Persistent gene expression in mouse vagina exposed neonatally to diethylstilbestrol

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Abstract

Developmental exposure to a synthetic estrogen, diethylstilbestrol (DES), induces carcinogenesis in human and laboratory animals. In mice, neonatal DES treatment induces persistent proliferation and keratinization of the vaginal epithelium, even in the absence of the ovaries, resulting in cancerous lesions later in life. To understand the mechanisms underlying this persistent cell proliferation and differentiation, we characterized the gene expression patterns in the neonatally DES-exposed mouse vagina using DNA microarray and real-time quantitative RT-PCR. We found that genes related to cellular signaling, which are candidates for mediating the persistent proliferation and differentiation, were altered, and genes related to the immune system were decreased in the neonatally DES-exposed mouse vagina. We also noted high expression of interleukin-1 (IL-1)-related genes accompanied by phosphorylation of JNK1. In addition, expression IGF-I and its binding proteins was modulated and led to phosphorylation of IGF-I receptor and Akt, which is one of the downstream factors of IGF-I signaling. This led us to characterize the expression as well as the phosphorylation status of IL-1 and IGF-I signaling pathway components which may activate the phosphorylation cascade in the vagina of mice exposed neonatally to DES. These findings give insight into persistent activation in the vagina of mice exposed neonatally to DES.

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Introduction

Estrogens tightly regulate cell proliferation and differentiation in target organs, particularly the reproductive tracts (oviduct, uterus and vagina) and mammary gland. The vaginal epithelium is a particularly intriguing model for estrogen-regulated gene expression. It undergoes characteristic changes from non-keratinized to a fully keratinized epithelium depending on the levels of the endogenous estrogen, estradiol (E_2), during the estrous cycle. Under this process, E_2 -induced cell proliferation is restricted to the basal layer. The suprabasal cells are no longer mitogenic but differentiate while moving up through the epithelium; finally, the apical cells exhibit keratiniz-

ation, losing their nuclei and cytoplasm and substituted by the keratin proteins. The fully stratified and keratinized epithelium in the vagina resembles epidermis in the skin, however, again, differentiation of vaginal epithelium is regulated by estrogen.

Long-term estrogenic stimulation is a known risk factor for carcinogenesis in laboratory animals and humans (Marselos & Tomatis 1992*a,b*). In humans, transplacental exposure to a synthetic estrogen, diethylstilbestrol (DES), which was routinely prescribed to pregnant women for preventing miscarriages in 1940s to the 1970s, induces vaginal clear-cell adenocarcinoma in young women (Herbst *et al.* 1971). As generations of women exposed to DES approach menopause, concern has

arisen about their health risk, because it has been hypothesized that *in utero* DES exposure influences the incidences of breast cancer, squamous neoplasia of the cervix and vagina, and vaginal clear-cell adenocarcinoma later in life (Herbst 2000, Hatch *et al.* 2001, Palmer *et al.* 2002). Rodent models of DES exposure have been developed to understand the mechanistic basis of DES effects in humans. In mice, developmental exposure to estrogens within a critical developmental period elicits various permanent alternations in female reproductive tracts. For example, in the vagina, neonatal estrogen administration induces persistent cell proliferation and cornification of vaginal epithelium even after ovariectomy (OVX), resulting in hyperplastic lesions later in life (Takasugi *et al.* 1962, Forsberg 1979, McLachlan *et al.* 1980). We previously reported that persistent erbB receptor phosphorylation and sustained expression of epidermal growth factor-like growth factors contributed to persistent action in the neonatally DES-exposed vagina (Miyagawa *et al.* 2004). However, the underlying mechanisms remain unknown, therefore, additional factors must be present. In order to understand the underlying mechanisms of persistent action, it is important to understand cellular events, such as mRNA/protein expression and the phosphorylation status of signaling molecules.

In this report, we characterized the mRNAs expression patterns using DNA microarray and real-time quantitative RT-PCR (Q-PCR). In the vagina of mice exposed neonatally to DES, expressions of various genes were modulated, and interleukin-1 (IL-1) and insulin-like growth factor-I (IGF-I) signaling were activated without estrogen stimulation. In particular, IGF-I receptor (IGF-IR) and its downstream factor, Akt, were phosphorylated, which may lead to persistent cell proliferation and differentiation in the vagina. In addition, genes related to the immune system were suppressed in the neonatally DES-exposed mouse vagina. These findings give insight into persistent phenomena in vaginas exposed neonatally to DES in mice, and also in humans.

Materials and methods

Animals and treatments

Female C57BL/6J mice were maintained under 12 h light:12 h darkness at 23–25 °C and fed

laboratory chow (CE-2; CLEA, Tokyo, Japan) and tap water freely. All procedures were approved by the Institutional Animal Care and Use Committee at the National Institute for Basic Biology, Okazaki National Research Institutes.

Female newborn mice were given a daily s.c. injection of 2.5 µg DES (Sigma)/g body weight (BW) per day dissolved in sesame oil (Kanto Chemical, Tokyo, Japan) or the vehicle alone beginning from day 0 (the day of birth) to day 4. These mice underwent OVX at day 46 and were killed at day 60 (referred to as neoDES mouse) (see Fig. 1A). For examining effects of estrogen and IGF-I, a single injection of 50 µg E₂ (Sigma) or 500 µg long R3 IGF-I (JRH Biosciences, Lenexa, TS, USA)/g BW was given to 60-day-old mice, which underwent OVX at day 46, and were killed 0.5, 1, 3, 6, 12, 24, 36 and 48 h (E₂) or 15 min (IGF-I) after the injection. Some mice underwent OVX at day 46 and were treated with a daily injection of 2.5 ng DES/g BW per day for 5 days, then killed 24 h after the last injection. This treatment was sufficient to induce stratified and keratinized vaginal epithelium in adult OVX mice. For tissue separation, neoDES vaginas were cut into small pieces, placed into 1% trypsin in Hanks' balanced salt solution and incubated at 4 °C for 90 min. Then, the vaginal epithelium and stroma were separated mechanically using fine surgical forceps. More than three mice were pooled per each experimental group.

Immunohistochemistry

Tissues were fixed in neutral buffered 10% formalin, embedded in paraffin and sectioned at 6 µm. Deparaffinized sections were incubated in 0.3% H₂O₂ in methanol for 15 min to eliminate endogenous peroxidases. After washing with PBS, the sections were stained with Histofine (Nichirei, Tokyo, Japan) according to the manufacturer-supplied protocol. Antibody for Ki-67 antigen was obtained from Novocastra Laboratories Ltd (Newcastle upon Tyne, UK). The sections were incubated at 1:1000 dilutions in PBS containing 1% BSA (Sigma) for 60 min at room temperature.

DNA microarray analysis

Preparation of cRNA and microarray analysis were essentially performed as described previously

(Watanabe *et al.* 2003). Total RNA was isolated with the TRIZOL reagent (Invitrogen) and purified using the RNeasy kit (Qiagen). Total RNA (10 µg) was converted into double-strand cDNA using the SuperScript Choice System (Invitrogen) with the T7-(dT)₂₄ primer (Amersham), which was used for first-strand cDNA synthesis. Biotin-labeled cRNA was synthesized using the ENZO BioArray HighYield RNA transcript labeling kit (Amersham). The cRNA was purified using RNeasy kit and partly hydrolyzed in fragmentation buffer (40 mM Tris, 100 mM potassium acetate and 30 mM magnesium acetate, pH 8.1) 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 Test 3 array (Affymetrix; Amersham) containing housekeeping genes. All preparations met Affymetrix's criteria for use on their expression arrays. After checking the quality of the cRNAs, 15 µg cRNA were hybridized to the high-density oligonucleotide arrays, Murine U74A GeneChip Expression Arrays (Affymetrix; the references to array design are available on the manufacturer's website, <http://www.affymetrix.com>) for 16 h at 45 °C. The arrays were then washed, stained with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR, USA) in an Affymetrix fluidix station and scanned with an argon-ion laser confocal scanner (Affymetrix).

Data were analyzed with GeneChip software Microarray Suite 5.0 (Affymetrix) as described elsewhere (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 level and cross-hybridization signals, the relative level of the gene expression is represented by differences between the levels of fluorescent intensity of the PMs and 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.

The signals obtained from experimental groups were performed twice, independently, and the differences in intensities were calculated. In order to maintain reproducibility, we only selected the genes whose expression level changes were less than

2-fold between two independent experiments under the same conditions and average values were used as gene expression levels. Genes showing 3-fold alteration in the neonatally DES-treated mice as compared with oil controls were selected and analyzed further.

Real-time Q-PCR

Changes in gene expression were confirmed and quantified using Q-PCR using the ABI Prism 5700 Sequence Detection System with SYBR Green Master Mix according to the manufacturer's instructions (Applied Biosystems, Foster City, CA, USA). Total RNA (2.5 µl) was used in the RT reaction carried out with SuperScript II reverse transcriptase (Invitrogen) and random primers (9-mer; Takara, Ohtsu, Japan) for 60 min at 42 °C. PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min in 15 µl volumes. The primers were chosen to amplify short PCR products (less than 100 bp) with the assistance of Primer Express software (Applied Biosystems). The representative primer sequences are as follows: IL-1α (X01450), AGG AGA GCC GGG TGA CAG TAT, TCA CTG AAA CTC AGC CGT CTC TT; IL-1β (X04964), TCA GGC AGG CAG TAT CAC TCA TT, GGA AGG TCC ACG GGA AAG A; IL-1 receptor type I (IL-1RI) (M20658), CCT GTG CGG GAC ACT AAG GA, GCC CAC TTT TGC CAT GCT A; IL-1 RII (X59769), GTT TAT CTC GGC TGC TTA CCC A, CAA AAA TCA GCG ACA CTT CCA C; IL-1 receptor antagonist (IL-1ra) (L32838), CCT TCT CAT CCT TCT GTT TCA TTC A, CTT GCA TCT TGC AGG GTC TTT; IL-1-like protein 1 (IL-1LI) (AJ250429), GGG CCA AGG AAT CAA AGA GC, CGG ATT CGA AGC TGG AGG TA; IGF-I (X04480), TTC AGT TCG TGT GTG GAC CGA G, TCC ACA ATG CCT GTC TGA GGT G; keratinocyte growth factor (KGF) (NM_008008), GAA AGG GAC CCA GGA GAT GAA, TGA TTG CCA CAA TTC CAA CTG; and ribosomal protein L8 (U67771), ACA GAG CCG TTG TTG GTG TTG, CAG CAG TTC CTC TTT GCC TTG T. The other primer sequences are available upon request. Melting curve analysis using 5700 software was performed to confirm the primer efficiency. Relative RNA equivalents for each sample were obtained by

standardization of ribosomal protein L8 levels. The expression of granulocyte-macrophage colony stimulation factor (GM-CSF) was quantified using the ABI Prism 7000 System with FAM-labeled Taqman probe and primers (Assays-on-Demand Gene Expression Products; Applied Biosystems) because an efficient primer set could not be established using the SYBR Green Q-PCR. The relative RNA equivalent for GM-CSF was obtained by standardization of ribosomal protein S5 levels. For quantifying gene expression on DNA microarray data, more than two pools of samples per group were run in triplicate to determine sample reproducibility, and the average relative RNA equivalents per sample were used for further analysis. All values are represented as fold change compared with the control treatment group average as 1.0. For analyses of IGF-I, KGF, GM-CSF and IL-1-related genes, three pools of samples per group were used. Statistical analysis was performed using Student's *t*-test or Welch's *t*-test followed by an F-test. Points of statistical significance vs control are indicated by asterisks (*), with $P < 0.05$.

Protein preparation and immunoblotting

Dissected mouse vaginas were immediately homogenized in buffer (20 mM Hepes, 2 mM EDTA, 2 mM EGTA, 250 mM sucrose, 2 mM Na_3VO_4 , 2 mM NaF, 100 mM β -glycerophosphate and protease inhibitor cocktail (Complete Mini; Boehringer Mannheim, Mannheim, Germany), pH 7.5). The homogenates were centrifuged at 900 *g* for 15 min at 4 °C. Supernatant fluids were recentrifuged at 105 000 *g* for 60 min and the resulting supernatant fluids were used for detection for stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) and Akt. The pellets were suspended in homogenization buffer without sucrose but containing 250 mM NaCl and 1% Triton X-100, and they were used for detection of IGF-IR. Protein contents were determined using the Bradford Assay (Protein Assay reagent; BioRad, Hercules, CA, USA).

After electrophoresing on SDS-polyacrylamide gels, proteins were subsequently transferred to nitrocellulose membranes. The membranes were preincubated with 3% BSA in Tris-buffered saline contained 0.1% Tween-20 (TBST) overnight at 4 °C. Incubations with each antibody were

performed at a dilution of 1:1000 at room temperature for 2 h in TBST (IGF-IR, SAPK/JNK, Akt, phospho-SAPK/JNK and phospho-Akt) or 1:1000 at 4 °C overnight in TBST and 5% BSA (phospho-IGF-IR). Anti-IGF-IR antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-SAPK/JNK, -Akt, -phospho-SAPK/JNK (Thr183/Tyr185 of human sequence corresponding to Thr183/Tyr185 of mouse sequence), -IGF-IR (Tyr 1131 to Tyr 1163 of mouse sequence) and -phospho-Akt (Ser 473 and Thr 308 of mouse sequence) antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). For experimental reproducibility, more than two pools of experimental groups were used. Signals were detected with an ECL kit (Amersham).

Results

Histology of the neoDES vagina

Persistent epithelial proliferation and keratinization were induced by five daily injections of DES from the day of birth as shown in Fig. 1A. The vaginal epithelium of neonatally oil-injected 60-day-old OVX mice (control) was composed of two or three layers of cuboidal cells, whereas the epithelium of the age-matched, neonatally DES-exposed, OVX mice (neoDES) exhibited stratification and superficial keratinization (Fig. 1B). In the neoDES vagina, basal epithelial cells showed high proliferative activity, which was confirmed by Ki-67 antigen immunostaining (Fig. 1C). In contrast, the basal epithelial cells of control animals did not.

Microarray analysis of gene expression changes in the neoDES vagina

Neonatal DES treatment causes persistent cell proliferation and differentiation, however, molecular mechanisms underlying estrogen-induced changes have not been elucidated. We compared gene expression of vaginas of OVX mice treated neonatally with either oil (control) or DES (neoDES) using DNA microarray. The oligo-microarray system (Affymetrix GeneChip – Murine U74A) was used for analysis of the gene expression pattern. The scanned data were analyzed and fold changes in expression levels were determined in the 9977 genes. The genes showing changes in expression (increased or decreased more than

Figure 1 (A) Treatment timeline of neonatal DES treatment and histological analysis. Female newborn mice were given five daily injections of 2.5 µg DES/g BW or the vehicle alone from day 0. They underwent OVX at day 46 and were killed at day 60. (B) Histology of vaginas from a 60-day-old OVX control (upper panel) and neoDES mice (lower) at low magnification. The sections were stained with hematoxylin and eosin. Scale bar=100 µm. (C) Immunohistochemical staining of Ki-67 antigen with methyl green. The samples are from control (upper) and neoDES vaginas (lower). Scale bar=100 µm.

3-fold) between control and neoDES vaginas were selected for further analysis. Thus, 115 increased genes (22 genes were expressed sequence tags (ESTs)) and 160 decreased genes (53 genes were ESTs) were identified by microarrays.

As expected, the expression of cytokeratin (CK) mRNAs and components of the cornified cell envelope were highly increased in the neoDES vagina (Table 1). Expression of marker genes related to keratinization was also increased; e.g. metallothionein IV (93.7-fold compared with controls) (Quaife *et al.* 1994), transglutaminase 3 (17.1-fold) (Kim *et al.* 1993), peptidylarginine deiminase IV (5.1-fold) (Ishigami *et al.* 1998). In

contrast, expression of CK8 and CK18, which are both preferentially expressed in simple epithelial cells and basal cells in stratified epithelium, was decreased (Bosch *et al.* 1988). Intriguingly, expression of several genes related to the immune system were decreased in neoDES vaginas, as compared with controls (Table 2).

In order to further characterize the neoDES vagina, we focused on aberrant signaling related to proliferation and differentiation of vaginal epithelium. Hence, genes involved in signal transduction were studied in detail. As to genes related to cell signaling from microarray data, Q-PCR was employed to validate the microarray data and

Table 1 List of increased and decreased genes related to the cytoskeleton in vaginas of mice exposed neonatally to DES

	Gene name	Fold changes
Accession no.		
AF053235	Cytokeratin 16 (CK16)	2352
Y09227	Small proline-rich protein (SPR) 3	1552
X91825	SPR 1b	326
X99251	Repetin	111
J03458	Filaggrin	104
AJ005566	SPR 2H	87.4
AJ005564	SPR 2F	64.0
AJ005561	SPR 2C	26.9
V00830	CK10	24.3
AB012042	CK6 β	24.3
K02108	Keratin complex 2, gene 6A	24.3
AJ005562	SPR 2D	23.4
X03491	CK4	19.0
U09189	Loricrin	17.8
AF057156	SPR 1A	16.0
AJ005559	SPR 2A	16.0
AJ005560	SPR 2B	12.6
M10937	CK1	12.6
AJ005568	SPR 2J	10.6
AJ005569	SPR 2K	8.9
U13921	CK13	8.6
AB012033	CK6 α	8.6
AJ005559	SPR 2A	7.7
AB017202	Nidogen-2	7.5
AJ005567	SPR 2I	6.7
AJ005565	SPR 2G	3.6
AJ005563	SPR 2E	3.5
M22832	CK18	-23.4
X15662	CK 8	-8.6
M12481	Actin beta	-6.1

quantify the expression of putative targets of neonatal DES exposure. Table 3 shows a list of genes whose expression was changed more than 3-fold as measured by Q-PCR. The list includes growth factors and cytokines, membrane receptors/proteins, intracellular/signaling factors, transcription factors and their regulators, nuclear proteins, protease inhibitors and miscellaneous genes.

We have analyzed the factors involved in vaginal cell proliferation and differentiation focusing on growth factors and cytokines (Miyagawa *et al.* 2004). The novel finding in the present study is that genes related to the IL-1 system (IL-1L1 and IL-1 RII) were increased in expression in the neoDES vagina. Thus, the IL-1 system is also possibly involved in the persistent vaginal epithelial cell proliferation and differentiation. We also found

that several growth factor-binding proteins were increased (IGF-binding protein (IGFBP)-2 and fibroblast growth factor (FGF)-binding protein (FGFBP)-1) and decreased (IGFBP-3 and latent transforming growth factor- β binding protein (LTBP)-2) in the neoDES vagina as compared with controls. As to IGF-I signaling, its receptor (IGF-IR) was decreased in the neoDES vagina. It is a common property of receptor tyrosine kinase that ligand binding and phosphorylation of receptor ultimately result in downregulation (Pastan & Willingham 1983). Although their ligands (IGFs) did not show 3-fold change on DNA array, these growth factors systems are possibly involved in persistent proliferation of epithelial cells in the neoDES vagina. To confirm this hypothesis, we further analyzed two signaling pathways, the IL-1 and IGF-I cascades, in the neoDES vagina.

Table 2 List of increased and decreased genes related to the immune system in vaginas of mice exposed neonatally to DES

Accession no.	Gene name	Fold changes
AF109905	MHC class III regions Hsc70t	7.5
L38444	T cell-specific guanine nucleotide triphosphate-binding protein (TGTP)	-8.0
AV092014	Peptidoglycan recognition protein (Pglyrp)	-6.7
AF039663	AC133 antigen homolog	-5.9
X13333	CD14 antigen	-5.7
V00746	MHC class I antigen H-2K	-5.5
U69488	Viral envelope like protein (G7e)	-5.3
AB001489	Phosphatidylinositol glycan class R	-4.9
AF076482	Peptidoglycan recognition protein precursor (Pgrp)	-4.9
M58156	MHC class I antigen H-2K-b	-4.4
X00246	MHC class I antigen with a Set 1 repetitive element	-4.4
X16202	MHC class I antigen Q4	-4.3
AF109906	MHC class III region RD	-4.1
X58609	MHC class I antigen Q2-k	-4.0
AJ132098	Vanin-1	-3.9
AF029215	MRC OX-2	-3.7
X58861	Complement component 1 q subcomponent α	-3.6
M18837	MHC class I Q4 beta-2-microglobulin	-3.5

IL-1 signaling in the neoDES vagina

Cytokine networks regulate epidermal tissue homeostasis and keratinocyte proliferation and differentiation (Schroder 1995). Using an *in vitro* skin equivalent model, IL-1 induction regulates epidermal proliferation and differentiation through the induction of KGF/FGF7 and GM-CSF in fibroblasts (Szabowski *et al.* 2000). Therefore, it seemed to be convincing that the IL-1 system also has functions in vaginal epithelial cell proliferation and differentiation.

The time-course of gene expression (IL-1 α , IL-1 β , IL-1RI, IL-1RII, IL-1ra and IL-1L1) was examined in vaginas from 60-day-old OVX control mice after a single injection of E₂ (Fig. 2A). E₂ administration rapidly induced IL-1RI expression within 1 h (5.1-fold compared with 0 h); IL-1RI expression subsequently declined slowly. Expression of the other genes was not changed but IL-1L1 expression increased from 24 h, peaking at 36 h (43.3-fold). In neoDES vaginas, expression of IL-1 α (4.2-fold) and IL-1 RII (30.1-fold) was increased together with high-level expression of IL-1L1 (Fig. 2B). To study the tissue distribution of these factors, epithelium and stroma from neoDES vaginas were separated by incubation in trypsin, followed by dissection and RT-PCR analysis. IL-1 α and IL-1L1 were found to be primarily expressed

in the epithelium whereas IL-1RI and IL-1RII were expressed in both epithelium and stroma (Fig. 2C).

To investigate the participation of IL-1 signaling in the neoDES vagina, we tested the activation status of SAPK/JNK, which is one of the downstream factors of IL-1 (Karin *et al.* 1997). As shown in Fig. 2D, SAPK/JNK (particularly JNK1) was phosphorylated in the neoDES vagina but only slightly phosphorylated in controls. Then, we tested the mRNA expression of KGF and GM-CSF in stromal tissue in the control and neoDES vaginas. In both control and neoDES vaginas, KGF and GM-CSF mRNA were expressed; however, unexpectedly, their expression levels showed no change (Fig. 2E).

IGF-I signaling in the neoDES vagina

In mouse uterus, locally produced uterine IGF-I mediates the effects of E₂ on growth and cell proliferation (Klotz *et al.* 2002). In the neoDES vagina, IGFBP-2 levels were increased, but IGFBP-3 and IGF-IR levels were decreased (Table 3). These data raised the possibility that the IGF-I pathway is modulated in the neoDES vagina, therefore, we further analyzed this signaling pathway in the vagina.

Table 3 List of increased genes related to signalling factors in the vagina of mice exposed neonatally to DES

Increased			Fold changes microarray/Q-PCR
Accession No.	Gene name		
Growth factors and cytokines			
Membrane receptors/proteins			
Intracellular/signaling factors			
Transcription factors and their regulators			
Proteases and protease inhibitors			
Miscellaneous genes			
	Interleukin-1 like protein 1 (IL-IL1)		7-7/124
	IL-1 receptor type II (IL-IRII)		4-6/30-1
	Mucin 1 (episialin)		8-0/3-5
	MECA39/branched chain aminotransferase 1 (BCAT)		4-4/42-5
	IGF-binding protein 2 (IGFBP2)		7-2/23-9
	FGF-binding protein 1 (FGFBP1)		6-7/8-0
	14-3-3 protein sigma		4-1/4-1
	Mad		6-1/14-1
	E74-like factor 5 (Elf5)		4-9/6-3
	Basic domain/leucine zipper transcription factor		4-0/4-1
	Single WAP motif protein 2		68-6/2688
	Squamous cell carcinoma antigen 2 (Scca2)		90-5/832
	T cell-specific protein (Tcl-30)		7-2/258
	Secretory leukoprotease inhibitor (SLPI)		27-9/146
	Bikunin		5-9/30-9
	Neuropsin		6-3/16-8
	Plasminogen activator inhibitor type II		5-1/7-4
	Maspin		4-9/6-5
	Farnesyl diphosphate synthetase		4-1/6-4
	Glutaredoxin		11-7/4-5
	Fatty acid binding protein (Fabpe)		7-2/4-3
	Ceroid-lipofuscinosis, neuronal 8 (Cln8)		6-5/4-0
	Flavin-containing monooxygenase		4-4/3-5
	Arachidonate 12-lipoxygenase pseudogene 2		14-4/3-4
	Cellular retinoic acid binding protein II		7-0/3-1

Table 3 (continued) List of decreased genes related to signalling factors in the vagina of mice exposed neonatally to DES

Decreased			Fold change microarray/Q-PCR
Accession No.	Gene name		
Growth factors and cytokines			
U27267	LPS-induced C-X-C chemokine (LIX)		-8.3/-11.4
M29464	Platelet-derived growth factor a (PDGF-a)		-5.5/-5.3
AF004874	Latent TGF-β binding protein-2 (LTBP2)		-7.7/-33.0
D86563	Rab4		-6.7/-8.2
X94998	Fibromodulin		-6.3/-7.3
M38337	Milk fat globule-EGF factor 8 protein (MFG-E8)		-5.5/-6.6
AF056187	IGF-1 receptor (IGF-1R)		-3.7/-4.2
A1840868	Nedd4-like		-3.9/-6.0
M93422	Adenyl cyclase type VI		-4.6/-5.6
U50631	Heat-responsive protein (HRP12)		-4.1/-4.6
A1851250	Sprouty protein with EVH-1 domain 2		-3.2/-4.5
X81581	IGFBP3		-9.6/-3.9
L07918	GDP-dissociation inhibitor (D4/LyGDI)		-3.9/-3.2
A1845584	Dual specificity phosphatase 6 (Dusp6)		-4.3/-3.0
U88327	Suppressor of cytokine signalling-2 (SOCS-2)		-3.5/-3.0
AJ223069	TCF-3		-6.5/-17.4
X80339	Six1		-7.5/-7.2
D26532	Core binding factor alpha 2 (PEBP2aB2)		-4.0/-5.9
U33005	TBC1		-4.9/-5.9
A1447619	Zinc finger protein 161		-4.4/-5.4
V00727	C-fos		-3.9/-5.2
M94623	Aryl-hydrocarbon receptor (AhR)		-4.6/-4.3
AF074600	LIM-only family of nuclear LIM protein 4		-4.1/-3.7
U32395	Mad4		-3.7/-3.7
AF017085	BAP-135 homolog (Diwslt)		-4.4/-3.2
A1854510	Similar to KIAA0130 gene product		-4.0/-3.1
A1849928	Cyclin D		-8.0/-6.3
AB028921	NAKAP95		-6.3/-4.3
X67209	Npdc-1		-3.9/-3.8
AW121336	WAP domain protein HE4 (He4)		-17.1/-28.3
X57437	L-histidine decarboxylase		-6.6/-13.0
M22679	Alcohol dehydrogenase class I		-9.8/-10.1
M77497	Cytochrome P450 2f2		-8.4/-6.1
D00466	Apolipoprotein		-4.1/-3.5
M83218	Intracellular calcium-binding protein (MRP8)		-6.6/-3.2
Transcription factors and their regulators			
DNA/RNA and nuclear proteins			
Proteases and protease inhibitors			
Miscellaneous genes

Figure 2 IL-1-related gene expression and its downstream signaling in the mouse vagina.

(A) Time-course analysis of IL-1-related gene expression. Samples were from vaginas of mice 0 (control), 1, 3, 6, 12, 24, 36 and 48 h after a single injection of 50 ng E₂/g BW. The right panel shows an enlarged view of the 0–6 h range of the left panel. More than three mice were used per each group. Error bars represent the standard error. (B) Expression profiles of IL-1-related genes in vaginas of 60-day-old control and neoDES mice. (C) Tissue distribution of IL-1 α , IL-RI, IL-1RII and IL-1L1 mRNA expression in the neoDES vagina. E, epithelium; S, stroma; W, whole tissue. (D) Activation status of JNK was detected by anti-phospho-JNK antibody. The samples are from control (lane 1) and neoDES mice (lane 2). (E) Expression of KGF and GM-CSF mRNA in vaginal stromal tissue in control and neoDES vaginas.

Figure 3 Activation status of the IGF signaling pathway in the neoDES vagina. (A) Expression of IGF-I mRNA in vaginal tissue in control and neoDES vaginas. More than three mice were used in each group. Error bars represent the standard error. (B) Phosphorylation of IGF-IR was detected by anti-phospho-IGF-IR antibody. The samples were from vaginas of OVX mice treated with oil (lane 1), DES (lane 2) and neoDES mice (lane 3). (C) IGF-I-induced phosphorylation of Akt in the adult OVX mouse vagina. The samples were from vaginas of OVX mice treated with saline (lane 1) and long R3 IGF-I (lane 2). (D) Activation status of Akt was detected by anti-phospho-Akt antibodies. The samples were from vaginas of OVX mice treated with oil (lane 1), DES (lane 2) and neoDES mice (lane 3).

IGF-I mRNA level in the whole tissue was not altered between control and neoDES vaginas (Fig. 3A and Miyagawa *et al.* 2004). However, when compared in the stromal component, IGF-I mRNA level was increased in the neoDES vagina (Fig. 3A). As shown in Fig. 3B, distinct phosphorylation of IGF-IR was detected in the neoDES vagina and it is similar to vaginas of mice treated with DES successively as a positive control. IGF-IR in vaginas of oil-treated control mice showed a low level of phosphorylation, although IGF-IR protein was expressed. To further characterize the factors downstream of IGF-I signaling in the neoDES mouse, we examined the activation status of Akt, which is known as one of the major regulatory factors in IGF-I signaling (Martin *et al.* 2000). As in the mouse uterus (Klotz *et al.* 2002), IGF-I administration induced phosphorylation of Akt in vaginas of adult OVX mice (Fig. 3C). Furthermore, we detected Akt activation in the vaginas of

neoDES and DES-treated mice but not in oil-treated controls (Fig. 3D).

Discussion

We examined the global expressions of mRNAs, focusing on factors involved in cell signaling in the neoDES vagina, which shows persistent vaginal hyperplasia and keratinization (Takasugi *et al.* 1962, McLachlan *et al.* 1980, Iguchi 1992). First, we intended to clarify the overall expression patterns of genes in the neoDES vagina which seemed to be useful for further analysis. Using DNA microarray, we found altered expression of several genes in the neoDES vagina compared with controls and detected genes showing altered expression which are candidates for mediating the persistent proliferation and differentiation. Nonetheless, each gene needs to be elucidated in detail; in this report, we

focused on the two signaling pathways, the IL-1 and IGF-I systems, which are possibly involved in cellular signaling in the vagina.

IL-1 signaling

The cytokine IL-1 elicits pleiotropic biological effects in various tissues, including the regulation of epidermal differentiation and inflammation in skin (Dinarello 1998). Although the function of IL-1L1 remains undescribed (Barton *et al.* 2000), its abundant expression in skin and vaginal epithelium suggests that it may be involved in epithelial cell differentiation (Mulero *et al.* 1999). IL-1L1 expression was elevated 24 h after E₂ administration, similar to other differentiation markers such as CK1 (data not shown). Intriguingly, expression of IL-1 α , which showed no change in expression after estrogen stimulation in adult OVX mice, was increased in the neoDES vagina. Although IL-1 α and IL-1L1 may not be estrogen-primed genes, these data suggested that IL-1 signaling has a functional role in the maintenance of epithelial cell differentiation, rather than estrogen-primed cell proliferation in the vagina. The negative regulator of IL-1 signaling, IL-1RII, was also highly expressed in the neoDES vagina. Stimuli that induce IL-1 gene expression in keratinocytes *in vitro*, also strongly stimulate IL-1RII expression (Groves *et al.* 1995), and a high level of IL-1RII is expressed in keratinocytes in psoriasis (Groves *et al.* 1994). Thus, the increased expression of IL-1RII in the neoDES vagina showing ovary-independent stratification and keratinization is not surprising.

IL-1 stimulates the activity of the transcription factors AP-1 and nuclear factor κ B (NF- κ B). These effects are mediated through activation of SAPK/JNK and inhibitor of NF- κ B kinases respectively. In the present study, we found that JNK1 was activated in the neoDES vagina. IL-1 induced in the epidermis is known to regulate KGF and GM-CSF in the dermis through AP-1 (Szabowski *et al.* 2000). We supposed that there is the presence of similar regulation by IL-1 signaling in both epidermis and vaginal epithelium. KGF and GM-CSF were expressed in both control and neoDES vaginas and their expression levels were not different. Therefore, it is unlikely that KGF and GM-CSF have direct positive roles in epithelial proliferation and differentiation in the vagina. Actually, estrogen treatment did not induce mRNA

expression of KGF and GM-CSF in the vagina (Masui *et al.* 2003, Miyagawa *et al.* 2004), and KGF-null mice are fertile and morphologically normal (Guo *et al.* 1996). This is in contrast to skin, where KGF effects are crucial. Defects in the skin and hair follicles were observed in KGF-null mice (Guo *et al.* 1996). Overexpression of KGF in transgenic mice induced altered epidermal growth and differentiation, including wrinkled skin and increased epidermal thickness (Guo *et al.* 1993). Thus, KGF contributed less to vaginal epithelial differentiation than to that of skin, however, non-physiological amounts of KGF treatment may affect vaginal keratinocyte differentiation. It was reported that neonatally KGF-treated mouse vaginas showed persistent epithelial differentiation (Hom *et al.* 1998). Although we indicated differences between skin and vagina, both showed stratification and keratinization in the epithelium, and the roles of the IL-1 system, KGF, GM-CSF and also JNK1 phosphorylation in the vagina remain unknown.

IGF-I signaling

In the mouse uterus and vagina, epithelial proliferation appears to be a paracrine event mediated by stromal soluble factors, but these factors have not been identified (Cooke *et al.* 1997, Buchanan *et al.* 1998). Although the stromal contribution to persistent activation of vaginal epithelium is less than that of normal E₂-induced action in the vagina (Cunha *et al.* 1977, Miyagawa *et al.* 2004), some stromal factors must be contributing to the persistent activation in the neoDES vagina. Such candidates were not directly detected on DNA array data, which is probably due to the conservative adoption of 3-fold differences in the present microarray analysis. In the present study, IGF-I mRNA derived from vaginal stroma was increased by estrogen treatment as compared with controls but was not changed at whole tissue levels. This IGF-I signaling may be enhanced by its growth factor-binding factors, which influence growth factor availability and activity, because IGFBP-3 levels were decreased. As a result, IGF-IR is phosphorylated, leading to Akt phosphorylation, which suggests that IGF-I signaling is active in the neoDES vagina, where it may regulate several biological processes. Of course, other growth factors have a potential involvement

in persistent signaling in the neoDES vagina. A large amount of epithelial cells makes it difficult to detect genes derived from stroma. We need to further analyze the gene expression from the stromal component. Still, IGF-I is one of the candidates of stromal factors which mediate the vaginal epithelial cell proliferation.

We found that IGFBP-3 was downregulated whereas IGFBP-2 was highly expressed in the neoDES vagina. IGFBP-2 generally regulates IGF-induced action by binding to IGFs. Although IGFBP-2 inhibits IGF action, IGFBP-2 is overexpressed in various malignant tissues, often correlating with tumorigenesis (Zumkeller 2001). It should be noted that some of the effects of IGFBP-2 overexpression on cell proliferation are proposed to be independent of IGFs (Slootweg *et al.* 1995, Badinga *et al.* 1999). Therefore, the elevated levels of IGFBP-2 we detected could be directly involved in the inappropriate epithelial proliferation in neoDES vaginas.

Akt phosphorylation

In the present study, we found that Akt was phosphorylated by estrogen in the mouse vagina, which was similar to the uterus, as described in the demonstration that Akt was a functional mediator of estrogen-induced cell proliferation (Klotz *et al.* 2002). In addition, expression of active Akt in MCF-7 cells results in estrogen-independent tumor growth (Faridi *et al.* 2003). Akt promotes cell survival through phosphorylation and inactivation of several pro-apoptotic targets, such as Bad and forkhead transcription factors which indirectly affect NF- κ B and p53 (Brunet *et al.* 1999, Downward 1999, Vivanco & Sawyers 2002). These observations indicate that Akt is involved in estrogen-dependent and/or -independent cell proliferation and even carcinogenesis. The present study showed that Akt phosphorylation occurred in the neoDES vagina even in the absence of estrogen stimulation by OVX, and it may be involved not only in the persistent cell proliferation but also in carcinogenesis in the neoDES vagina later in life.

Other cellular events in the neoDES vagina

We detected additional genes that are expected to be involved in vaginal epithelial cell differentiation. For example, various proteases and protease

inhibitors were highly expressed in neoDES vaginas. The protease inhibitors are involved in keratinocyte differentiation or epidermal wound repair (Tamechika *et al.* 1996, Cui *et al.* 1999, Katz & Taichman 1999, Li *et al.* 2000, Zhu *et al.* 2002). The serine protease, neuropsin, is expressed in superficial layers of keratinized epithelium in the mouse vagina (Katsu *et al.* 2002). These genes are thought to be involved in fibrinolysis, inflammation, cell migration and growth factor proteolysis by regulating the activity of other proteases (Travis & Salvesen 1983, Carrell *et al.* 1989, Potempa *et al.* 1994). Thus, proteases and protease inhibitors identified in this study may be involved in the differentiation of mouse vaginal epithelium.

Application to DES syndrome in the human

Although estimates vary, about four million women have been exposed to DES during pregnancy (Newbold 1993). Epidemiological studies indicate an increased incidence of breast cancer, squamous neoplasia of the cervix and vagina, and vaginal clear-cell adenocarcinoma in women exposed to DES *in utero* (Herbst 2000, Hatch *et al.* 2001, Palmer *et al.* 2002). In addition to the aberrant cellular signal transduction, a hypothesis for the association between DES and high-grade disease is that DES *in utero* possibly induced permanent alteration in the immune system. Profound alterations in the immune system, especially of natural killer cells, were found in mice treated neonatally with DES (Kalland 1982). In the present study, we demonstrated that expression of genes related to the immune system was decreased, which could lead to a locally reduced immune function in neoDES mice. In humans, permanent alterations in the immune system may lead to a reduced ability to fight off a genital infection, such as human papilloma virus, thereby increasing the susceptibility to secondary risk factors for carcinogenesis. Further characterization of the perinatally estrogen-exposed mouse model is needed to fully understand the potential risk of the carcinogenic effects of estrogens.

In conclusion, we characterized the gene expression pattern of the neoDES vagina. In such vaginas, the IGF-I signaling cascade was activated, resulting in Akt phosphorylation. Aberrant Akt phosphorylation is one of the key regulators of the vagina showing persistent proliferation. We also

found JNK-1 phosphorylation status and expression of various genes were changed. These complicated expressions of genes and protein phosphorylation status lead to vaginal changes and the present findings give insight into the persistent activation in vaginas from neonatally DES-exposed mice.

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