Analysis of Estrogen-Responsive Genes in Reproductive Organs of Female Mice

マウス雌性生殖器官における

器官特異的エストロゲン応答遺伝子の解析

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Preface

Exposure to estrogen or diethylstilbestrol (DES), a synthetic estrogen, during perinatal development results in various reproductive abnormalities in mouse, such as oviductal tumors, uterine epithelial metaplasia, persistent vaginal stratification and keratinization, vaginal adenosis and cervico-vaginal carcinomas. In human, DES exposure *in utero* was reported to case the vaginal carcinoma and uterine abnormalities in babies (Herbst *et al.*, 1971).

The previous studies in mouse suggest the reproductive abnormalities were resulted from the reduction of morphological genes, Wnt7a and Hoxa-10 (Satokata *et al.*, 1995; Benson *et al.*, 1996; Parr *et al.*, 1998). Gene expressions related to morphogenesis are regulated differently at developmental stage and which defines cell fate followed by organ specificity (Miller *et al.*, 1998a). The timing of the morphological gene expression may be closely related to a critical period of normal development in the reproductive tract of mice. In the previous reports, however, the knockout mice of Wnt7a and Hoxa10 failed to explain over various reproductive abnormalities.

DNA microarray is an useful tool as a screening of novel functional genes.

Estrogen-responsive genes were reported in the abnormal vagina of mice given DES neonatally and in the uterus of normal females (Miyagawa *et al.*, 2004a; Watanabe *et al.*, 2002). A series of estrogen-responsive genes was represented as squalene, cholesterol metabolism product, cell cycle regulators in adult uterus (Watanabe *et al.*, 2002). By contrast, in the mouse vagina, epidermal growth factor (EGF) receptor and EGF-like growth factors were up-regulated by DES given neonatally (Miyagawa *et al.*, 2004a). There is little evidence on the estrogen-responsive genes relating to organ specificity and critical period during perinatal development. I studied, therefore, estrogen-responsive genes using DNA microarray to understand organ specificity in Müllerian duct (Chapter1), age-specificity including with critical period in the vagina (Chapter2) and the organ specificity in the mature organs derived from the Müllerian duct, uterus and vagina, or not, mammary gland (Chapter3).

In order to understand the molecular background of the Müllerian duct abnormalities induced by DES, gene expression was examined on gestational day (GD) 19 following a 8-day exposure of DES to mothers. I focused on Eph receptor family genes and Wnt antagonist, Dkk2, Nkd2 and sFRP1, selected by the microarray analysis, and Wnt and Hox genes for further study (Chapter 1).

Second, I examined the mechanisms underlying the reversible and irreversible cell proliferation in vaginae induced by DES before and after the critical period. Global gene expression and proliferation after a single DES injection were examined in mouse vaginae at 0, 5, 20 and 70 days of age (Chapter2).

Third, I examined the differences of mitosis and estrogen-responsive genes among the uterus, vagina and mammary gland of mice. The organ specificities of these gene expressions were correlated with mitosis in the uterus, vagina and mammary gland (Chapter3).

II.Chapter 1

Gene expression change in the Müllerian duct of the mouse fetus exposed to diethylstilbestrol *in utero*

Introduction

Prenatal diethylstilbestrol (DES) exposure induces persistent malformations of male and female reproductive organs in mice. Female mice exposed perinatally to DES showed non-coiled oviduct, uterine metaplasia, disorganization of uterine circular muscles and ovary-independent vaginal epithelial stratification and cornification (Newbold *et al.*, 1983; Iguchi *et al.*, 1987; 1988; 1992; 2002; Ozawa *et al.*, 1991).

DNA microarray has been successfully used to analyze estrogen-responsive genes in the mouse uterus and vagina, and genes possibly related to persistent vaginal proliferation induced by neonatal DES exposure (Watanabe *et al.*, 2002; 2003a,b, 2004; Miyagawa *et al.*, 2004; Suzuki *et al.*, 2006). Therefore, we studied global gene expression including signal transduction and organogenesis genes in the Müllerian duct after DES exposure *in utero* using microarray at GD19 and selected several genes for further study. We focused on the expression of ephrin, Eph family, Wnt, Wnt-antagonists and Hoxa genes.

Hox genes, expressed in spinal cord, limb and reproductive tracts, determine anterior to posterior body axis as the same genetic line on chromosomal loci. Abdominal-B genes, the most of 5' Hox genes, are expressed in the Müllerian duct

along the axis; a-9, a-10, a-11, and a-13 from anterior to posterior at gestation day (GD) 15.5 (Taylor *et al.*, 1997). With these positional expressions of Hox genes along anterior to posterior axis, Müllerian duct differentiates into three reproductive organs such as oviduct, uterus and upper vagina. Lack of positional Hox gene expressions is considered to result in the reproductive abnormalities because of the loss of organ specificity (Satokata *et al.*, 1995; Benson *et al.*, 1996). While, lack of Hoxa-13 expression caused the failure of differentiation in caudal Müllerian duct (Warot *et al.*, 1997). DES repressed the expression of Hoxa-10 and a-11 in mouse uterus at GD 17 followed by reduced reproductive performance, including embryo implantation, assessed in adult offspring (Ma *et al.*, 1998; Block *et al.*, 2000). Failure of the segment-related positional identity in vertebrates was reported widely in the lack of Hox genes disturbed the body axis in limb, spinal cord, hind brain and reproductive tracts (Satokata *et al.*, 1995; Benson *et al.*, 1996; Carpenter *et al.*, 1993, 2002; Kmita *et al.*, 2005; Daftary *et al.*, 2006). However, mechanism of the morphogenetic regulation by Hox genes in DES-exposed Müllerian duct is unknown.

In limbs, Hoxa-13 knockout mice showed the down-regulation of Eph receptor A7 and inhibition of mesenchymal cell adhesion and apoptosis (Stadler *et al.*, 2001).

Eph receptors are tyrosine-kinase protein receptors and they regulate cellular movement underlying critical events of development by binding to ephrin ligands (Wilkinson *et al.*, 2001; Kullander *et al.*, 2002). Eph receptor-ephrin regulates cell migration, formation of the tissue boundary and path finding of axons in vertebrates (Coulthard *et al.*, 2002). Moreover, Eph signaling induces cytoskeletal regulation, mitogenic response via ERK/MAPK, and fluid homeostasis in cell-cell communication (Kullander *et al.*, 2002). Functions of Eph families in Müllerian duct, however, have not been reported yet.

Epithelial-mesenchymal differentiation in the Müllerian duct is regulated by Wnt signaling correlated with Hox genes. In female reproductive organs, Wnt-4, -5a and -7a are expressed (Miller *et al.*, 1998c). Lack of Wnt-7a induced uterine metaplasia like DES-exposed mice *in utero* (Parr *et al.*, 1998). 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 early development of female reproductive tracts (Vainio *et al.*, 1999).

In the Müllerian duct, functions of Wnt antagonists have not been clarified yet. Serected frizzled related protein (sFRP) competes with Wnt receptor and frizzled (Fz) receptors (Jones *et al.*, 2002), and sFRP2 was down-regulated by estrogen in adult

mouse uterus (Hou *et al.*, 2004). Dicknock (Dkk) inhibits Wnt pathway indirectly since it induces endocytosis of Wnt-Fz receptor complex and binds a second receptor, LRP5/6 (Glinka *et al.*, 1998; Zorn *et al.*, 2001; Kawano *et al.*, 2003). Dkk1 promotes head formation in *Xenopus* (Glinka *et al.*, 1998). Dkk1, 2 and 3 expressions were reported in mouse embryo heart, tooth, kidney, palate, limb bud and neural epithelium for epithelial-mesenchymal cell transformation (Monaghan *et al.*, 1999). Naked cuticle (Nkd) inhibits Wnt signaling via Dishevelled receptor and it also one of the last segment polarity genes (Jones *et al.*, 2002; Zeng *et al.*, 2000; Rousset *et al.*, 2001). Nkd1 was expressed in forelimb and neural crest of mouse embryos, and Nkd1 and 2 are expressed in tail bud and subepithelial mesenchyme of tongue, soft plate, snout and skin in the mouse embryos (Wharton *et al.*, 2001). However, Wnt antagonist such as Dkk, Nkd and sFRP have not been reported in the fetal mouse uterus.

In order to understand molecular mechanisms underlying reproductive tract abnormalities in female mice induced by prenatal DES exposure, we analyzed expression changes in Eph family, Wnt, Wnt-antagonists and Hoxa genes after DES exposure.

Materials and Methods

Animals

Mice of ICR/Jcl strain kept under 12 h light/12 h dark at 23-25˚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 National Institutes of Natural Sciences. The day on which a vaginal plug was found was considered as gestation day (GD) 0. Diethylstilbestrol (DES, Sigma Chemical Co., St. Louis, MO, USA) was dissolved in sesame oil. Pregnant mice were given daily injections of 67 μg DES/kg maternal body weight or the oil vehicle alone from GD 10 to 18 as described previously (Suzuki *et al.*, 2002). These experiments were repeated 3 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 labled 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 described (Watanabe *et al.*, 2002). Microarray analysis was performed triplicate using 3 different samples.

Data Analysis. Scanned data were analyzed with GeneChip Suit Analysis Software ver.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 (Li *et al.*, 2001), 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, USA) 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 were divided by those from control samples. These selected genes were listed on http://www.nibb.ac.jp/bioenv1/suzuki/suzukidata004.html. These raw data were loaded into NCBIs Gene Expression Omnibus as the dataset GSE1886 (GEO,

http://www.ncbi.nlm.nih.gov/geo/). Categories in DES-regulated genes were determined from GEO database.

Quantitative real-time PCR

Total RNA was purified as described above. cDNA was synthesized from purified total RNA with Superscript II RT(-) (Invitrogen), and random primers at 42 ˚C for 60 min. PCR reactions were performed in the PE Prism 5700 Sequence Detection System (PE Biosystems, Tokyo, Japan) with SYBR-Green PCR core reagents (Applied Biosystems Japan, Tokyo, Japan) in the presence of appropriate primers, according to the manufacturer's instruction. 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 at triplicate in the following conditions: 2 min at 50 ˚C and 10 min at 95 ˚C, followed by a total of 40, two-temperature cycles (15 s at 95 ˚C and 1 min at 60 ˚C). Model 7000 software was used to construct amplification plots from extension-phase fluorescent emission data collected during PCR amplification. Threshold (Cτ) 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 analysis in Q-PCR was conducted by ANOVA test.

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 were average 0.980 (minimum: 0.967, maximum: 0.996). Genes showing at least 2-fold expression change in DES-exposed mice were listed in http://www.nibb.ac.jp/bioenv1/suzuki/suzukidata004.html.

To examine the gene expression changes by DES in the three organs of the Müllerian duct origin, we analyzed clustering pattern of DES-regulated genes. Clustering analysis in controls revealed that organ specificity of gene expression. Genes in the uterus were close to the oviduct than the vagina. However, clustering

analysis in DES-exposed mice revealed that DES-regulated genes in the three organs show less organ specificity as compared to controls (Fig. 1).

DES up-regulated 387,387 and 225 genes, and down-regulated 177,172 and 75 genes in the oviduct, uterus and vagina, respectively (Fig. 2). 72 up-regulated genes and 15 down-regulated genes were commonly found in the three organs. In the oviduct, DES up-regulated and down-regulated genes were 233 and 105, respectively. In the uterus, DES up-regulated and down-regulated genes were175 and 86, respectively. While, the vagina showed 77 DES up-regulated and 26 down-regulated genes (Fig. 2).

We focused on genes related to signal transduction and organogenesis in DES-exposed Müllerian duct (Table 2). Expressions of RAB 20 and E74-like factor 3 were up-regulated in all DES-exposed organs. While, expressions of prostaglandin E receptor 3, tumor necrosis factor receptor superfamily member 19, Eph receptor A7 and naked cuticle 2 (Nkd2) were down-regulated in all DES-exposed organs (Table 2).

Several organ-specific genes in DES-exposed mice were expressed (Table 2). Forkhead box J1 (foxj1), expressed in ciliated cells in the oviduct (41), was one of oviduct-specific genes in DES-exposed oviduct. While, insulin-like growth factor-I (IGF-I) and homeo box, msh-like 1 (Msx1) and fibroblast growth factor 9 (Fgf9) were

uterus-specific genes in DES-exposed uterus. In DES-exposed oviduct and uterus, Dickkopf (Dkk) homolog 2 and 3 were up-regulated, and ephrin B2, growth differential factor 10 and serected frizzled-related sequence protein 1 (sFRP1) were down-regulated (Table 2)

Hoxa-11 and Hoxd-10 were down-regulated in DES-exposed oviduct. Moreover, expression of Hoxd-9 was down-regulated in DES-exposed oviduct and uterus (Table2). In Wnt family genes, Wnt-4 was up-regulated only in DES-exposed vagina. While, Wnt-6, Wnt-7a, and Wnt-11 genes were commonly down-regulated in DES-exposed uterus.

Four genes of Eph families, ephrin B2, Eph receptor A3, A4 and A7, and three Wnt antagonists showed altered expressions in DES-exposed female reproductive tracts (Table 2). Thus, we further studied Hoxa, Wnt, Eph families and Wnt antagonists genes by Q-PCR.

The Expression of Hox and Wnt Genes in Female Reproductive Tracts by Q-PCR

At GD19, Q-PCR revealed that DES down-regulated Hoxa-10 mRNA in the oviduct (Fig. 3). Hoxa-9 mRNA was up-regulated but Hoxa-10 was down-regulated by

DES in the uterus. DES didn't alter the expression of Hoxa-11 in all organs, and Hoxa-13 in the vagina. Up-regulation of Hoxa-13 mRNA expression was observed in the oviduct and uterus by DES. Expression of Wnt-7a mRNA was down-regulated by DES (Fig.4). While, expression of Wnt-5a mRNA was elevated by DES in all organs. The expression of Wnt-4 was apparently down-regulated in the oviduct. In the DES-treated uterus, the expression of Wnt-4 was also down-regulated but this did not reach statistical significance (p-0.06, vs. organ-matched control). Whilst, up-regulation of Wnt-4 mRNA expression was observed in the DES-treated vagina, but this did not also reach statistical significance (p-0.06, vs. organ-matched control)(Fig. 4).

Gene Expression of Eph Family and Wnt Antagonists in the Müllerian Duct

After DES exposure, ephrin B2 mRNA was down-regulated in the oviduct and uterus but not in the vagina. Eph receptor A3 expression was up-regulated in the oviduct and vagina but not in the uterus. Eph receptor A4 and A7 mRNA were down-regulated by DES in all organs (Fig. 5).

In DES-exposed mice, expression of Dkk2 mRNA was up-regulated in the oviduct and

uterus but it was down-regulated in the vagina (Fig. 6). Expression of Nkd2 mRNA was down-regulated by DES in all organs. Expression of sFRP1 mRNA was down-regulated by DES in the oviduct and uterus (Fig. 6).

Some of the microarray data (Table 2) and Q-PCR data (Figs. 5 and 6) were not consistent for Eph receptor A3, Eph receptor A4, Nkd2 and sFRP1. Therefore, we relied on the Q-PCR data for discussion.

Discussion

In utero exposure to DES induced reproductive abnormalities in mice and human (Suzuki *et al.*, 2002; Herbst *et al.*, 1971; McLachlan *et al.*, 1980). DES-induced malformation of reproductive organs has been considered to be caused by disruption of Hoxa genes expression along A/P axis in the Müllerian duct (Ma *et al.*, 1998). While, Wnt signalings regulate and maintain Hoxa gene expression in the Müllerian duct (Miller *et al.*, 1998a, b, c). DES-induced repression of Wnt-7a gene has been linked to developmental effects on mouse reproductive tracts (Ma *et al.*, 1998; Couse *et al.*, 2001; Huang *et al.*, 2005).

Previous studies reported down-regulation of Hoxa-10 and a-11 at GD 17 in DES-exposed uterus and down-regulation of Hoxa-9 in DES-exposed oviduct (Ma *et al.*,

1998; Block *et al.*, 2000). In the present study, we confirmed the decrease in Hoxa-10, but we did not observe a change in uterine Hoxa-11. Four antisense cDNAs for Hoxa-11 have been described in a cDNA library from mouse embryo limb (Hsieh-Li *et al.*, 1995). It may be that changes in uterine Hoxa-11 mRNA were not detected by our Q-PCR because of the presence of the anti-sense strand DNA.

In the present study, DES did not down-regulate expression of Hoxa-13 mRNA in the vagina at GD 19. The same DES treatment *in utero* induced ovary-independent vaginal stratification and cornification in mice (Suzuki*et al.*, 2002). Thus, the ovary-independent vaginal changes may not be related to Hoxa-13. Interestingly, cluster analysis performed herein revealed that the pattern of gene expression in the vagina, either in control or DES-treated animals, differed significantly from those of the oviduct and uterus. Hoxa-10 expression is required for oviductal formation and uterine growth (Satokata *et al.*, 1995; Benson *et al.*, 1996). Thus, molecular mechanism of growth and differentiation in the caudal Müllerian duct is different from other regions.

Dkk2 acts as an antagonist of Wnt signaling to induce endocytosis of Wnt-Fz receptor complex and is activated by β-catenin (González-Sancho *et al.*, 2005). DES down-regulated Dkk2 expression in the vagina but up-regulated in the oviduct and

uterus in the present study. In the growth and differentiation of vagina, Wnt signaling regulates vaginal growth influenced by epithelial-mesenchymal interaction. Importance of epithelial-stromal interaction has been reported in the developmental effects of estrogens including DES on neonatal mouse vaginal epithelium, which are mediated through stromal estrogen receptor (Bigsby *et al.*, 1990; Cunha *et al.*, 2004). Loss of Wnt-7a caused vaginal adenosis and concretions (Miller *et al.*, 1998a, b) and loss of Wnt-5a caused absence of vagina (Mericskay *et al.*, 2004). Developing vagina during perinatal period expressed Wnt-5a and -7a, but not Wnt-4. Expression of Wnt-7a in the vagina disappeared by 10 days of age, and adult vagina expressed Wnt-4 and -5a genes in the epithelium (Miller *et al.*, 1998c). In normal neonatal vagina, Wnt-7a regulates the reduction of Wnt-4 (Parr *et al.*, 1998). However, in DES-exposed vagina, the reduction of Wnt-7a may cause the reduction of Dkk2 expressions. Thus, vaginal epithelium in DES-exposed fetus differentiate into squamous cells like adult cells, followed by repression of Dkk2.

DES repressed expression of Nkd2 and sFRP1 in the oviduct and uterus, and Nkd2 in the vagina. This is the first report showing expression of Wnt antagonists and their estrogen regulation in tissues derived from the Müllerian duct. Further studies are

needed to clarify the role of Wnt antagonists during development of the Müllerian duct.

Eph receptor-ephrin signaling is a trigger regulating developmental patterning (Coulthard *et al.*, 2002). Eph family genes are down-stream genes of Hox genes (Stadler *et al.*, 2001). Hoxa-9 directly regulates the transcription of Eph receptor B4 in endothelial cells followed by increased cell migration and tube formation (Bruhl *et al.*, 2004). In embryo limb, miss-expression of Hoxa-13 caused down-regulation of Eph receptor A7 resulting in inhibition of apoptosis (Stadler *et al.*, 2001). In the present study, DES-induced down-regulation of ephrin B2 mRNA as well as Hoxa genes was found in the oviduct and uterus. Moreover, down-regulation of Eph receptor A4 and A7 was found in all three organs by DES. Eph family of proteins may regulate pattern development in the Müllrerian duct by inducing changes in cytoskeleton dynamics, mitogenesis and integrin signaling, as they are reported in other organs (Kullander *et al.*, 2002).

Figure 7 summarized the expression change of Eph families, Wnt, Wnt antagonist and Hox genes induced by DES *in utero*. Further studies are needed to understand functional relationship of these genes in the developing mouse reproductive tracts and relation to reproductive tract abnormalities induced by DES.

Some of the microarray data and Q-PCR data were not consistent for Hoxa-11 in oviduct, Eph receptor A3 in vagina, Eph receptor A4 in all organs, Nkd2 in vagina and sFRP1 in oviduct. Therefore, we relied on the Q-PCR data for discussion. Recently, a new microarray method has been proposed to use "per cell" normalization method for mRNA measurement (Kanno *et al.*, 2006), which will give us consistent data between microarray and Q-PCR.

In conclusion, microarray analysis revealed the presence of organ-specific genes in the oviduct, uterus and vagina, and candidate genes related to reproductive abnormalities for further study. About 400 genes were up-regulated and 200 genes were down-regulated in the oviduct and uterus by DES *in utero*. Vagina showed less than half of the number of DES-regulated genes than those found in the oviduct and uterus. Down-regulation of ephrin B2, Eph receptor A4, A7 and Nkd2, accompanied with changes in Hox and Wnt gene expression, may lead to abnormalities of segment-related positional identity in DES-exposed the upper part of the Müllrerian duct. In addition, down-regulation of Dkk2 mRNA in DES-exposed vagina is possibly correlated with persistent vaginal epithelial stratification.

Figure Legends

Fig. 1. Cluster analysis of DES-regulated genes in the oviduct, uterus and vagina at GD19. Red, DES-up-regulated genes; Black, unchanged genes; Green, DES-down-regulated genes. Related expression patterns are grouped.

Fig. 2. Venn diagrams of number of DES-regulated genes in GD19 oviduct, uterus and vagina at gestational days. a) Number of up-regulated genes by DES in the Müllerian duct. Number of DES-up-regulated genes was the smallest in the vagina. b) Number of down-regulated genes by DES in the Müllerian duct. Number of DES-up-regulated genes was the smallest in the vagina. DES-down regulated genes revealed large number of oviduct-specific genes.

Fig. 3. Quantification of Hoxa-9, a-10, a-11 and a-13 mRNA expressions in the oviduct, uterus and vagina at GD 19 by Q-PCR. Results were normalized by ribosomal L8. Ratios were calculated relative expression in the control uterus. Ov: oviduct, Ut: uterus, Vg: vagina. #, $p< 0.05$ vs. control uterus; *, $p< 0.05$ v.s. organ-matched control groups

Fig. 4. Quantification of Wnt-4, -5a and -7a mRNA expressions in the oviduct, uterus and vagina at GD 19 exposed to DES at DG10-18 by Q-PCR. Results were normalized by ribosomal L8. Ratios were calculated relative expression in the control uterus. Ov: oviduct, Ut: uterus, Vg: vagina. #, $p< 0.05$ vs. control uterus; *, $p< 0.05$ v.s. organ-matched control groups

Fig. 5. Quantification of ephrin B2, Eph receptor A3, A4 and A7 mRNA expressions in the oviduct, uterus and vagina at GD19 exposed to DES at GD10-18 by Q-PCR. Results were normalized by ribosomal L8. Ratios were calculated relative expression in the control uterus. Ov: oviduct, Ut: uterus, Vg: vagina. #, $p< 0.05$ v.s. control uterus; *, $p< 0.05$ v.s. organ-matched control groups

Fig. 6. Quantification of Dkk2, Nkd 2 and sFRP1 mRNA expressions in the oviduct, uterus and vagina at GD19 exposed to DES at GD10-18 by Q-PCR. Results were normalized by ribosomal L8. Ratios were calculated relative expression in the control uterus. #, $p< 0.05$ v.s. control uterus; *, $p< 0.05$ v.s. organ-matched control groups

Fig. 7. Summary of mRNA expression of Eph family, Wnt, Wnt antagonists and Hoxa genes in DES-exposed Müllerian duct. Eph: Eph receptor.

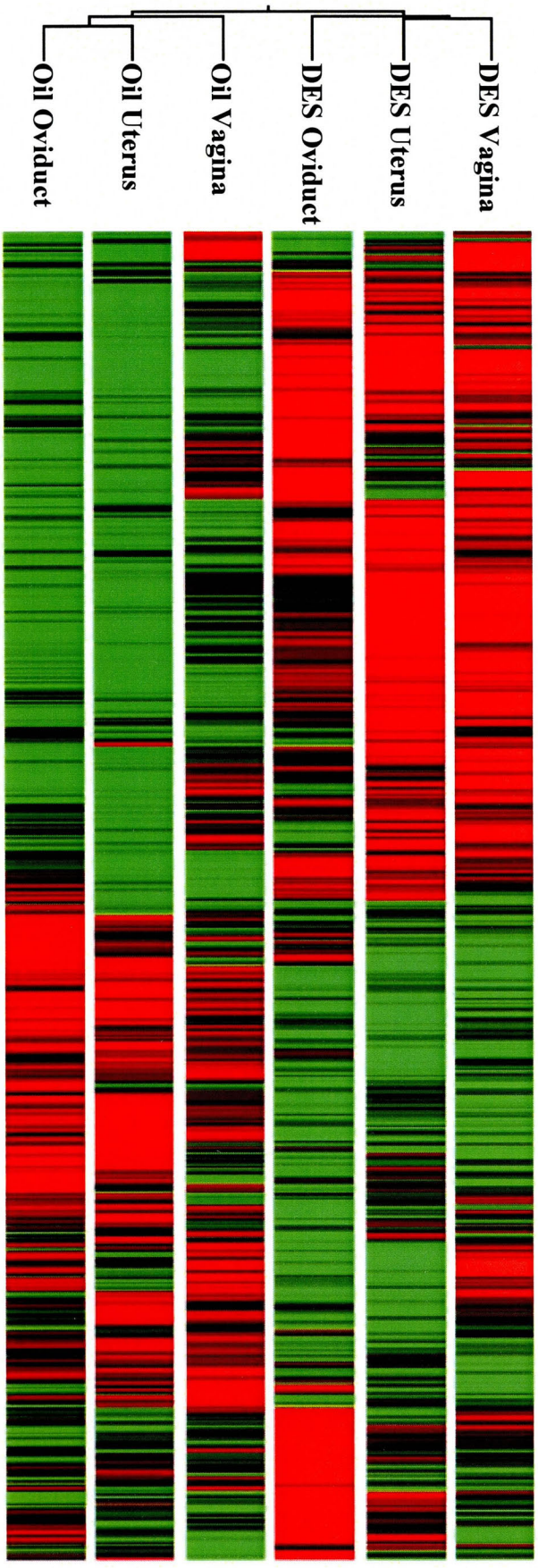
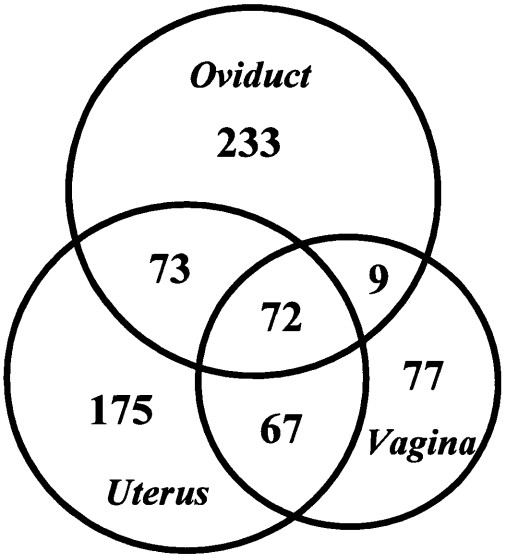

Oil Oviduct
Oil Uterus
Oil Vagina
DES Oviduct
DES Uterus
DES Vagina

Figure 1

a. Up-regulated genes



b. Down-regulated genes

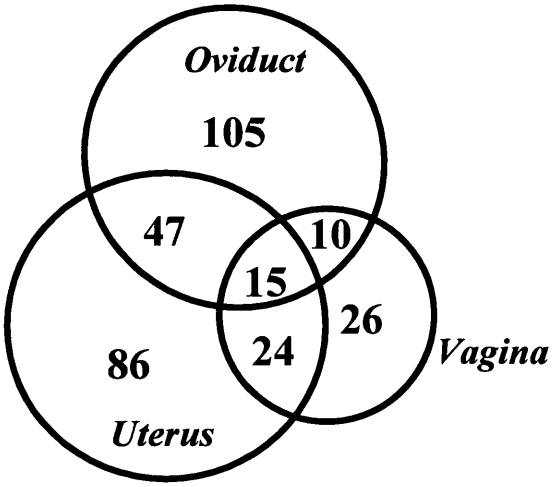
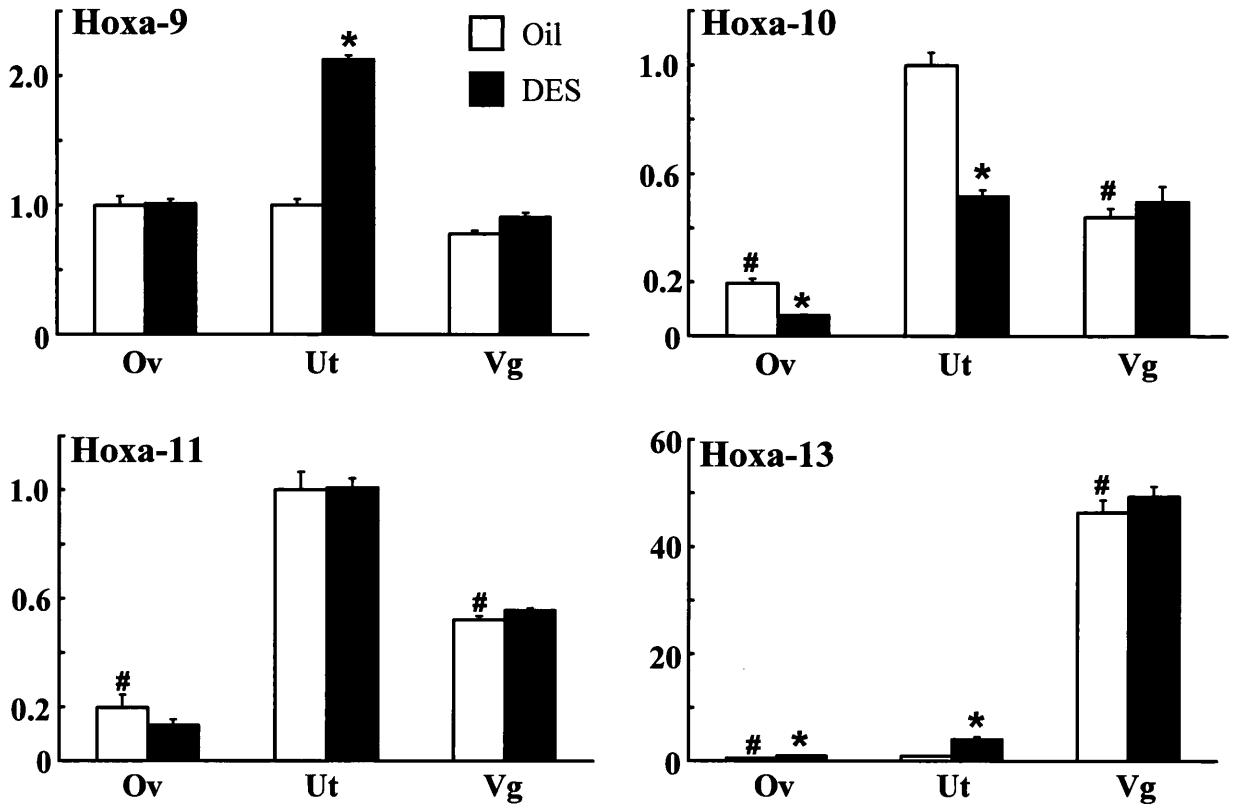


Figure 2


Hoxa-9
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DES
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1.0
0
Ov
Ut
Vg
Hoxa-10
1.0
0.6
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Hoxa-11
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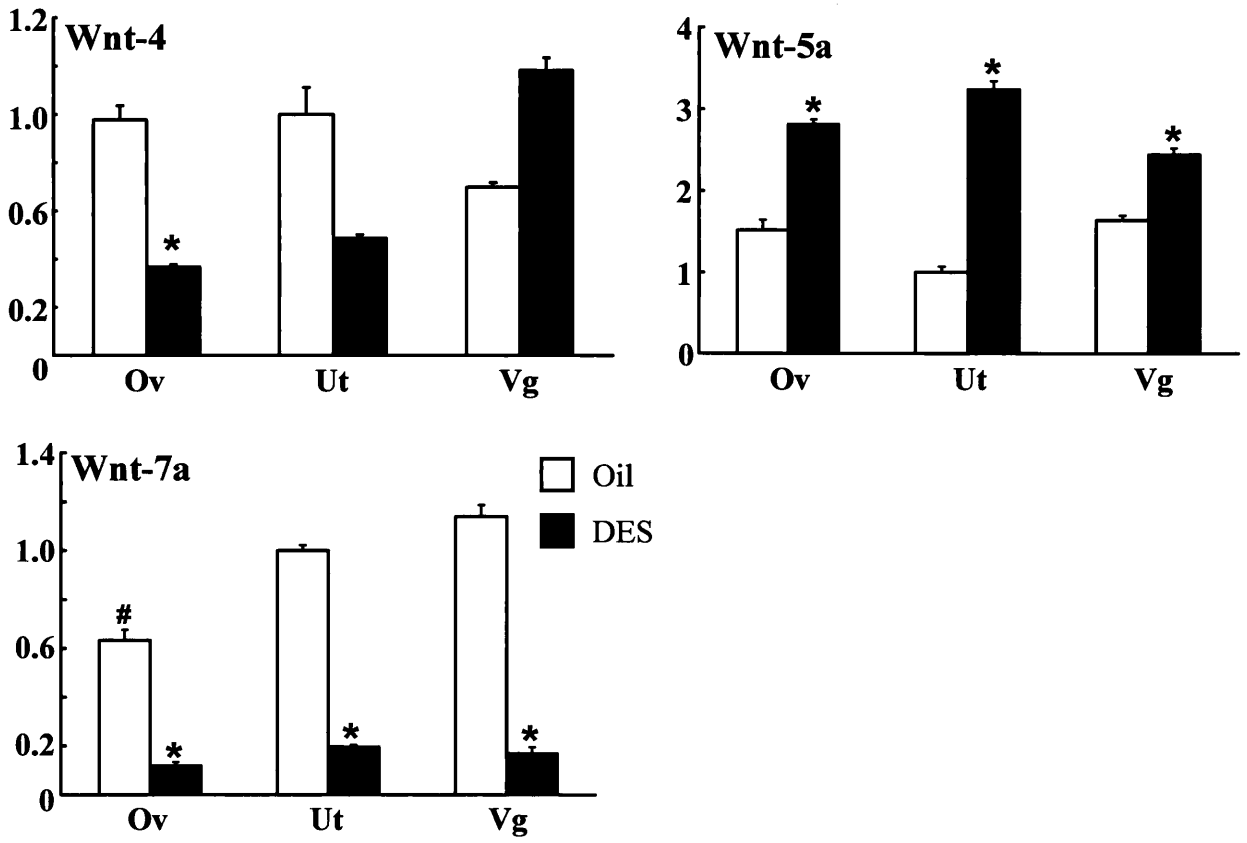
0.6
0.2
0
Hoxa-13
60
40
20
0

Figure 3


Wnt-4
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1.0
0.6
0.2
0
Ov
Ut
Vg
Wnt-5a
4
3
2
1
0
Ov
Ut
Vg
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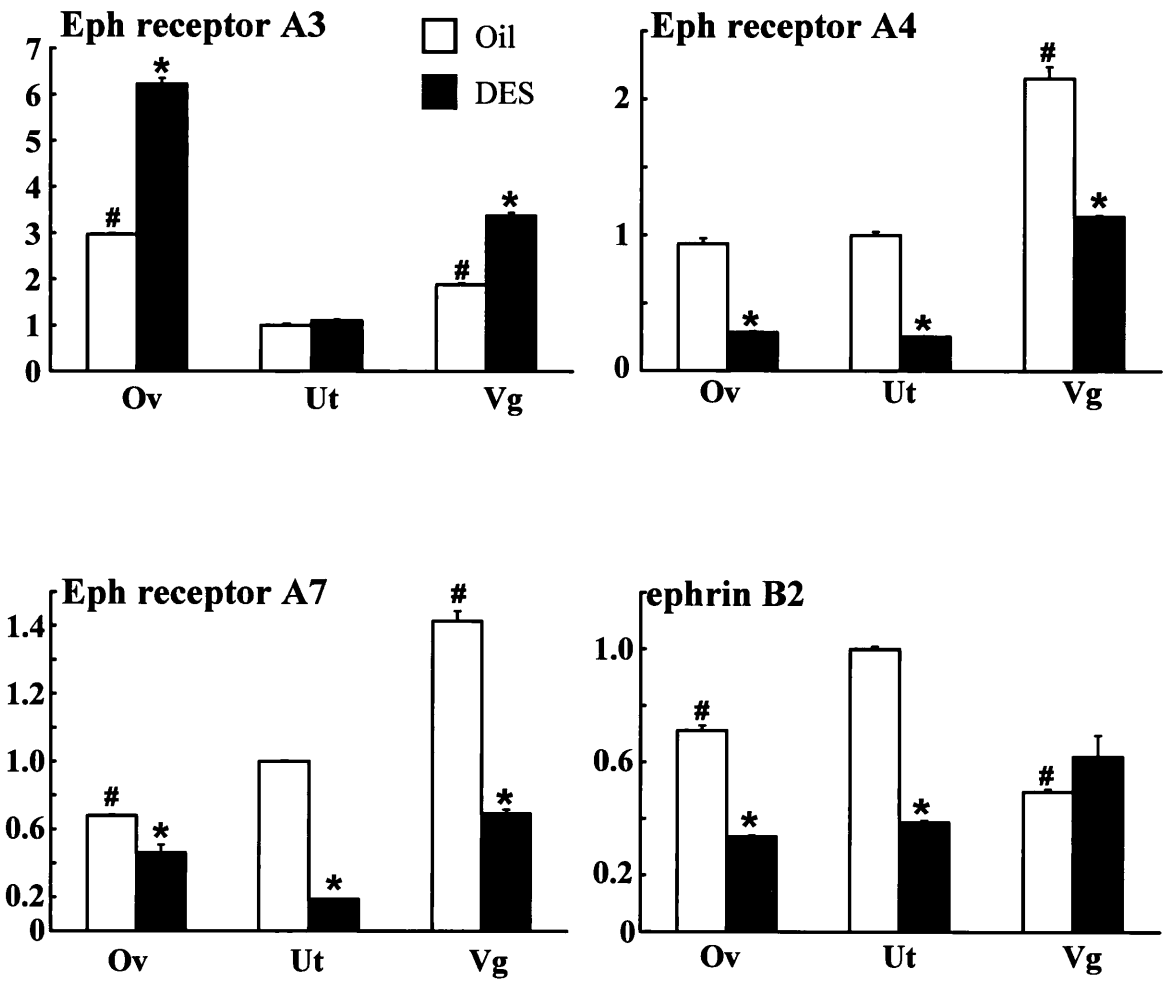
0.6
0.2
0
Ov
Ut
Vg
Oil
DES

Figure 4


Eph receptor A3
Oil
DES
7
6
5
4
3
2
1
0
Ov
Ut
Vg
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2
1
0
Ov
Ut
Vg
Eph receptor A7
1.4
1.2
1.0
0.6
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0.2
0
Ov
Ut
Vg

Figure 5

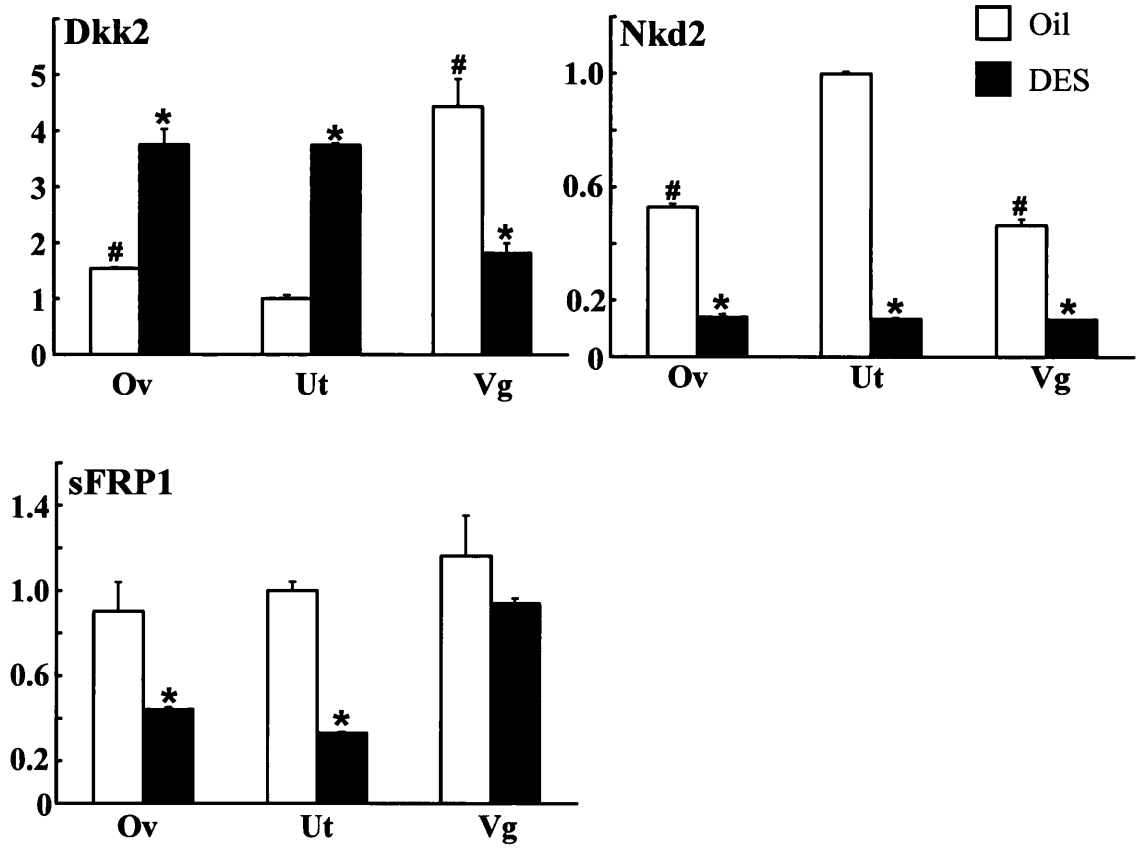

Figure 6

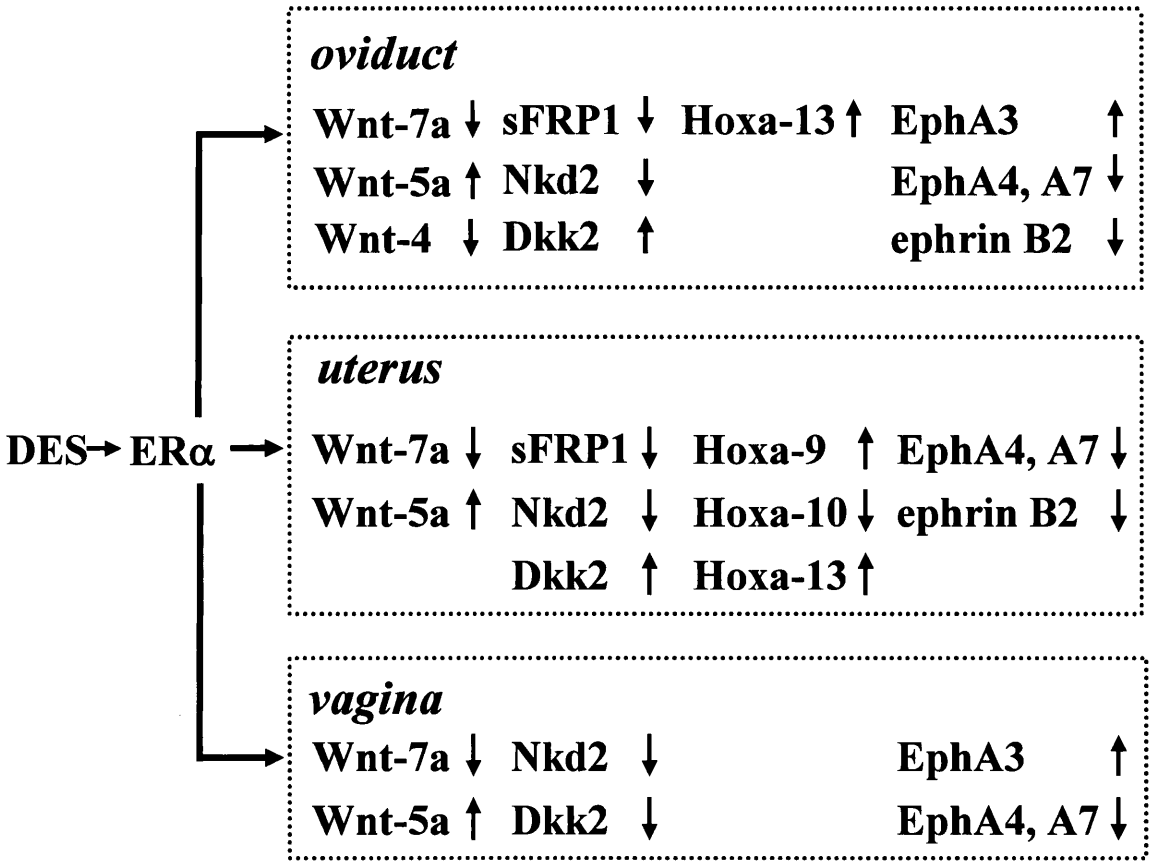
oviduct
Wnt-7a ↓ sFRP1 ↓ Hoxa-13 ↑ EphA3 ↑
Wnt-5a ↑ Nkd2 ↓ EphA4, A7 ↓
Wnt-4 ↓ Dkk2 ↑ ephrin B2 ↓
uterus
DES → ERα
Wnt-7a ↓ sFRP1 ↓ Hoxa-9 ↑ EphA4, A7 ↓
Wnt-5a ↑ Nkd2 ↓ Hoxa-10 ↓ ephrin B2 ↓
Dkk2 ↑ Hoxa-13 ↑
vagina
Wnt-7a ↓ Nkd2 ↓ EphA3 ↑
Wnt-5a ↑ Dkk2 ↓ EphA4, A7 ↓

Figure 7

Table 1. Sequences for primers using quantitative RT-PCR

Genebank 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

Table 2. DES-regulated genes related to signal transduction and organogenesis in Müllerian duct at GD 19

Gene accession No.	Fold change			Name
	Ovi	Ut	Vg	
Signal transduction				
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
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	NC	forkhead box O1
BB151515	NC	6.0	2.2	nerve growth factor receptor
BB759833	NC	NC	NC	forkhead box C1
NM_009523	NC	NC	3.5	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	homeo box, 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	homeo box, 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

Ovi, oviduct; Ut, uterus; Vg, vagina; Fold change means ratio v.s. organ-matched oil controls;NC means no change included with lesser than 2-fold change and more than 0.5-fold change

III. Chapter 2

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

Introduction

Estrogens induce cell proliferation and differentiation whereas estrogen depletion results in atrophy accompanied by apoptosis in adult reproductive tracts such as the uterus and vagina (Evans *et al.*, 1990; Suzuki *et al.*, 1996; Sato *et al.*, 2003). Estrogen exposure during a critical period in the early development induces persistent proliferation and keratinization in the vaginal epithelium (Takasugi *et al.*, 1962; 1964). Diethylstilbestrol (DES), a synthetic estrogen used to prevent miscarriage during the 1940's to the early 1970's, induced vaginal clear cell carcinoma and uterine abnormalities in daughters of mothers exposed to DES during pregnancy (Herbst *et al.*, 1971). Similar abnormalities were reported in mice exposed to estrogens during a perinatal critical period (Takasugi *et al.*, 1962; 1964; Forsberg *et al.*, 1969). In female mice, various abnormalities, such as polyovular follicles, oviductal tumors, uterine epithelial metaplasia, persistent vaginal stratification and keratinization, vaginal adenosis and cervico-vaginal carcinomas, were induced by perinatal exposure to estrogens including DES (Takasugi *et al.*, 1962; 1964; Forsberg *et al.*, 1969; Dunn *et al.*, 1963; Newbold *et al.*, 1982; 1985; Iguchi *et al.*, 1986; 1992).

During the normal estrous cycle, vaginal epithelial cell proliferation and keratinization

occur at the estrous stage (Evans *et al.*, 1990), whereas keratin 1 (K1) and progesterone receptor expressions were induced at the proestrous stage (Ohta *et al.*, 1993; Kamiya *et al.*, 1996). 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 (Kamiya *et al.*, 1996; Miyagawa *et al.*, 2004a, b). Reduced expression of estrogen receptor (ER) mRNA and persistent expression of c-fos and c-jun mRNAs were observed in the vaginae of neonatally DES-exposed mice, even after ovariectomy (Kamiya *et al.*, 1996). Persistent phosphorylation of erbB receptors, including epidermal growth factor (EGF) receptor, and sustained expression of EGF-like growth factors were found in neonatally DES-exposed mouse vaginae (Miyagawa *et al.*, 2004a). Neonatal exposure to a fibroblast growth factor family member, keratinocyte growth factor (KGF), resulted in persistent vaginal epithelial stratification (Hom *et al.*, 1998a). The induction of EGF by estrogens may play important roles in the proliferation of epithelial cells in the uterus and vagina (Hom *et al.*, 1998b).

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 (Miyagawa *et al.*, 2004b).

The critical periods for the induction of abnormalities by estrogenic chemicals during mouse development varies by organ (Iguchi *et al.*, 2002). 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 day (PND) 0, 5, 20 and 70. Mice were maintained under 12 h light/12 h dark at 23-25 ˚C 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 National Institutes of Natural Sciences.

Treatments

Diethylstilbestrol (DES, Sigma, 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 3 litters), PND 5 (7-12 mice from 3 litters), PND 20 (8 mice) and PND 70 (8 mice) were given a single subcutaneous (sc) injection of 2 μg DES/g bw or oil vehicle alone. PND 70 mice 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 (Q-PCR). In order to identify early genes induced by DES, tissues were dissected 6 h after the injection as described previously (Watanabe *et al.*, 2002).

In addition, 10 mice each were given a single sc injection of 2 μg DES/g bw or oil vehicle alone for bromodeoxyuridine (BrdU) experiment and immunostaining of Klf4 and 14-3-3 sigma.

DNA microarray analysis

Total RNA from vaginae was extracted using TRIZOL (Invitrogen, Tokyo, Japan) and purified using an RNeasy mini kit (Qiagen, Tokyo, Japan). Purified RNA was labeled with biotin according to the manufacturer's protocol and hybridized with a mouse genome U74Av2 array (Affymetrix Japan, Tokyo, Japan). After washing, the array was scanned to measure fluorescent intensity.

The fluorescent intensity of each probe was further analyzed using a model-based expression analysis program and expression levels were estimated. For the analysis, a perfect match (PM)-only model was used (Li *et al.*, 2001). The estimated values (gene expression levels) were transferred to the GeneSpring software program (Silicon Genetics, Redwood City, CA, USA) and analyzed. To deduce credible gene expression levels from DNA microarray analysis, we independently repeated each experiment at least twice, with averaged values being used for the analysis.

For the clustering analysis, genes activated more than two-fold by DES were genes were selected and similarities between experiments and expression levels were measured by standard correlation using the GeneSpring program as described (Watanabe *et al.*, 2002).

Putative target genes were validated by Q-PCR.

Quantitative RT-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 min. 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 min at 50 ˚C, 10 min at 95 ˚C, followed by a total of 40 two temperature cycles (15 sec at 95 ˚C and 1 min at 60 ˚C). Gene expression levels were normalized to the expression levels of L8 mRNA (U67771) and gel electrophoresis and melting curve analyses were performed to confirm correct amplicon size and the absence of non-specific bands. The primers were chosen to amplify short PCR products of less than 100 base pairs and their sequences are listed in Table 1. 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 2μg DES were killed 24 h after the injection. Two h before dissection, 0.05mg/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 min at room temperature (RT) to block endogenous peroxidase activity. They were washed with 0.5% Tween20 in PBS, incubated with 2N HCl in water for 20 min at RT. They were washed with 0.5% Tween20 in PBS, incubated with 1% bovine serum (BSA) for 20 min at RT and with anti-BrdU antibody (Roche, Mannheim, Germany) at a dilution of 1:15 in 1% BSA at 4°C over night. Washing with 0.5% Tween20 in PBS, sections were incubated with mixture of 3,3'-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2. Counter stain was conducted with methylgreen. 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.

Immunostaining of Klf4 and 14-3-3 sigma

After deparaffinized and washed with PBS, sections were incubated with 3% H_2O_2 in methanol for 15 min. Some sections were heated using microwave in the presence of citric acid for 14-3-3 sigma staining. Primary antibodies, 14-3-3 sigma (Immuno-Biological Laboratories Co., Tokyo, Japan) and Klf4 (H-180, Santa Cruz Biotechnology Inc., CA, USA), incubated for 1 h were detected using the Histofine SAB-PO(R) kit (Nichirei, Tokyo, Japan) and counterstained with hematoxylin.

Results

Gene expression in the vaginae of mice treated with DES at different ages

The number of detected genes in the mouse vagina was not different among animals examined at different ages in oil-injected controls; newborn (PND 0)=4988, PND 5=4937, PND 20=4881, 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 on 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 at PND 0, 5,

20 and 70 in vaginae, respectively (Fig. 1). The number of genes induced by DES at PND5 (208) 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. PND 5 specific DES-induced genes were 58 (28%) (Fig. 1). Five genes (including 2 ESTs) were specific to PND 0 (Fig. 1 and Table 2).

The expression of some estrogen responsive genes selected in the present study was confirmed by Q-PCR (Table 3). Many of these genes showed up-regulation by a single injection of DES from PND 0 to 70. Twenty-five genes including MAD2, G1 to phase transition 1, myb oncogene, c-fos, early growth response 1 (Egr-1) and Kruppel-like factor 4 (Klf4) were induced by DES exposure at all ages examined (Table 3). MAD2 is an estrogen responsive gene and assembles the mitotic spindle at G2/M checkpoint (Shah *et al.*, 2000). Myb, c-fos and Egr-1 were reported to be estrogen responsive genes in the mouse uterus and/or mammary gland (Kamiya *et al.,* 1996; Watanabe *et al.*, 2002; Guerin *et al.*, 1990). Klf4, an inhibitor of G1/S phase, plays a role in keratinocyte differentiation (Forster *et al.*, 2000; Chen *et al.*, 2003), 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 DES-repressed genes at PND 5 (30%) were also found at PND 20. Thirty-nine of 117 DES-repressed genes (33%) at PND 20 were also found at PND 70. While, the number of age specific DES-repressed genes was 5 (56%), 39 (59%), 64 (55%) and 107 (70%) at PND 0, 5, 20 and 70, respectively (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 (Ziegler *et al.*, 1993).

Clustering patterns and category of DES-regulated genes in the vaginae of mice at different ages

729 genes showing more than a 2-fold change following a single injection of DES at PND 0, 5, 20 and 70 were used for clustering. These genes can be grouped as PND 0 and PND 5 and 70 in controls, and PND 0, PND 5-70 in DES-exposed vagina (Fig. 2) because the clustering patterns of genes in DES-exposed vagina at PND 5 was more similar to that of PND 20 and 70 than PND 0 (Fig. 2).

These genes could be categorized into several groups (Fig. 3). Genes involved in cell proliferation (15%) and protein modification (17%) were found in the DES-induced genes at PND 0 (Fig. 3). 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 (Fig. 3). DES-regulated genes categorized in organogenesis were repressed by DES at PND 5, 20 and 70.

Confirmation of gene expression by quantitative RT-PCR

Expression of several genes showing up-regulation by DES in mouse vagina at different ages was confirmed using Q-PCR. Expressions of 14-3-3 sigma, Gadd45α, Klf4, Sprr2f and EST (AI121305) were increased with age in control mice. Gadd45α promotes G1 phase and acts at G2/M checkpoint (Fan *et al.*, 1999; Wang *et al.,* 1999). Expressions 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 (Fig. 4). Expressions of these genes, except for Klf4 and EST (AI121305), were induced by DES at PND 5, 20 and 70, but not at PND 0 (Fig. 4). Expression of Klf4 and EST (AI121305) was up-regulated by DES at PND 0. Expression of Sprr2f mRNA was induced by DES at PND 5 and 70 (Fig. 4).

Immunostaining of BrdU, Klf4 and 14-3-3 sigma

In oil treated controls, ratios of BrdU-positive cells were not different among postnatal ages both in the epithelial cells and stromal cells. BrdU-posive cells in the vaginal epithelium were increased at PND 20 and PND 70 in the DES treated vaginae as compared to the oil controls. In contrast, BrdU-positive cells in the stroma were higher in PND 0 and PND 5 (Fig. 5).

Immunoreactions of Klf4 and 14-3-3 sigma were observed in the vaginal epithelium and stroma at PND 0. Klf4 staining appeared randomly in the nuclei of vaginal epithelial and stromal cells. 14-3-3 sigma immunoreactivity was detected in the cytoplasm of vaginal epithelial cells than stromal cells. 14-3-3 sigma and Klf4 stainings were not altered 24 h after the DES injection at PND 0 as compared to the controls. Strong staining of 14-3-3 sigma and Klf4 was found in the vaginal epithelium of mice at PND70 with or without DES (data not shown).

Discussion

Estrogen, androgen and KGF exposure for 5 days from the day of birth induces persistent vaginal epithelial stratification in mice (Takasugi *et al.*, 1962; Forsberg *et al.*, 1969; Iguchi 1992; Miyagawa *et al.*, 2004a, b; Hom *et al.*, 1998a). The persistent vaginal

epithelial stratification with superficial keratinization induced by perinatal estrogen exposure was reported to be accompanied by persistent expression of several growth factors (Miyagawa *et al.*, 2004a, b; Hom *et al.*, 1998a , b; Masui *et al.*, 2003; Sato *et al.*, 2004). 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 (Sato *et al.*, 2004; Nelson *et al.*, 1994) and phosphorylation of ERα, EGFR and erbB in mouse vaginae (Miyagawa *et al.*, 2004a, b).

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 70. ER staining was found from PND 0 in mouse vaginal epithelial and stromal cells (Yamashita et al., 1989; Sato *et al.*, 1994). Thus, neonatal mouse vaginae seems 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 PND 5, 20 and 70. The PND 0 mouse vagina is still under development even without estrogenic stimulation (Takasugi *et al.*, 1964; Iguchi *et al.*, 1976). Thus, vagina at PND 0 is less sensitive to estrogenic stimulation than at later

stages in terms of gene expression.

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 (PND 5, 20 and 70). Vaginal stromal cells showed proliferative response to DES only at PND 0 and 5, but not at PND 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 (Iguchi *et al.*, 2002). The underlying mechanism of the differences in responsiveness between vaginal epithelial cells and stromal cells at different ages need to be analyzed in the near future for understanding 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 keratinization in mouse uteri (Watanabe *et al.*, 2002; 2003a, b; Hewitt *et al.*, 2003). Increased expression of mRNAs of cell cycle regulators were reported after 17β-estradiol treatment in the uteri of adult ovariectomized

mice (Hewitt *et al.*, 2003). 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.

p21 delaying S phase progression (Sherr, 1994), Gadd45α acting at G2/M checkpoint and 14-3-3 sigma inhibiting activation of cyclin B (Fan *et al.*, 1999; Sherr, 1994; Hermeking *et al.*, 1997) were induced by DES from PND 5. Thus, induction of these cell cycle regulators at mitotic phase checkpoint 6 h after DES stimulation may play roles 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 (Huang *et al.*, 2005). Sprr family genes are expressed in all squamous cells, such as epithelial cells of skin, vaginal and digestive tracts (Song *et al.*, 1999; Patel *et al.*, 2003). Sprr2a, 2b and 2f are expressed in uteri and vaginae (Tesfaigzi *et al.*, 1999). Sprr2f is expressed most intensely in uteri and vaginae (Tesfaigzi *et al.*, 1999). Sprr2f, Keratin complex 1(K1) and K2 mRNAs were elevated in vaginae 6 h 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 of vaginal epithelial cell proliferation is probably related to

expression of Sprr2f, K1 and K2 genes at PND 20 and PND 70. Sprr2a and 2b may be correlated with keratinization in vaginal epithelial cells.

Klf4 is a transcription factor of Sprr2 (Patel *et al.*, 2003; Segre *et al.*, 1999) and plays a role in keratinocyte differentiation (Chen *et al.*, 2003). DES induced Klf4 expression in vaginae even at PND 0 in the present study. Klf4 protein was expressed in vaginal epithelial cells at PND70 in the present study. The inductions of Klf4 and Sprr2a expressions in DES-exposed uteri at PND 5 were reported previously (Huang *et al.*, 2005). 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 (Hermeking *et al.*, 1997), and is induced in squamous cell carcinoma of the urinary bladder (Moreira *et al.*, 2004). 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 (Miyagawa *et al.*, 2004b), 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 PND 20 and 70, and PND 0 and 5, respectively. The

number of genes induced by 6 h after DES exposure in mouse vaginae at PND 0 was lower compared to that of PND 5, 20 and 70. 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, Sprr2f, were induced by DES in vaginae from PND 5 to adult. Thus, microarray analysis revealed that 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.

Figure legends

Fig. 1. a) The number of induced genes in vaginae 6 h after a single injection of 2 μg DES/g bw at PND 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 h after a single injection of 2 μg DES/g bw at PND 0, 5, 20 and 70. The numbers of DES-repressed genes increased linearly with age.

Fig. 2. Clustering analysis of DES-responsive genes in mouse vaginae selected in the present study. Mice of PND 0, 5, 20 and 70 were stimulated by a single injection of DES. Genes showing more than a 2-fold change in expression 6 h after a single injection of 2 μg DES/g bw at all ages were used for clustering analysis. 779 genes were selected. Control mice exhibited separated trees between neonatal period (PND 0 and 5) and adult (PND 20 and 70). Branching of clustered genes in DES-exposed vaginae at PND 5 was closer to that of PND 0.

Fig. 3. Functional categories of DES-responsive genes in mouse vaginae selected in the

present study. Clustered genes were categorized into 12 groups. Total number of clustered genes in each category was 100%.

Fig. 4. Ratio of gene expression of cell cycle and keratinocyte differentiation regulators was confirmed by quantitative-PCR. Results are the mean and SEM of three experiments. Each experiment was performed in triplicate. $p<0.05$ v.s. age-matched controls

Fig. 5. 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. *, $p<0.05$ v.s. age-matched controls.

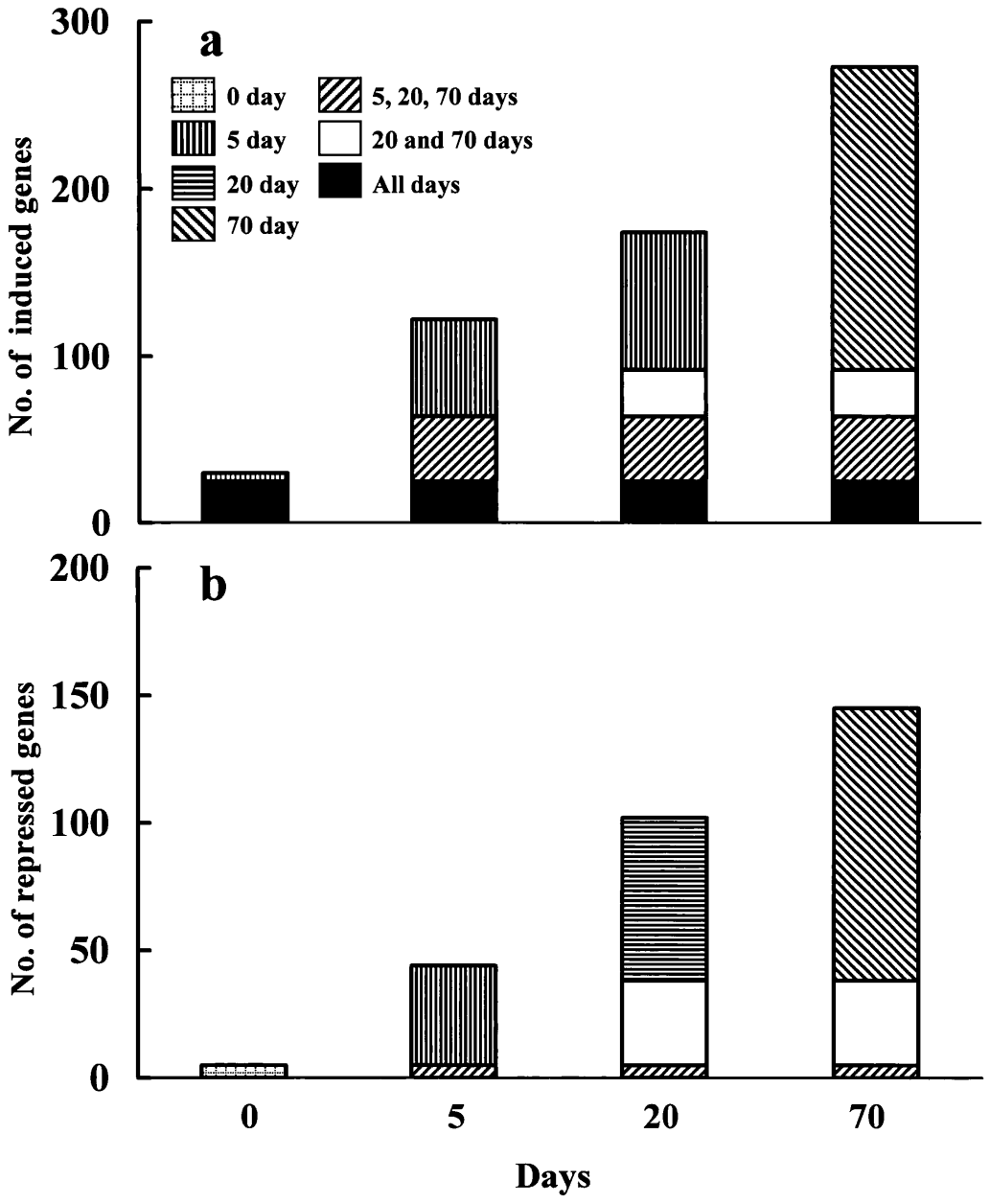
a
0 day
5 day
20 day
70 day
5, 20, 70 days
20 and 70 days
All days
No. of induced genes
300
200
100
0
b
No. of repressed genes
200
150
100
50
0
0
5
20
70
Days

Figure 1

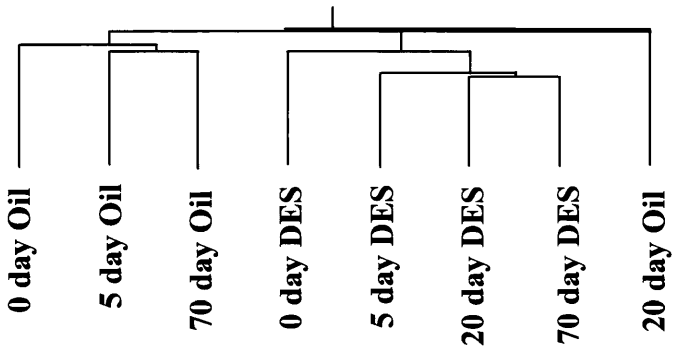
0 day Oil
5 day Oil
70 day Oil
0 day DES
5 day DES
20 day DES
70 day DES
20 day Oil

Figure 2

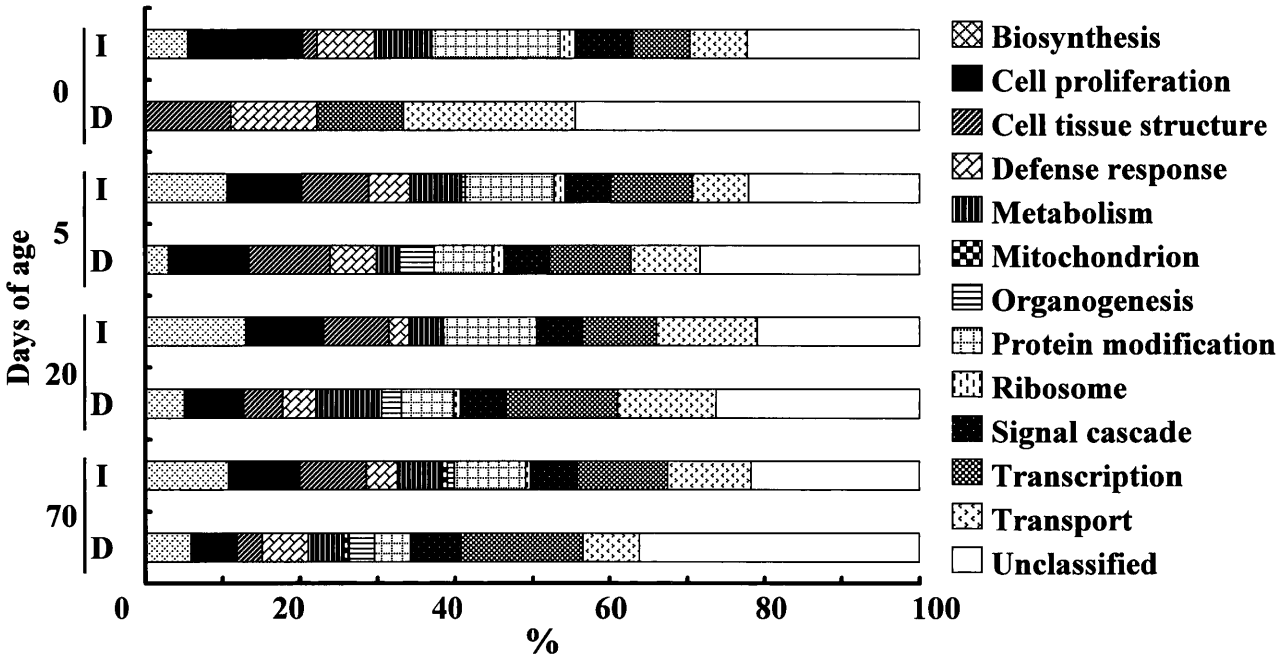
Days of age
0
5
20
70
I
D
0
20
40
60
80
100
%
Biosynthesis
Cell proliferation
Cell tissue structure
Defense response
Metabolism
Mitochondrion
Organogenesis
Protein modification
Ribosome
Signal cascade
Transcription
Transport
Unclassified

Figure 3

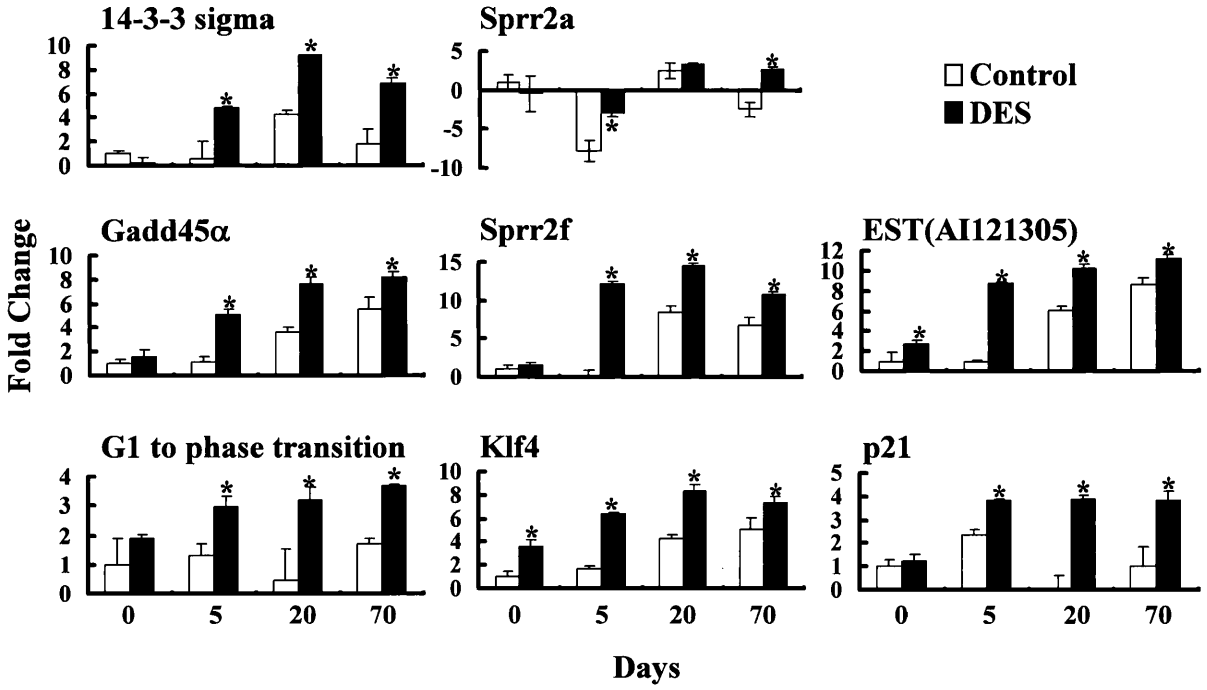
14-3-3 sigma
Sprr2a
Control
DES
Gadd45α
Sprr2f
EST(AI121305)
G1 to phase transition
Klf4
p21
Fold Change
Days
0
5
20
70

Figure 4

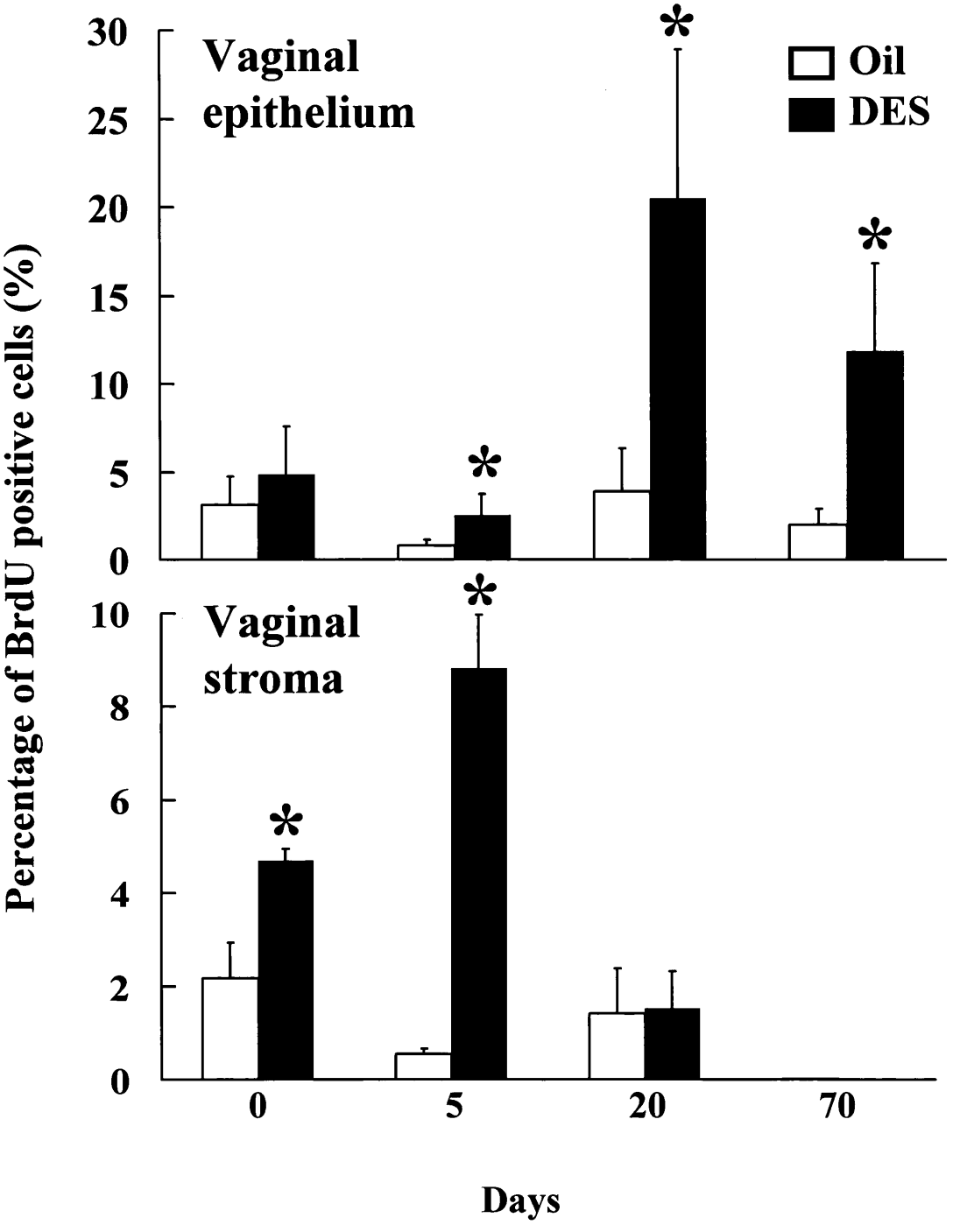
Vaginal epithelium
Oil
DES
Vaginal stroma
Percentage of BrdU positive cells (%)
30
25
20
15
10
5
0
10
8
6
4
2
0
0
5
20
70
Days

Figure 5

Table 1. Sequences for primers used for a quantitative RT-PCR

Genebank 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	CAAGTATGCATTGCGCGTTTA	CCCATCTGAGGGAAGTCCTTAA
AI121305	EST	TTATGTCCTCAGTCCGCAGCT	TAGTGTTGCAGGTCTGTGGTCC
AW048937	p21	TGAGACGCTTACAATCTGAGTGG	AACATGTATTGTGGCTCCCTCC
U00937	Gadd45α	GAAGAAGGAAGCTGCGAGAAAA	CCTGGCCATCCTAAATTAGCAGT
U67771	Ribosomal protein L8	ACAGAGCCGTTGTTGGTGTTG	CAGCAGTTCCTCTTTGCCTTGT

Sprr, small proline-rich protein; Gspt1, G1 to S phase transcript 1; p21, cyclin-inhibitor 21; GADD45 α, growth and DNA damage 45 α;

Table 2. Induced or repressed genes in vagina 6 h after a single injection of DES at only PND 0

Genebank No.	FC	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 super family 3

Table 3. Induced or repressed genes in vagina 6 h after a single injection of DES at PND 0, 5, 20 and 70 using DNA microarray.

Genebank No.	Gene	Age in days			
		0	5	20	70
Induced genes					
Cell proliferation					
AB003502	G1 to phase transition 1 (Gspt1)	2.2	2.8	2.1	3.3
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	-	3.4	3.4	2.7
AW048937	p21	-	2.4	3.2	3.7
U00937	GADD45 α	-	6.0	4.5	4.5
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	4.2	2.1	2.7
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	15.1	3.7	3.6
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	-	2.2	-	4.9
AJ005560	Sprr2b	-	-	-	4.4
AJ005564	Sprr2f	-	15.0	4.0	7.5
Repressed genes					
Transport					
D16215	flavin containing monooxygenase 1 (Fmo1)	0.5	0.3	0.3	0.5

Gas 6, growth arrest specific 6; Map2k1, mitogen activated protein kinase kinase 1; p21, cyclin-inhibitor 21; GADD45 α, growth and DNA damage 45 α; MAD2, mitotic arrest deficient; Sprr, small proline-rich protein; K, keratin complex; -, no change

IV. Chapter 3

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

Introduction

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 (Korach *et al.*, 1996), the mammary gland requires progesterone and prolactin in addition to estrogen to complete the architecture (Horseman *et al.*, 1999; Kelly *et al.*, 2002; Shayamala, 1999). Ovariectomy and termination of weaning induce apoptosis in epithelial cells in the uterus, vagina and mammary gland (Kojima *et al.*, 1996; Sato *et al.*, 1996). These estrogen target organs are controlled by estrogen receptors (ERα and ERβ) in the epithelial and stromal cells (Cunha *et al.*, 1997; Kurita *et al.*, 2000; Mueller *et al.*, 2002). It is known, moreover, that growth factor(s) from the stroma are involved in epithelial proliferation of these organs (Buchanan *et al.*, 1998; Cooke *et al.*, 1997; Cunha *et al.*, 1997). 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)(Buchanan *et al.*, 1999;

Cooke *et al.*, 1997; Huet-Hudson *et al.*, 1990; Klotz *et al.*, 2002). Prolactin also induces expression of IGF-2 mRNA in the developing mammary gland (Hovey *et al.*, 2003). 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 (Margeat *et al.*, 2003; Shang *et al.*, 2002). 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 (Frasor *et al.*, 2003; Watanabe *et al.*, 2002, 2003a and b). However, any comparisons of gene expression in the estrogen target organs have not been reported. Gene expression reached a maximum 6 h after E_2 administration in the uterus of ovariectomized adult mice without any histological changes (Watanabe *et al.*, 2002). Thus, we examined global gene expression 6 h 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.

Materals 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 h light/12 h dark at 23-25 ˚C, fed with a commercial diet (CE-2, CLEA) and provided tap water *ad libitum*. All experiments and animal husbandry protocols were approved by the animal care committee of National Institutes of Natural Sciences.

Treatments

17β-Estradiol (E2, 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 E2 levels were reduced. Six h after E2 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 h after the injection for bromodeoxyuridine (BrdU)-labeling study.

DNA microarray analysis

Total RNA was extracted from tissues (4 mice each) 6 h 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 (Watanabe *et al.*, 2002). 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 h 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 5700 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 h 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 in borate buffer

(0.1M 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 2000-3000 epithelial and 10000-20000 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 analyzed 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 h 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 both 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 mRNAs were increased by E_2 in the vagina (Fig. 3). Klk1 mRNAs was significantly increased by E_2 in the vagina.

BrdU incorporation in the uterus, vagina and mammary gland 24 h 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 in 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 h after the E_2 injection as compared with each control. In the mammary gland, however, BrdU-positive cells were not evident in the epithelium 24 h 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 organ 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 (Richards *et al.*, 1996; Sato *et al.*, 2002), 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) (Marshman *et al.*, 2003). IGFBP2 and IGFBP5 promote apoptosis in the prostate cancer cells and mammary gland cells (Marshman *et al.*, 2003; Plath-Gabler *et al.*, 2001; Schneider *et al.*, 2002; Richardsen *et al.*, 2003). Hence, proliferations of uterine and vaginal cells 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 (Watanabe *et al.*, 2002). 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 (Corthorn *et al.*, 1997). Klk is regulated hormonally (Corthorn *et al.*, 1994, 1997) and the gene expression was found in human ovarian, prostate and breast cancer cells and in mouse vagina (Jacobsen *et al.*, 2003; Obiezu *et al.*, 2001; Yousef *et al.*, 1999). 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 h 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 (Charpentier *et al.*, 2000; Frasor *et al.*, 2003; Gruvberger *et al.*, 2001). 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 (Gruvberger *et al.*, 2001). Moreover, using the serial analysis of gene expression (SAGE) method, 3 up-regulated genes were reported in MCF-7 cells 10 h after E_2 treatment *in vitro* (Charpentier *et al.*, 2000). 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 h to respond to E_2 *in vivo.* Mammary gland has various types of cells, such as epithelial cells, myoepithelial 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 (Mueller *et al.*, 2002). The mammary gland seems to be regulated by progesterone and prolactin rather than estrogen (Clevenger *et al.*, 2003; Horseman *et al.*, 1999; Jacobsen *et al.*, 2003; Kelly *et al.*, 2002; Mueller *et al.*, 2002; Shayamala, 1999). 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 (Jacobsen *et al.*, 2003). Prolactin induced expression of IGF-2 mRNA in the developing mammary gland (Hovey *et al.*, 2003). Thus, mammary gland is possibly regulated largely by progesterone and/or prolactin. This may account for no expression of estrogen responsive genes observed 6 h after the E_2 exposure and absence of definite mitogenic response in the mammary gland of ovariectomized adult mice 24 h 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.

Figure legends

Fig. 1. Gene expression profiles in the uterus and vagina 6 h after a single injection of 5 μg E_2/kg body weight. The Venn diagram indicates 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

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.

Fig. 3. Ratio of mRNA expressions of IGF-1 family and Kallikrein 1 in the three organs 6 h 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

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

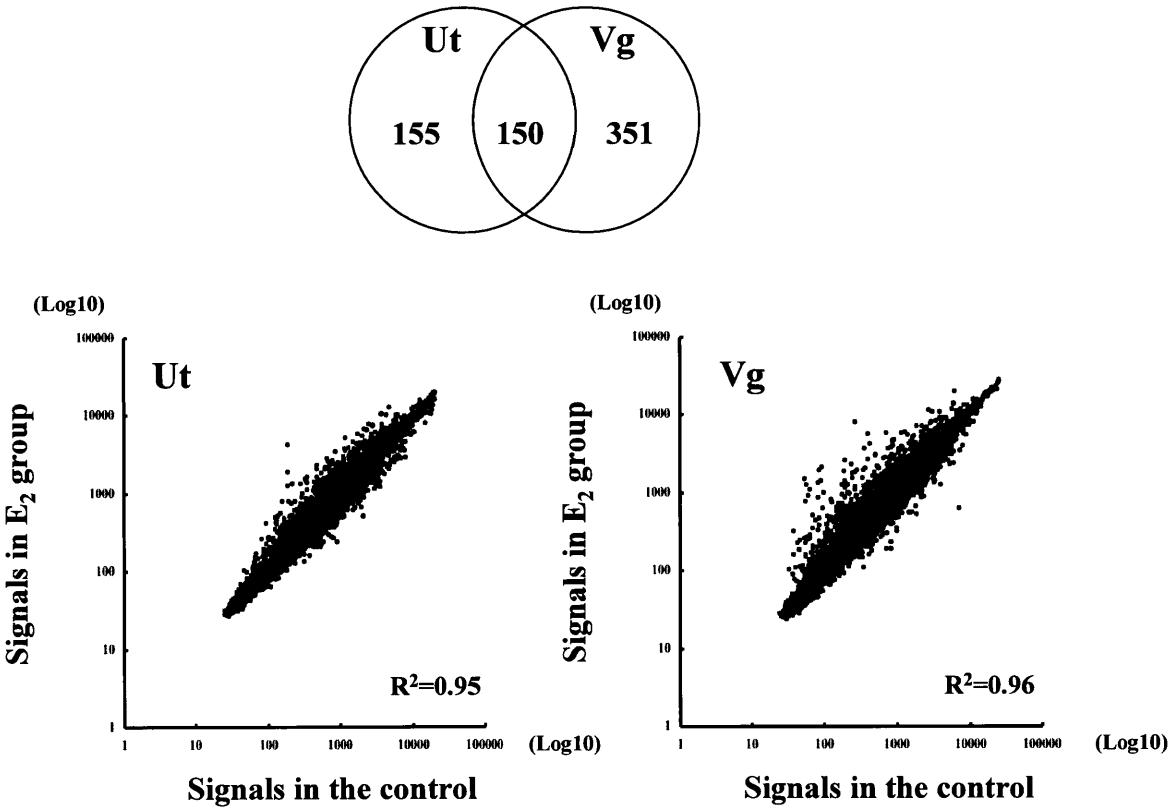
Ut
Vg
155
150
351
(Log10)
Ut
Signals in E_2 group
R^2=0.95
(Log10)
Signals in the control
(Log10)
Vg
Signals in E_2 group
R^2=0.96
(Log10)
Signals in the control

Fig. 1

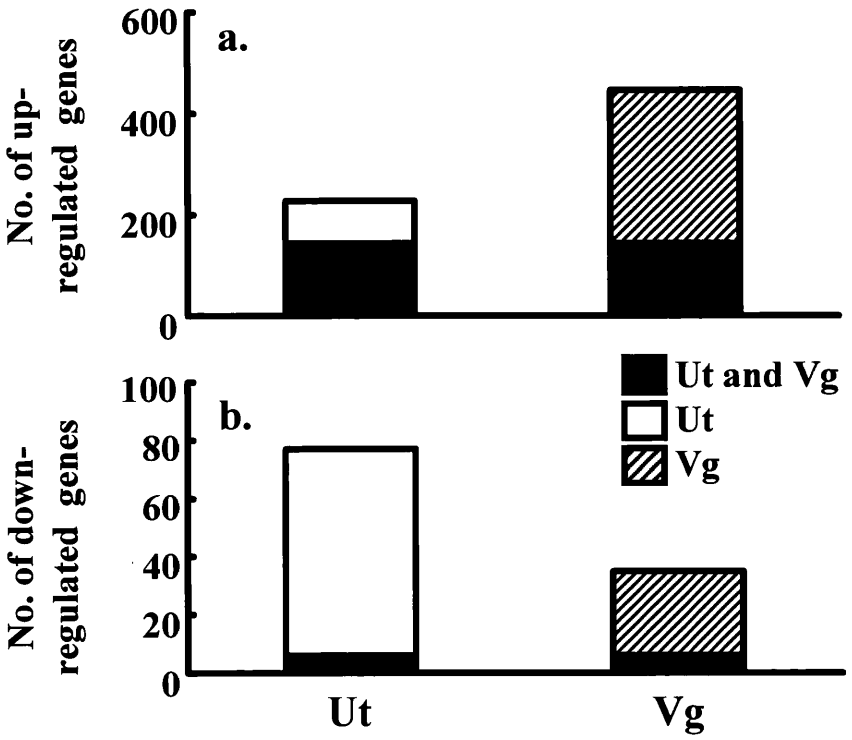
a.
600
400
200
0
No. of up-regulated genes
b.
100
80
60
40
20
0
No. of down-regulated genes
Ut and Vg
Ut
Vg
Ut
Vg

Fig. 2

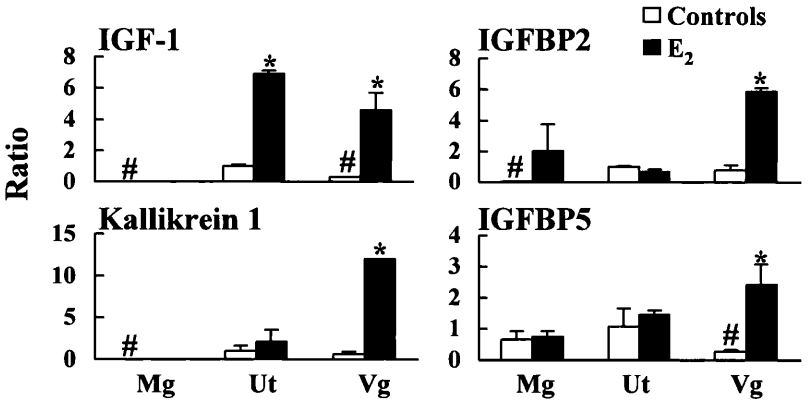

Fig. 3

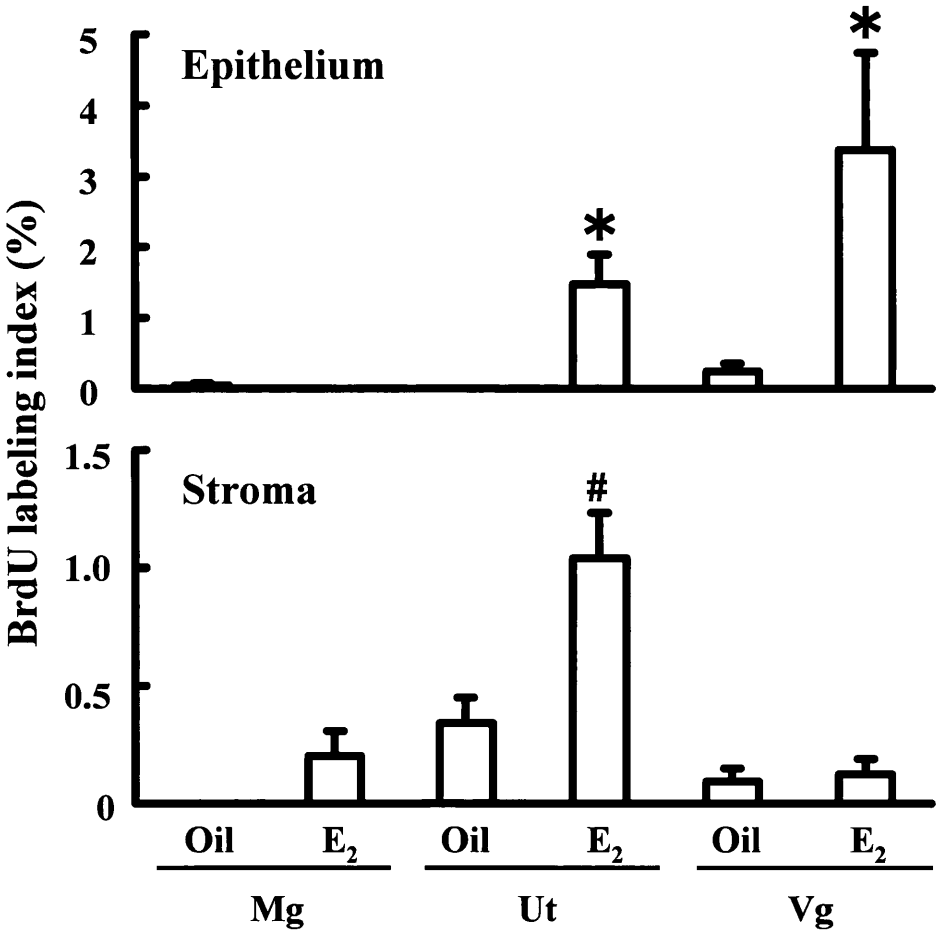
Epithelium
Stroma
BrdU labeling index (%)
5
4
3
2
1
0
1.5
1.0
0.5
0
*
*
#
Oil
E2
Oil
E2
Oil
E2
Mg
Ut
Vg

Fig. 4

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

Table2 List of estrogen responsive genes related development, cell growth and apoptosis in the uterus and vagina by microarray analysis.

Accession No.	Name	Ratio Ut	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
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.

V. Summary and Conclusion

I investigated the estrogen-responsive genes in mouse reproductive organs at perinatal and adult period using DNA microarray in the present study.

First, I investigated gene expression change in the Müllerian duct of the mouse fetus exposed to diethylstilbestrol *in utero* (Chapter1). DES up-regulated appoximately 400 genes and down-regulated 200 genes in the oviduct and uterus by DES *in utero*. However, DES-regulated genes in the vagina were half of those in the oviduct and uterus. Clustering analysis of DNA microarray revealed DES loose the organ-specific gene expressions in the Müllerian duct. From results of Q-PCR, DES repressed Wnt-4 gene in the oviduct and Wnt-7a gene in all three organs, but it induced expression of Wnt-5a gene in the oviduct and uterus. Hoxa-9 gene was induced by DES, while Hoxa-10 gene in the oviduct and uterus was repressed by DES. In the vagina, DES failed to disrupted Hoxa13 gene. The clustering analysis and Q-PCR suggested the downstream candidates of Hox and Wnt genes were included with ephrin B2, Eph receptor A4, A7, Nkd2, Dkk2 and sFRP genes.

Second, I investigated global gene expression in mouse vaginae exposed to DES at different ages (Chapter2).DNA microarray analysis exhibited 54 DES-induced

genes and 9 DES-repressed genes in vaginae at PND 0. While, more than 200 DES-induced genes were found in vaginae at PND 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 close to the adult type rather than those at PND 0. Genes related to keratinocyte differentiation, such as Gadd45α, p21, 14-3-3 sigma, Sprr2f and Klf4, were induced by DES. The proliferation of vaginal epithelium at PND5 was similar to adult one than that at PND 0. The number of DES-induced genes during the critical period, PND 0, was smaller than that found after the critical period.

Third, I investigated comparison of estrogen-responsive genes in the mouse uterus, vagina and mammary gland (Chapter3). In the mammary gland, gene expressions and mitosis were almost unchanged 6 h after the E_2 exposure. 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 IGF-1 genes in the uterus and vagina. In the vagina, E_2 up-regulated the expression of IGF binding proteins (IGFBP2 and IGFBP5). These results suggest that expression of IGF-1 and morphogenesis genes is regulated by E_2 in an organ-specific manner, which was supported by the results of BrdU labeling showing E_2-induced mitosis in the uterus and vagina except the mammary gland.

Clustering analysis of global gene expressions using DNA microarray analysis revealed the obvious organ-specificity and age-specificity in mouse reproductive tracts. DES disrupted the organ and age specificity of global gene expressions in mouse reproductive tracts during perinatal period, especially morphologic and keratinocyte differentiation genes. Interestingly, estrogen exerts little effect on the mammary gland as adults. The epigenetic mechanism of estrogenic transcription may be different in mammary gland as compared to those in mature uterus and vagina.

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