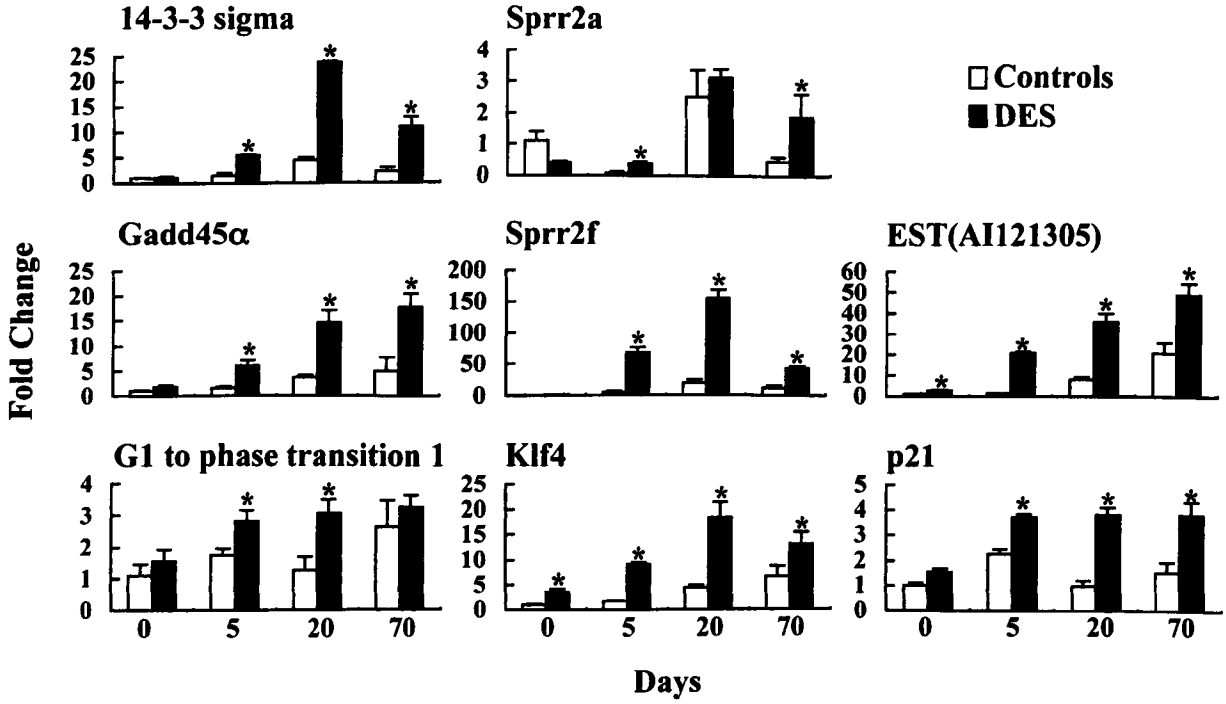

Figure 3. Changes in gene expression of cell cycle and keratinocyte differentiation regulators were confirmed by QRT-PCR. Fold change of gene expression by QRT-PCR was estimated based on the value of each gene at PND 0 as one. Results are the mean and SEM of three experiments. Each experiment was performed in triplicate. $P < 0.05$ vs. age-matched controls.

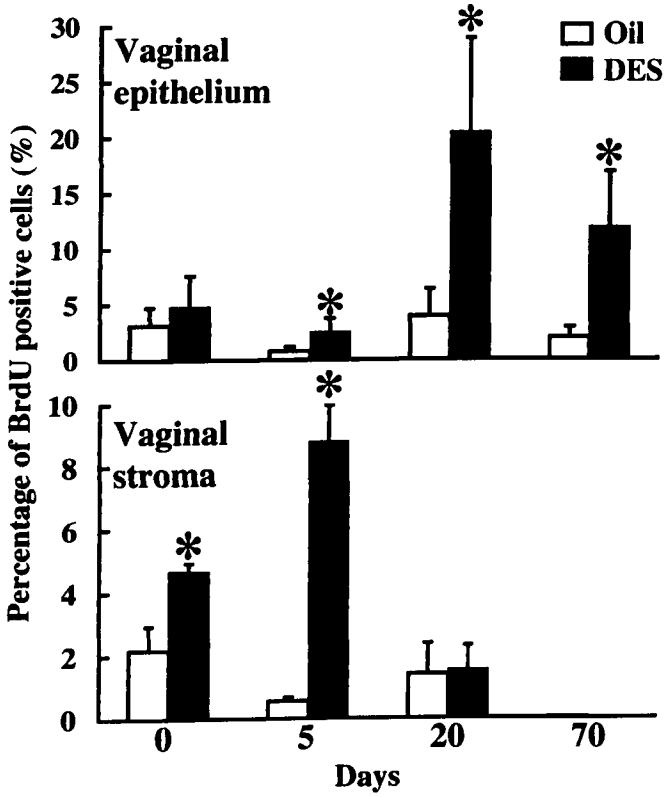

Figure 4. Percentage of BrdU-positive cells in vaginal epithelial cells and stromal cells at different ages. Mean and SEM. Note reverse of vaginal cell proliferation in epithelium and stroma after PND 5. *$P <$ 0.05 vs. age-matched controls.

studies examining differences in gene expression in Müllerian and urogenital sinus vagina in response to DES are essential.

Clustering analysis of estrogen-responsive genes in DES-exposed mouse vaginae showed that they could be broadly categorized into two types; a neonate type (PND 0) and an adult type (PNDs 5, 20, and 70). Vaginal stromal cells showed proliferative response to DES only at PNDs 0 and 5, but not at PNDs 20 and 70. In contrast, vaginal epithelial cells showed proliferative response to DES at PND 20 and PND 70 in the present study. The critical window for induction of estrogen-independent persistent changes in vaginae is within 3–5 days after birth (22). The underlying mechanism of the differences in responsiveness between vaginal epithelial cells and stromal cells at different ages needs to be analyzed in the near future to understand the molecular basis of the critical window. In mouse vaginae, proliferative response to DES in epithelial cells and stromal cells were reversed after PND 5, which may indicate the critical window of the mouse vagina.

Estrogens induce expression of genes related to cell cycle regulators, chromatin remodeling, IGF-I signaling, apoptosis, and keratin expression in mouse uteri (23–25, 42). Increased expression of mRNAs of cell cycle regulators were reported after 17β-estradiol treatment in the uteri of adult ovariectomized mice (42). In the present study, these cell cycle regulators except for cyclin G1 and E1, were induced in adult vaginae; thus vaginae responded to estrogen similar to uteri at the gene expression level.

From PND 5 onward, DES induced the following cell regulatory genes: p21, which delays S phase progression (43); Gadd45α, which acts at the G2/M checkpoint; and 14–3–3 sigma, which inhibits activation of cyclin B (32, 43, 44). Thus, induction of these cell cycle regulators at the mitotic phase checkpoint 6 hrs after DES stimulation may play a role in DNA synthesis required for vaginal cell proliferation.

Induction of keratinocyte differentiation regulators, such as Sprr1a, Sprr2a, and keratin complexes, was reported in estrogen-exposed uteri (45). Sprr family genes are expressed in all squamous cells, such as epithelial cells of skin, vagina, and digestive tract (46, 47). Sprr2a, Sprr2b, and Sprr2f are expressed in uteri and vaginae (48). Sprr2f is expressed most intensely in uteri and vaginae (48). Sprr2f and Keratin complex 2 (K2) mRNAs were elevated in vaginae 6 hrs after DES exposure from PND 5 to PND 70. Genes related to epithelial cell differentiation responded to DES earlier than genes related to proliferation at PND 5. The increase in expression of Sprr2f and K2 genes at PND 20 and PND 70 is probably related to proliferation of vaginal epithelial cells. Sprr2a and Sprr2b may be correlated with keratinization in vaginal epithelial cells.

Klf4 is a transcription factor of Sprr2 (47, 49) and plays a role in keratinocyte differentiation (30). DES induced Klf4 expression in vaginae even at PND 0 in the present study. The inductions of Klf4 and Sprr2a expressions in DES-exposed uteri at PND 5 were reported previously (45). In vaginae, Klf4 is an early estrogen-responsive gene and a candidate for persistent vaginal stratification by perinatal DES exposure.

14–3–3 Sigma also regulates the cell cycle by inhibiting G2/M progression-dependent p53 (44) and is induced in squamous cell carcinoma of the urinary bladder (50). Expression of 14–3–3 sigma was found in DES-stimulated mouse vaginae at PND 5 in the present study and also in neonatally DES-exposed vaginae (17), suggesting that this gene will be a candidate for further study in the persistent vaginal stratification and keratinization induced by perinatal estrogen exposure.

In conclusion, vaginal epithelial cells and stromal cells showed proliferation after a single injection of DES at PNDs 20 and 70, and at PNDs 0 and 5, respectively. The number of genes induced 6 hrs after DES exposure in mouse vaginae at PND 0 was lower than those induced at PNDs 5, 20, and 70. The DES-induced gene expression pattern in vaginae at PND 5 was closer to the adult type. Several cell cycle regulators such as Gadd45α, G1 to S phase transition 1, and p21; and keratinocyte differentiation factors, 14–3–3 sigma and Sprr2f, were induced by DES in vaginae from PND 5 to adult. Thus, microarray analysis revealed that the gene expression pattern in vaginae during the critical period was different from that after the critical period. Further studies are essential to examine the time course of gene expression to discover late genes induced in mouse vaginae by DES exposure at different ages.

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