Studies on Molecular Mechanisms of Persistent Vaginal Abnormalities Induced by Neonatal Estrogen Exposure in Mice

（出生直後のエストロゲン暴露により誘起されたマウス膣の恒久的異常に関する分子機構の研究）

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Contents

I. Preface

An integrated network of hormones regulates the reproductive systems in most animal species. Estrogens, one class of hormones, are responsible for the development of reproductive organs, and the induction of behavioral and physiological processes. *In utero* exposure to a synthetic estrogen, diethylstilbestrol (DES), induces vaginal clear-cell adenocarcinoma in humans (Herbst et al., 1971; Herbst and Bern, 1981). Estrogens act via intracellular estrogen receptors (ERα and ERβ) which are members of the nuclear receptor super family of transcription factors. Upon ligand binding, ERs enhance the rate of transcriptional initiation by assembling and recruiting transcription regulatory complexes to the promoter regions of estrogen responsive genes. Estrogens are responsible for estrous cycles, and the development and maintenance of female sex characteristics, including behavioral and physiological processes. Estrogen exhibits acute and transient actions on its target organs. For example, estrogen administration promotes cell proliferation and differentiation in adult female reproductive tracts, resulting in weight gains, while estrogen withdrawal by ovariectomy induces rapid involution of the uterus and vagina, resulting in return to the unstimulated states. These reversible and strict responses to estrogen are important in animals to maintain female reproductive organ homeostasis at various physiological states, and are required for normal health and reproduction.

On the other hand, estrogen administration to the neonatal mouse induces persistent harmful effects on the reproductive organs; estrogen-independent proliferation and cornification in the vaginal epithelium, hyperplastic lesions, carcinogenesis and hypospadias in the vagina; uterine hypoplasia, epithelial metaplasia

and cancers; oviductal cancers; and polyovular follicles and anovulation (Taksugi et al., 1962; Takasugi, 1976; Forsberg, 1979; Iguchi, 1992; Iguchi *et al.*, 2002). Again, these morphological and functional defects in female reproductive organs are induced only when the mouse is given estrogen within the early neonatal period (a critical period). However, little is known about the relative contribution of the individual ER subtypes (ERα or ERβ) in the induction of abnormalities. Therefore, I have analyzed the effects of neonatal exposure to ER subtype selective ligands and a synthetic estrogen, diethylstilbestrol (DES) on female mouse reproductive tracts (Chapter 1).

Cell proliferation and differentiation of mouse vaginal epithelium are strictly regulated by endogenous estrogen during the estrous cycle. There have been many reports in regard to the acute and reversible effects of estrogens, however, studies on persistent developmental effects to estrogen exposure have never shed light on those induction mechanisms (McLachlan, 1980). Miyagawa et al. (2004a, b) reported the persistent phosphorylation of ERα, erbB receptors and JNK1, and sustained expression of EGF-like growth factors, interleukin-1 (IL-1)-related genes and IGF-1 mRNA (*Igf1*) in the neonatally DES-exposed mouse vagina, suggesting an involvement of various signaling pathways in the persistent cell proliferation in the mouse vagina induced by neonatal DES exposure. These results suggest that neonatal estrogen-exposure induces the imprinting in gene expressions, imbalances of cell proliferation and cell death, and induction mechanisms of those irreversible phenomena. Therefore, I have performed global analyses of the gene expressions in the mouse vagina exposed neonatally to DES using microarray technology, and selected candidates of genes and pathways possibly related to the persistent vaginal changes induced by neonatal DES exposure (Chapters 2 and 3).

Moreover, I have analysed the differences in gene expressions, and estrogen-dependent signalling pathways between the vagina and uterus (Chapter 4). The estrogen-responsive genes in the vagina identified by gene profiling provide an important foundation for understanding functional mechanisms of estrogen regulated morphogenesis and maintenance of the mouse vagina and uterus. However, no comprehensive studies have been conducted on mRNA expressions in these organs in mice after estrogen exposure. Therefore, I have analyzed expression of genes in the vagina and uterus in ovariectomized mice given a single injection of 17β-estradiol, and analysed sequential changes in several estrogen-related signalling pathways.

II. Chapter 1

Estrogen Receptor Subtypes Selectively Mediate Female Mouse Reproductive Abnormalities Induced by Neonatal Exposure to Estrogenic Chemicals

1. Introduction

Estrogens tightly regulate cell proliferation and differentiation particularly in the oviduct, uterus, vagina and mammary gland of the female reproductive tracts. Long-term estrogenic stimulation is a known risk factor for carcinogenesis in humans and laboratory animals (Marselos and Tomatis, 1992a,b). Transplacental exposure to the synthetic estrogen, diethylstilbestrol (DES), which was routinely prescribed to pregnant women for preventing miscarriages in 1940's-1970's, induces vaginal clear-cell adenocarcinoma in young women (Herbst et al., 1971). It has been hypothesized that *in utero* DES exposure influences the incidence 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). Therefore, as generations of women exposed to DES approach menopause, concerns about possible long-term health risks of DES exposure grow.

Rodent models of DES exposure have been developed to understand the mechanistic basis of DES effects on humans. In mice, developmental exposure to estrogens within a critical developmental period elicits various permanent alternations in the female reproductive tract (Herbst and Bern, 1981). For example, neonatal estrogen administration induces persistent epithelial cell proliferation and superficial keratinization in the vagina, even after ovariectomy. This results in hyperplastic lesions later in life, as well as smooth muscle disorganization and epithelial squamous metaplasia in the uterus (Takasugi et al., 1962; McEwen et al., 1977; Forsberg, 1979; Iguchi, 1992).

Miyagawa et al. (2004a,b) previously reported that persistent phosphorylation of estrogen receptor α (ERα), erbB receptors and JNK1 and sustained expression of

EGF-like growth factors, interleukin-1 (IL-1)-related genes and IGF-1 mRNA (*Igf1*) contributed to persistent activity of these signaling pathways in the neonatally DES-exposed vagina. Neonatal treatment of female rats and mice with estrogens and estrogenic chemicals induces anovulation and persistent estrus as a consequence of insufficient phasic secretion of gonadotropins from the hypothalamic-pituitary axis (Takewaki, 1962; Takasugi, 1976; Iguchi, 1992; Kato et al., 2003).

Estrogens act primarily through the nuclear estrogen receptors, ERα and ERβ in mammals. ERα and ERβ can be detected in a broad spectrum of tissues. In some organs, both ER subtypes are expressed at similar levels, whereas in others, ERα or ERβ predominate. In addition, both ER subtypes may be present in the same tissue, but in different cell types. ERα is mainly expressed in the uterus, prostate (stroma), ovary (theca cells), testis (Leydig cells), epididymis, bone, breast, various regions of the brain, liver and white adipose tissue. ERβ is expressed in colon, prostate (epithelium), testis, ovary (granulosa cells), bone marrow, salivary gland, vascular endothelial cells and certain regions of the brain (Weihua et al., 2003; Dahlman-Wright et al., 2006).

ERα knockout (αERKO) mice were used to study the action of DES. In wild-type mice, uterine expression of the genes *Hoxa10*, *Hoxa11* and *Wnt7a* exhibited significant decreases shortly after DES treatment (Ma et al., 1998; Kitajewski and Sassoon, 2000), whereas this effect was not observed in the αERKO mice (Couse et al., 2001). Expression was induced in an estrogen dose-dependent manner for most of the genes noted. However, expression of these genes was not altered following estrogen treatment in αERKO mice (Watanabe et al., 2002). Adult αERKO mice were completely resistant to the chronic effects of neonatal DES exposure such as atrophy, decreased weight, smooth muscle disorganization, and squamous metaplasia in the

uterine epithelium, proliferative lesions of the oviduct, and persistent vaginal keratinization (Couse et al., 2001). Thus, the lack of DES effects on gene expression and on tissue differentiation in the αERKO mouse provides unequivocal evidence supporting an obligatory role for ERα in mediating the detrimental actions of neonatal DES exposure in the murine reproductive tract. Couse et al. (2003) reported that ERα, but not ERβ, is indispensable in the negative-feedback effects of estradiol that maintain proper LH secretion from the pituitary. ERα appears to be the predominant ER in the adult mouse uterus, vagina, oviduct and mammary gland (Couse et al., 2000; Korach et al., 2003). Immunohistochemical localization of ERβ was demonstrated only in differentiating granulosa cells of the ovary where ERα was observed prominently in interstitial cells. ERα mRNA was expressed in the female reproductive tract at all ages examined with little or no significant levels of ERβ, except on postnatal day 1 when a low level of message appeared (Jefferson et al., 2000). ERβ was detectable in the uterus of both wild-type and αERKO mice, but only at very low levels (Korach et al., 2003). The significance of ERβ in the induction of polyovular follicles by genistein in mice has been reported by Jefferson et al. (2002). Bodo et al. (2006) demonstrated that involvement of both ERα and ERβ in the sexual differentiation of the anteroventral periventricular area in the mouse hypothalamus.

On the other hand, estrogenic chemicals in the environment have been concerned to have potential adverse effects on animals and humans exposed during embryonic developmental stage (Damstra et al., 2002). Most of estrogenic chemicals bind to ERα better than ERβ, but some chemicals bind to ERβ better than ERα (Kuiper et al., 1997). Thus, importance of ER subtypes need to be studied in induction of adverse effect by estrogenic chemicals.

Recently, ERα- and ERβ-specific ligands have been synthesized and characterized using transactivation assays (Harris et al., 2002; Frasor et al., 2003; Katzenellenbogen et al., 2003). In this report, I studied the importance of each ER subtype in the induction of anovulation through the hypothalamic-pituitary axis with persistent estrus, permanent vaginal epithelial cell proliferation, disorganization of uterus, and in the induction of polyovular follicles in mice exposed neonatally to selective ER ligands or to DES.

2. Materials and Methods

2.1. Reagents

Diethylstilbestrol (DES), 17β-estradiol (E_2) and ethinylestradiol (EE_2) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Estrogen receptor α (ERα) specific ligand, 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (propyl pyrazole triol, PPT) and ERβ specific ligand, 2,3-bis(4-hydroxyphenyl)-propionitrile (diarylpropionitrile, DPN) were obtained from Tocris Bioscience (Ellisville, MO, USA). Sesame oil and dimethyl sulfoxide (DMSO) were obtained from Kanto Chemical (Tokyo, Japan).

2.2. Estrogen Receptor Transactivation Assay

CHO-K1 cells were seeded in 24-well plates at 5×10^5 cells/well in phenol-red free Dulbecco's modified Eagle's medium (Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with 10% charcoal/dextran treated fetal bovine serum (Hyclone, South Logan, UT, USA). The cells were transfected overnight with 400 ng of pGL3-Basic-4×ERE-tk-luc, 100 ng of pRL-tk-luc (as an internal control to normalize

variation in transfection efficiency; contains the *Renilla reniformis* luciferase gene with the herpes simplex virus thymidine kinase promoter; Promega, Madison. WI, USA), and 200 ng of pTARGET-mouse ERα (mERα) or mERβ using Fugene 6 transfection reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer-recommended protocol.

After 18 h, doses ranging from 10^{-12}-10^{-6} M of DES, PPT, DPN, E_2 or EE_2 were administered to the culture media. The cultures were treated with ligands for 24 h, then the cells were collected and the luciferase activities of the cells were measured by a chemiluminescence assay with Dual-Luciferase Reporter Assay System (Promega). Luminescence was measured using a Turner Designs Luminometer TD-20/20 (Promega). Promoter activity was calculated as firefly (*Photinus pyralis*)-luciferase activity/sea pansy (*R. reniformis*)-luciferase activity (Katsu et al., 2006). Transfection assays were repeated five times.

2.3. Animals and Treatments

Female C57BL/6J mice were maintained under 12 h light/12 h dark at 23-25°C and fed laboratory chow (CE-2, CLEA, Tokyo, Japan) and tap water *ad libitum*. All procedures and protocols were approved by the Institutional Animal Care and Use Committee at the National Institute for Basic Biology, National Institutes of Natural Sciences.

Three female newborn mice were sacrificed and the hypothalamus, ovary, uterus and vagina were dissected to measure expression levels of ERα and ERβ mRNA. The other female newborn mice were given 5 daily subcutaneous (s.c.) injections of 0.025, 0.25 or 2.5 μg DES/g body weight (bw) dissolved in sesame oil,

0.25, 2.5 or 25 μg/g bw PPT or DPN, dissolved in 5.6% DMSO or the vehicle alone beginning from day 0 (the day of birth). These mice were ovariectomized at 13 weeks and sacrificed at 15 weeks of age.

Vaginal smears were recorded from 11 weeks of age for 4 weeks. After ovariectomy, the dissected ovaries were weighed and fixed in 10% neutral buffered formalin. At 15 weeks of age, 6 mice in each experimental group treated with the highest concentrations of DES, PPT and DPN, and oil controls were given a single injection of 50 μg of BrdU/g bw 2 h before sacrifice. The vagina was cut in half longitudinally and one horn of each uterus was weighed. Half of the tissue was fixed in 10% neutral buffered formalin and the other half was frozen in liquid nitrogen for analysis of gene expression.

In addition, 8-19 newborn female mice were given 5 daily injections of 2.5 μg DES/g bw, 25 μg PPT or DPN/g bw or the vehicle alone. These mice were used for analysis of polyovular follicles in the ovary at 30 days of age.

2.4. Hematoxylin and Eosin (HE) Staining and BrdU Immunostaining

Tissues were embedded in paraffin, sectioned at 8 μm, following by standard HE staining and analysis of the ovaries, uteri and vaginae. Parts of deparafinized sections were incubated in 0.3% H_2O_2 in methanol for 30 min, then immersed in 2N HCl for 20 min in order to denature the genomic DNA. After washing with PBST (PBS in 0.5% Tween), the sections were incubated with anti-BrdU antibody (Boehringer Mannheim, Mannheim, Germany) diluted 1:20 in PBS containing 1% BSA overnight at 4˚C.

The sections were subsequently incubated with 3,3-diaminobenzidine tetrahydrochloride containing hydrogen peroxide. BrdU-labeling index was estimated by counting the number of BrdU-incorporated cells per h in the basal layer of cells in the vaginal epithelium as described previously (Miyagawa et al., 2004a). Polyovular follicles containing more than one oocyte in a follicle bigger than 50 μm were histologically examined and counted as described previously (Iguchi et al., 1986).

2.5. Real-time Quantitative RT-PCR

Changes in gene expression were confirmed and quantified using the ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, USA). Total RNA, isolated with RNeasy kit (QIAGEN, Chatsworth, CA, USA) from each group, was used in RT-PCR reactions carried out with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. PCR conditions were as follows: 50˚C for 2 min, 95˚C for 10 min, and 36 cycles of 95˚C for 15 sec and 60˚C for 1 min in 15 μl volumes. Relative RNA equivalents for each sample were obtained by standardization of ribosomal protein L8 levels. Sequences of gene primer sets are given in Table 1. More than three 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. Error bars represent the standard error, with all values represented as fold change compared to the control treatment group normalized to an average of 1.0.

2.6. Statistical Analysis

Statistical analyses were performed using one-way or two-way of analysis of variances, Fisher's exact probability test, Student's *t*-test or Welch's *t*-test followed by F-test as appropriate. Differences with $P<0.05$ were considered significant.

3. Results

3.1. Estrogen Receptor Transactivation Assay

Transactivation assays with mouse ERα revealed that DES, E_2 and EE_2 showed high activity at 10^{-10} M, whereas the ERα-specific ligand, PPT, showed the highest transactivation activity at 10^{-9} M. The estrogenic activity of PPT toward mERα was 10 times less than DES, E_2 and EE_2. The ERβ-specific ligand, DPN, showed no significant estrogenic activity to mERα, confirming that PPT is an ERα specific ligand (Fig. 1A).

Transactivation of mERβ showed the highest activity of DES at 10^{-10} M, and E_2 and EE_2 at 10^{-9} M. DPN showed highest transactivation activity at 10^{-9} M. As with PPT and mERα, the estrogenic activity of DPN toward mERβ was 10 times less than DES, E_2 and EE_2. PPT showed no significant estrogenic activity toward mERβ, confirming that DPN is an ERβ specific ligand (Fig. 1B). Based on these results, the doses of PPT and DPN for neonatal mouse were set 10 times higher than those of DES.

3.2. Vaginal Smear Observation

Vaginal smears were observed daily from 11 to 15 weeks of age (from 2 weeks before ovariectomy until 2 weeks after ovariectomy). Control mice showed regular estrous cycles before ovariectomy and diestrous type smears after the ovariectomy. All mice given 0.025-2.5 μg DES showed constant estrous smears before ovariectomy and

4 of 11 mice exposed to 0.025 μg, and all of 0.25 and 2.5 μg DES exposed mice showed constant estrous smears even after ovariectomy. Seven of 11 mice exposed to 0.025 μg DES showed diestrous type smears after ovariectomy. Three of 10, 10 of 11 and all 16 mice treated neonatally with 0.025, 0.25 and 2.5 μg PPT, respectively, showed constant estrous smears before ovariectomy. The remaining 7 of 10 and 1 of 11 mice treated with 0.25 and 2.5 μg PPT, respectively, showed estrous cycles before ovariectomy. After ovariectomy, 9 of 10 and 9 of 11 mice at the 0.25 and 2.5 μg PPT showed diestrous smears after ovariectomy. The remaining 1, 2 and 16 mice treated with 0.25, 2.5 and 25 μg PPT, respectively, showed constant estrous smears even after ovariectomy. In neonatally DPN treated mice, 1 of 10 mice at the 0.25 μg, and 5 of 16 at the 25 μg showed constant estrous smears. The remaining mice showed regular estrous cycles before ovariectomy. After ovariectomy, 2 of 16 mice at the 25 μg DPN showed constant estrous smears, the rest showed diestrous smears.

3.3. Ovarian Histology

Ovaries dissected at 13 weeks of age were examined histologically. All control mice showed corpora lutea in the ovary indicating that ovulation had occurred. However, all DES-exposed mice lacked corpora lutea in the ovary, demonstrating anovulation even at the lowest (0.025 μg/g bw) concentration. A significantly higher incidence of anovulation was found in mice exposed neonatally to 2.5 and 25 μg PPT, however, no significant increase in the number of mice showing anovulation was induced by 0.25 μg of PPT or by any dose of DPN exposure (Table 2, Fig. 2). Hyperplastic interstitial cells and lack of corpora lutea were encountered in the ovaries of mice exposed neonatally to DES (0.025-2.5 μg) and 2.5 and 25 μg PPT. In all

treatment groups, mice showed regular estrous cycles before ovariecomy had corpora lutea, whereas, mice showing constant estrous smears lacked corpora lutea in the ovary.

3.4. Uterine and Vaginal Histology

The uterine epithelium was composed of a single layer of low columnar cells with several uterine glands and circular and longitudinal muscle layers in ovariectomized control and 0.25-25 μg DPN-exposed mice. Disorganization of stromal cells and muscle layers, such as hypoplasia of circular muscle and decrease in density of longitudinal muscle, was encountered in mice treated neonatally with 0.025-2.5 μg DES and 2.5 and 25 μg PPT (Table 2, Fig. 2).

The vaginal epithelium of neonatally oil-injected, 15-week-old ovariectomized control and 0.25-25 μg DPN-exposed mice was composed of 2-3 layers of cuboidal cells. The vaginal epithelium of the age-matched, neonatally 0.25-2.5 μg DES- and 25 μg PPT-exposed, ovariectomized mice exhibited stratification and superficial keratinization (Table 2, Fig. 3). In the vagina showing ovary-independent epithelial stratification, the basal cells in the epithelium showed high proliferative activity (18-19%), which was confirmed by BrdU immunostaining. In contrast, the basal cells in the vaginal epithelium of ovariectomized controls and DPN-exposed mice showed very low incidence (1.7%) of BrdU incorporation (Fig. 3E).

3.5. Persistent Expression of Growth Factor and IL-1-related Genes

mRNA expression of *Areg (amphiregulin)*, *Ereg (epiregulin)*, *Hbegf (heparin-binding epidermal growth factor)*, *Il1r2 (interleukin-1 receptor type II)*, *Il1f5*

(interleukin-1 family member 5 delta), ESR1 (ERα) and *ESR2 (ERβ)* in the vagina was analyzed using real-time quantitative RT-PCR in mice exposed neonatally to 2.5 μg DES, 25 μg PPT and 25 μg DPN. The vaginae of mice exposed neonatally to DES and PPT showed persistent expression of these genes, but not the vaginae of mice exposed to oil or to DPN (Fig. 3F). In addition, the expression of ERα mRNA in the vagina of ovariectomized control mice were 1000 times higher than that of ERβ (data not shown), exhibiting that ERα is the predominant ER in the vagina of ovariectomized adult mice. A piece of vagina used for mRNA analysis was also histologically analyzed. I confirmed the epithelial stratification in neontally DES- and PPT-exposed mice, but not in controls and mice exposed neonatally to DPN.

3.6. Induction of Polyovular Follicles

Ovaries dissected at 30 days of age were histologically examined. A high incidence of polyovular follicles (PFs) was found in ovaries of mice exposed neonatally to 25 μg PPT and DPN (3.1% and 4.3%, respectively), although, no significant difference was found in the incidence of PFs between mice exposed to PPT or DPN. Mice exposed to 2.5 μg DES exhibited the highest incidence of PFs in the ovary (14%), showing that DES is the most potent inducer of PFs among chemicals used in this experiment (Table 3, Fig. 4).

3.7. Expression of ERα and ERβ in Various Tissues of Female Newborn Mice

The ratio of ERα to ERβ mRNA in the hypothalamus, ovary, uterus and vagina were analyzed using real-time quantitative RT-PCR in newborn female mice. ERα mRNA expression was higher than that of ERβ mRNA in all tissues examined:

hypothalamus, 15.0 ± 2.4; ovary, 17.8 ± 4.9; uterus, 33.7 ± 6.1 and vagina, 27.7 ± 5.8 (the value indicates the ratio of ERα mRNA to ERβ mRNA, Mean ± S.E.), demonstrating that ERα is the predominant ER in these tissues in female newborn mice.

4. Discussion

I confirmed the selective activation of ER subtypes reviewed previously by Katzenellenbogen et al. (2003) using transactivation assays with mouse ERα and ERβ (Katsu et al., 2006). In the mERα assay, E_2 and DES maximally activated the reporter gene at 10^{-10} M and PPT activated it at 10^{-9} M. In the mERβ transactivation assay, E_2, DES and DPN maximally activated the reporter gene at 10^{-10} M. Based on these data, the dose of PPT and DPN to be used for *in vivo* studies was set 10 times higher than DES.

In rodents, administration of aromatizable androgen or estrogen to neonatal females induces anovulatory sterility (Barraclough, 1961; Takewaki, 1962; Gorski, 1963; Takasugi, 1976; Iguchi et al., 1988; Aihara and Hayashi, 1989; Kincl, 1990; Iguchi, 1992), whereas castration of neonatal male rats evokes the capacity for sexual cyclicity and lordosis behavior that is characteristic of the female rat. These treatments are effective only during the ‘‘critical period’’ of perinatal life, and the steroids given are considered to masculinize or defeminize the brain (Goy and McEwen, 1980; Iguchi et al., 1988). ERα and ERβ expression has been demonstrated in the mouse brain (Mitra et al., 2003). ERα knockout (αERKO) mice are sterile, βERKO mice are fertile, but the average number of offspring is less than that of wild type mice (Couse and Korach, 1999; Couse et al., 2003). This demonstrates the critical importance of ERα in

the normal development of the hypothalamic-pituitary-ovarian (HPG) axis. Couse et al. (2003) further demonstrated that ERα is indispensable to the negative-feedback effects of estradiol that maintain proper LH secretion from the pituitary.

Plastic component, bispheno-A (BPA) and phytoestrogen, genistein, bind to ERβ about 7 times better than they do ERα (Kuiper et al., 1997). Neonatal exposure to BPA induced anovulatory sterility in female rats (Kato et al., 2003). Also, neonatal exposure to BPA, or to genistein affected sexual differentiation of the anteroventral periventricular nucleus of the hypothalamus (Patisaul et al., 2006). I, therefore, studied effects of neonatal exposure to ER selective ligands on the hypothalamus. In the present study, vaginal smears of mice exposed neonatally to 0.025-2.5 μg DES and to 2.5 and 25 μg of the ERα specific ligand, PPT, showed constant estrus. However, mice exposed to the ERβ specific ligand, DPN, showed cyclic smear patterns. Mice showing constant estrous smear patterns that were exposed neonatally to DES and PPT had no corpus luteum in the ovary, indicating anovulatory sterility. These results clearly suggest that ERα, but not ERβ, mediates most of the estrogenic effects of chemicals on the HPG axis during critical developmental stages.

In newborn mice, ERα is localized in the uterine stromal cells, but not in the epithelial cells whereas it is expressed in both epithelial and stromal cells in the vagina (Sato et al., 1992). The present study confirmed that ERα is the predominant form of ER in the uterus and vagina as reported previously (Jefferson et al., 2000; Couse and Korach, 2004). The present study demonstrated that the ratio of ERα/ERβ is bigger in the adult vagina than the newborn vagina.

In tissue recombination experiments, ERα-negative uterine epithelium (derived from the αERKO mouse uterus) recombined with ERα-positive stroma, showed

proliferation following estrogen stimulation, whereas wild type epithelium recombined with ERα-negative uterine stroma did not proliferate (Cooke et al., 1997; Buchanan et al., 1998, 1999). These reports suggested that epithelial cell proliferation could be mediated indirectly by ERα in the stroma. In cell culture conditions, estrogen did not stimulate vaginal or uterine epithelial cell proliferation (Iguchi et al., 1983; Iguchi, 1985), however, estrogen stimulated DNA synthesis in human endometrial epithelial cells co-cultured with stromal cells in a transfilter system (Pierro et al., 2001). Estrogen stimulated vaginal and uterine stromal cell proliferation in culture (Inada et al., 2006). Thus, ERα activity in stromal cells is essential for estrogen-mediated epithelial cell proliferation in mouse reproductive tracts. Perinatal treatment with estrogens (e.g., E_2, DES, EE_2), aromatizable and non-aromatizable androgens, or BPA induce ovary-independent persistent proliferation of vaginal epithelium with superficial keratinization (Takasugi, 1976; Iguchi, 1992; Suzuki et al., 2002; Inada et al., 2006). No such changes in the vagina were induced in the neonatally DES-exposed αERKO mice (Couse and Korach, 2004), indicating the essential role of ERα in the induction of ovary-independent vaginal changes induced by estrogens. Here I showed that persistent vaginal epithelial cell proliferation with the superficial keratinization was induced by neonatal treatment with 0.25-2.5 μg DES or 25 μg PPT, but not DPN. Neonatal treatment with 0.025-2.5 μg DES or 2.5-25 μg PPT induced disorganization of circular muscle in the uterus; however, DPN did not induce this abnormality. These results also indicate that ERα action is essential for induction of uterine muscular disorganization and ovary-independent persistent vaginal epithelial cell proliferation caused by estrogens during the critical developmental stage.

In the persistently proliferating vaginal epithelial cells in mice exposed neonatally to DES, it has been reported that phosphorylation of ERα and erbB2 receptor, and persistent expression of genes related to epidermal growth factor, such as amphiregulin (*Areg*), epiregulin (*Ereg*), heparin-binding EGF (*Hbegf*), interleukin-1 (IL-1) receptor type II (*Il1r2*), IL-1 family member 5 (delta) (*Il1f5*), tumor necrosis factor-α and insulin-like growth factor-I (Miyagawa et al., 2004a,b). The present results show that the persistent expression of *Areg*, *Ereg*, *Hbegf, Il1r2* and *Il1f5* in vagina of mice treated neonatally with DES and PPT, but not DPN. These results indicate that ERα action is also essential for the induction of persistent molecular changes in the vagina.

Perinatal treatment with estrogens such as E_2, DES, EE_2 and genistein induces polyovular follicles (PFs) in the ovary (Iguchi, 1985; Iguchi and Takasugi, 1986; Iguchi et al., 1986; Jefferson et al., 2002; Kirigaya et al., 2006; Kipp et al., 2007). Neonatal treatment with a large dose of BPA also induced PFs in mice (Suzuki et al., 2002). The critical period for induction of PFs is within 3 days after birth in mice (Iguchi et al., 2002). ERα is localized in interstitial and thecal cells, whereas ERβ is localized in granulosa cells in the ovary (Jefferson et al., 2002). ERβ is the predominant form in the ovary (Jefferson et al., 2002; Couse and Korach, 2004). ERβ is critical in granulosa cell differentiation and the ovulatory response to gonadotropins (Couse et al., 2005). Neonatal exposure of genistein induced PFs in wild-type and αERKO female mice, but not in βERKO females, and the induction of PFs in the ovary is dependent on the presence of functional ERβ within the ovary (Jefferson et al., 2002). Our results show that neonatal treatment with PPT or DPN equally induce PFs in ovaries; therefore, both ERα and ERβ are involved in the induction of PFs.

However, expression of ERα mRNA was higher than that of ERβ mRNA in the ovary of newborn mice. E_2, progesterone and genistein disrupt nest breakdown and primordial follicle formation, which may result in the PFs in mouse ovary (Chen et al., 2007). Kipp et al. (2007) showed that PFs induced by neonatal DES or E_2 exposure were accompanied by decreased levels of activin β-subunit mRNA and protein. This resulted in loss of phosphorylation of Smad 2 protein, a marker of activin-dependent signaling, in the estrogen-treated ovaries. Therefore, both ER subtypes may be involved in these molecular and histological changes in the newborn mouse ovary.

Estrogenic chemicals in the environment potentially have adverse effects on animals including humans when exposed during development (Damstra et al., 2002). Most of estrogenic chemicals bind to ERα better than ERβ, but some chemicals such as genistein, coumestrol, BPA etc. bind to ERβ better than ERα (Kuiper et al., 1997). Ratio of ERα/ERβ is different among tissues as demonstrated in the present study. The present results suggest that chemicals having affinity to ERβ as well as chemicals having affinity to ERα can induce adverse effects when exposed during critical sensitive periods, which differ among tissues (Iguchi et al., 2002). ER specific ligands can be used to understand the involvement of ER subtypes in various estrogen-mediated diseases such as mammary cancer and endometriosis.

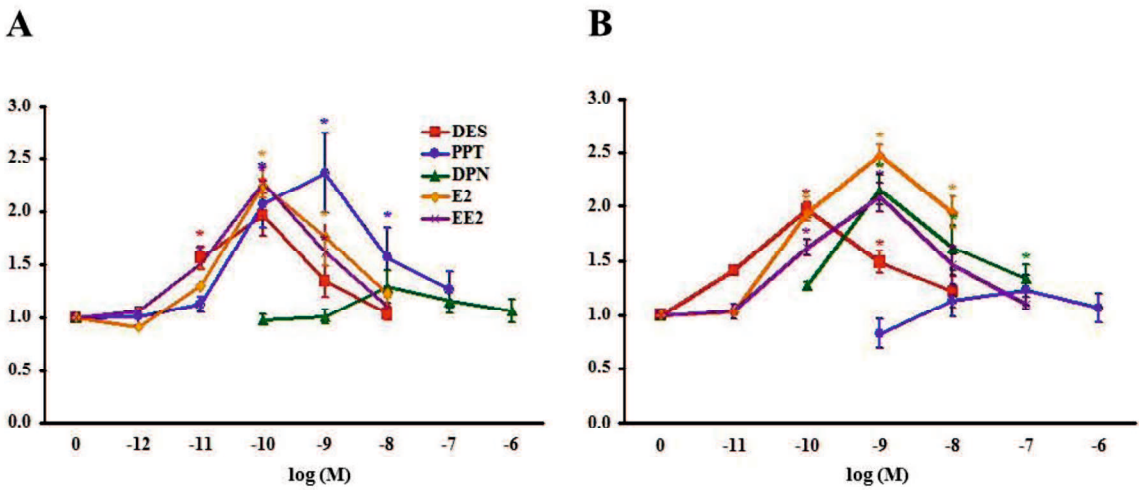


Fig. 1. Estrogenic activities of ER selective ligands, natural and synthetic estrogens for mouse ERα (A) and mouse ERβ (B) in reporter gene assays. DES (diethylstilbestrol), PPT (propyl pyrazole triol); ERα specific lignad, DPN (diarylpropionitrile); ERβ specific ligand, E_2 (17β-estradiol) and EE_2 (ethinylestradiol). $^{*}P<0.05$ vs controls (two-way ANOVA).

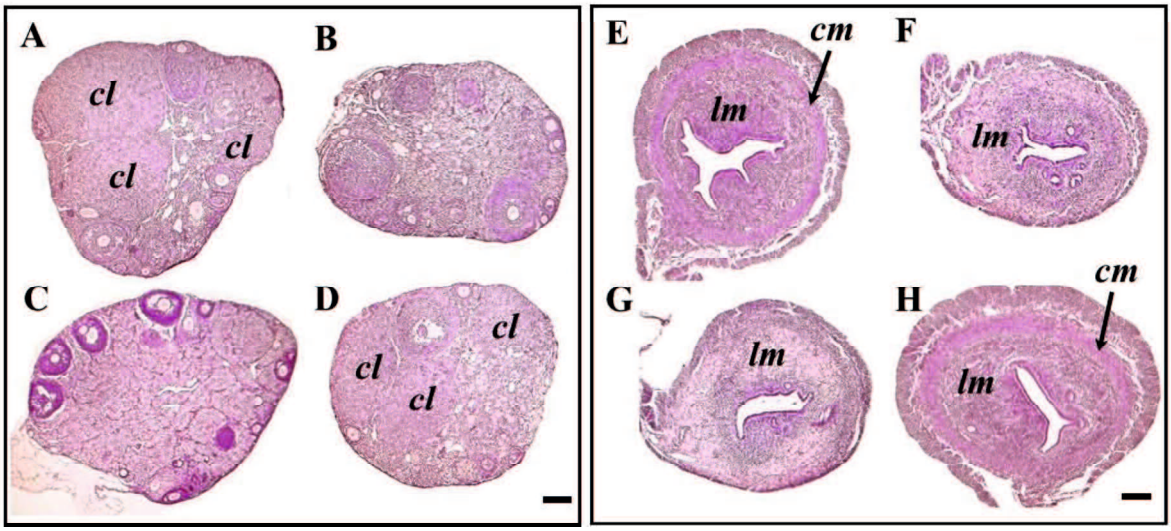


Fig. 2. Histology of ovaries in 13-week-old mice exposed neonatally to oil (A), 2.5 μg DES (B), 25 μg PPT (C) and 25 μg DPN (D) for the first 5 days. Note corpora lutea (*cl*) in oil control mouse and DPN-treated mouse. Histology of uteri of 15-week-old, ovariectomized mice exposed neonatally to oil (E), 2.5 μg DES (F), 25 μg PPT (G) and 25 μg DPN (H) for the first 5 days. Note disorganization of muscle layers in DES- and PPT-treated mice. *cm*: circular muscle, *lm*: longitudinal muscle. Bar: 100 μm.

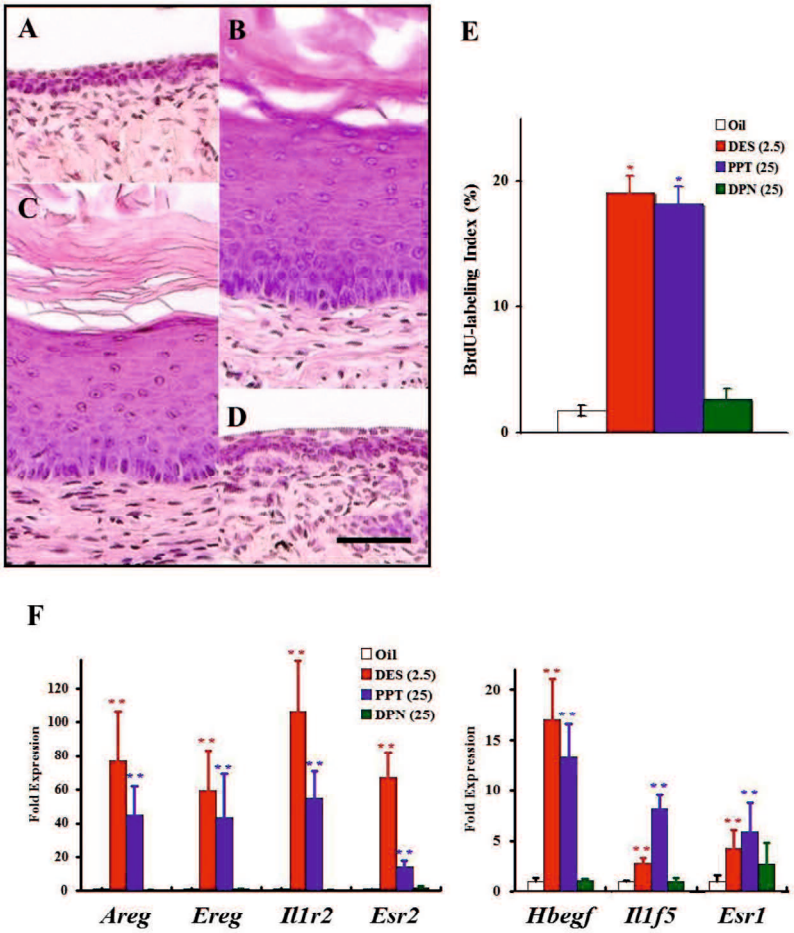


Fig. 3. Histology of vaginae of 15-week-old, ovariectomized mice exposed neonatally to oil (A), 2.5 μg DES (B), 25 μg PPT (C) and 25 μg DPN (D) for the first 5 days. Note ovary-independent persistent proliferation of vaginal epithelium in DES- and PPT-treated mice. Bar: 50 μm. Incidence of BrdU-incorporation (%) in basal cells of vaginal epithelium of mice exposed neonatally to oil, 2.5 μg DES, 25 μg PPT and 25 μg DPN (E). $^{*}P<0.05$ vs controls (one-way ANOVA). Persistent expression of mRNAs of growth factors, interleukin-1-related genes and estrogen receptors in mouse vagina exposed neonatally to oil, 2.5 μg DES, 25 μg PPT or 25 μg DPN for the first 5 days (F). Expression of *Areg* (amphiregulin), *Ereg* (epiregulin), *Hbegf* (heparin-binding epidermal growth factor), *Il1r2* (interleukin-1 receptor type II), *Il1f5* (IL-1 family, member 5), *Esr1* (estrogen receptor α, ERα) and *Esr2* (ERβ) in vagina show higher levels in DES- and PPT-treated mice than those of controls and DPN-treated mice. The expression of each mRNA in vagina of the oil-treated control mice was regarded as the basal level (1.0). $^{**}P<0.05$ vs controls (Student's *t*-test or Welch's *t*-test followed by F-test).

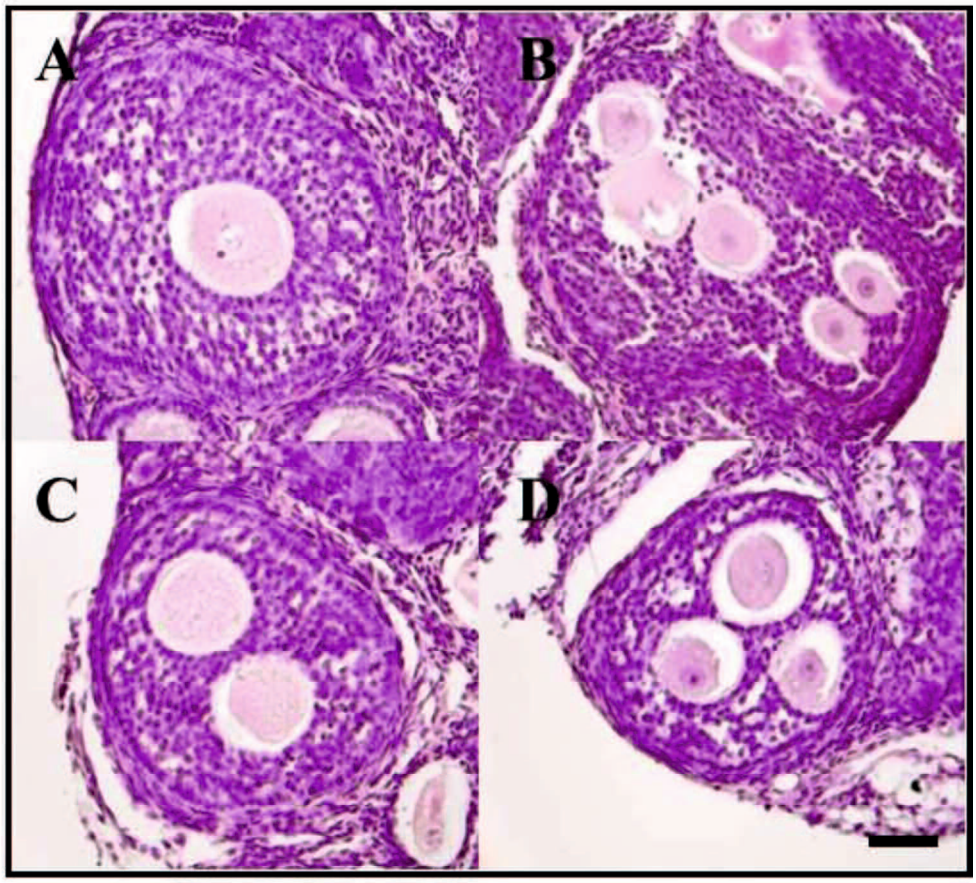


Fig. 4. Histology of ovaries in 30-day-old mice exposed neonatally to oil (A), 2.5 μg DES (B), 25 μg PPT (C) and 25 μg DPN (D) for the first 5 days. Note polyovular follicles in ovaries of DES-, PPT- and DPN-treated mice. Bar: 100 μm.

Table 1. Sequences of gene primer sets for real-time quantitative RT-PCR

Gene	Primer (5'-3')[a]	Product size (bp)	GenBank accession no.
Arfg	F: CATTATGCAGCTGCTTTGGA R: TTTCGCTTATGGTGGAAACC	124	NM_009704
Erfg	F: CGCTGCTTTGTCTAGGTTCC R: GGGATCGTCTTCCATCTGAA	122	NM_007950
Hbegf	F: GACCCATGCCTCAGGAAATA R: GGCATTTGCAAGAGGGAGTA	89	NM_010415
Il1r2	F: GTTTATCTCGGCTGCTTACCCA R: CAAAAATCAGCGACACTTCCAC	101	NM_010555
Il1f5	F: GGGCCAAGGAATCAAAGAGC R: CGGATTCGAAGCTGGAGGTA	69	NM_019451
Esr1	F: AATGAAATGGGTGCTTCAGG R: AAGGACAAGGCAGGGGTATT	98	NM_007956
Esr2	F: CTACAGTGTTCCCAGCAGCA R: GCATAGAGAAGCGATGATTGG	136	NM_010157

Areg (amphiregulin), *Erfg* (epiregulin), *Hbegf* (heparin binding-epidermal growth factor), *Il1r2* (interleukin-1 receptor type II), *Il1f5* (IL-1 family, member 5), *Esr1* (estrogen receptor α, ERα) and *Esr2* (ERβ), [a]F, forward; R, reverse.

Table 2. Effects of neonatal exposure of ERα- and ERβ-ligands on ovary (13 weeks), uterus and vagina (15 weeks) in mice

Treatments (μg/g BW)	No. of mice used	No. of mice with		
		Ovary without corpora lutea	Uterine muscle disorganization	Vaginal epithelial stratification
Oil	14	0	0	0
0.025 DES	11	11*	11*	4
0.25 DES	9	9*	9*	9*
2.5 DES	14	14*	14*	14*
0.25 PPT	10	3	3	1
2.5 PPT	11	10*	9*	2
25 PPT	16	16*	16*	16*
0.25 DPN	10	1	0	0
2.5 DPN	12	0	1	0
25 DPN	16	5	0	2

*$P<0.05$ vs controls (Fisher's exact probability test).

Table 3. Incidence of polyovular follicles (PFs) in 30-day-old mice treated neonatally with ERα- and ERβ-ligands

Treatments (μg/g BW)	No. of mice examined	PFs frequency[a] (No. of mice with PFs)	Incidence of PFs (%)
Oil	13	77 (10)	0.6 ± 0.14[b] (2)[c]
DES (2.5)	8	100 (8)	14.0 ± 0.84[*] (2-7)
PPT (25)	15	100 (15)	3.1 ± 0.43[*] (2-6)
DPN (25)	19	100 (19)	4.3 ± 0.58[*] (2-3)

[a]Ratio of mice with PFs (%), [b]Mean ± S.E., [c]Range of the number of oocytes/PF in parentheses, [*]P<0.05 vs controls (one-way ANOVA).

III. Chapter 2

WNT Family Genes and Their Modulation in the Ovary-independent and Persistent Vaginal Epithelial Cell Proliferation and Keratinization Induced by Neonatal Diethylstilbestrol Exposure in Mice

1. Introduction

Estrogen-induced cell proliferation and differentiation in female reproductive organs such as oviduct, uterus and vagina are long being studied by several group of researchers (Takasugi et al., 1962; Dunn and Green, 1963; Takasugi and Bern, 1964; Forsberg, 1969; Herbst et al., 1971; McLachlan et al., 1980; Newbold and McLachlan, 1982; Newbold et al, 1985; Iguchi et al., 1986, Iguchi, 1992). Diverse biological effects of estrogens are primarily mediated via the activation of nuclear estrogen receptors, ERα and ERβ, which are ligand-inducible transcription factors (Tsai and O'Malley, 1994; Beato et al., 1995). Increase in specific gene expressions via ERα or ERβ after estrogen exposure in mice has been silenced by an ER antagonist, ICI 182,780 (Miyagawa et al., 2004a,b).

Vaginal epithelium is an intriguing model for analyzing the estrogen action in mice. It undergoes characteristic changes from a non-keratinized to a fully keratinized epithelium depending on the levels of the endogenous estrogen, estradiol (E_2), during the estrous cycle (Miller et al., 1998).

Estrogen exposure, during a critical period in the early development in mice, induces persistent, ovary-independent proliferation and keratinization in the vaginal epithelium at adulthood (Takasugi et al., 1962; Takasugi and Bern, 1964). In humans, trans-placental exposure to a synthetic estrogen, diethylstilbestrol (DES), which was routinely prescribed to pregnant women for prevention of miscarriages from the 1940s to 1970s in the USA and European countries, resulted in vaginal clear-cell adenocarcinoma in young women (Herbst et al., 1971). Although perinatal estrogenic chemical exposure induces various abnormalities, i.e., polyovular follicles, oviductal tumors, uterine epithelial metaplasia, persistent vaginal stratification and keratinization,

vaginal adenosis, and cervico-vaginal carcinomas (Takasugi et al., 1962; Dunn and Green, 1963; Takasugi and Bern, 1964; Forsberg, 1969; Newbold and McLachlan, 1982; Newbold et al, 1985; Iguchi et al., 1986; Iguchi, 1992; Suzuki et al., 2002), the critical period of estrogen action during mouse development varies from organ to organ (Iguchi et al., 2002). DES exposure during critical developmental period results in alterations of the response to estrogens in mouse vagina, leading to a set of subsequent abnormalities. Among them, vaginal epithelial proliferation persists even after ovariectomy in mice exposed to sufficient doses of DES during the early neonatal period (Takasugi et al., 1962; Takasugi and Bern, 1964).

Wnt genes are the vertebrate homologs of *wingless*, the Drosophila segment polarity gene comprised of 16 members. They are a large group of highly conserved secreted glycoproteins, and play crucial roles in embryonic developmental processes (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998; Smalley and Dale, 1999), tumorigenesis (Tsukamoto et al., 1988; Smalley and Dale, 1999; Lustig and Behrens, 2003) and reproduction (Parr and McMahon, 1998; Vanino et al., 1999) mostly via Frizzled (Fz) receptor (Dale, 1998). Fzs constitute a large family of seven transmembrane G protein-coupled receptors and possess an extracellular cysteine-rich domain (CRD) for Wnt/binding (Wang et al., 1996; Liu et al., 1999). Among several Wnt-mediated intracellular signaling pathways (Willert and Nusse, 1998; Huelsken and Birchmeier, 2001; van Noort and Clevers, 2002), the canonical Wnt β-catenin pathway has been well studied.

The Wnt singling is highly responsive to variable hormone concentration and location (Wever-Hall et al., 1994). It is well known that Wnt singling plays roles in epithelial-mesenchymal interactions and cellular organization during embryonic and

postembryonic development, involving in cell proliferation and differentiation, cell fate specification and cell-to-cell communication (Cadigan and Nusse, 1997; Wodarz and Nusse, 1998; Smalley and Dale, 1999). Wnt signaling also plays a key role in murine female reproductive tract development (Miller et al., 1998; Daikoku et al., 2004), and has been suggested as a target for potential endocrine disruptors (Sassoon, 1999). Miller et al. (1998) reported that three Wnt family genes, *Wnt4*, *Wnt5a* and *Wnt7a*, were expressed in the uterus and cervix in specific epithelial-mesenchymal interactions during postnatal development and in the adult. However, the expression of Wnt genes in vagina has not yet been elucidated.

Previously, Miyagawa et al. (2004a,b) examined the global expression of mRNA, focusing on factors involved in cell signaling in the vagina of mice exposed neonatally to DES showing persistent hyperplasia and the superficial keratinization. In the present study, I report that neonatal exposure of DES and ERα specific ligand induced persistent up-regulation of *Wnt4* and persistent down-regulation of *Wnt11* in mouse vagina. In addition, to clarify the role of Wnt4 in vaginal histological modulation by estrogen, I used Wnt4 hetero ($Wnt4^{+/-}$) mice, since $Wnt4^{-/-}$ mice exhibit fetal lethality (Stark et al., 1994; Vainio et al., 1999). *Wnt4* expression was correlated to epithelial keratinization, in mouse vagina exposed neonatally to DES.

2. Materials and Methods

2.1. Reagents

Diethylstilbestrol (DES) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Estrogen receptor α (ERα) specific ligand, 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (propyl pyrazole triol, PPT),

ERβ specific ligand, 2,3-bis(4-hydroxyphenyl)-propionitrile (diarylpropionitrile, DPN) and estrogen receptor antagonist, ICI 182,780, were obtained from Tocris Bioscience (Ellisville, MO, USA). Sesame oil and dimethyl sulfoxide (DMSO) were obtained from Kanto Chemical (Tokyo, Japan).

2.2. Animals and treatments

C57BL/6J mice and 129^{+Ter}/Sv mice were purchased from CLEA Japan (Tokyo, Japan). Wnt4 mutant mice (129^{+Ter}/Sv strain) were from Jackson Laboratory (Bar Harber, ME, USA) through Prof. K.-I. Morohashi. They were maintained under 12 h light/12 h dark at 23-25°C and fed laboratory chow (CE-2, CLEA) and tap water *ad libitum*. All procedures and protocols were approved by the Institutional Animal Care and Use Committee at the National Institute for Basic Biology, National Institutes of Natural Sciences.

C57BL female newborn mice were given 5 daily subcutaneous (s.c.) injections of 0.025 (n=6), 0.25 (n=6) or 2.5 μg (n=6) DES/g body weight (bw) dissolved in sesame oil or the oil vehicle alone (n=6) beginning from day 0 (the day of birth). Ovariectomy was performed in all mice exposed neonatally to DES, since the aim of the present study was to understand the underlying molecular mechanisms of ovary (estrogen)-independent persistent vaginal changes. These mice ovariectomized at 8 weeks and sacrificed at 10 weeks of age were used for DNA microarray analysis, reverse transcriptase polymerase chain reaction (RT-PCR), histology and immunohistochemistry. In addition, mice exposed to 2.5 μg DES/g bw neonatally and ovariectomized as adults (n=8) were given 5 daily intraperitoneal injections of 5 μg ICI 182,780/g bw or oil vehicle alone beginning from day 65 and killed 24 h after the last

injection. Tissues were used for real-time quantitative RT-PCR and histological examination for counting number of vaginal epithelial cell layers.

Newborn female C57BL mice were given 5 daily s.c. injections of 2.5 μg DES/g bw (n=4), 25 μg/g bw PPT (n=4) or DPN (n=4) dissolved in 5.6% DMSO or the vehicle alone (n=4) beginning from day 0. These mice ovariectomized at 13 weeks were sacrificed at 15 weeks of age, and used for real-time quantitative RT-PCR and histology.

$Wnt4^{+/+}$ and $Wnt4^{+/-}$ newborn mice were given 5 daily s.c. injections of 2.5 μg DES/g bw dissolved in oil (n= 10 or 4, respectively) or the oil vehicle alone (n=5 each). These mice ovariectomized at 8 weeks were sacrificed at 10 weeks of age, and analyzed *Wnt4* mRNA expression and histology.

2.3. DNA microarray analysis

Total RNA from vaginae exposed neonatally to 0.025, 0.25 or 2.5 μg DES/g bw or oil vehicle alone were extracted using TRIZOL (Invitrogen, Carlsbad, CA, USA) and purified using an RNeasy mini kit (QIAGEN, Chatsworth, CA, USA). Quality and quantity of total RNA were confirmed by the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). cRNA probes were prepared from the purified RNA using an Affymetrix cRNA probe kit (Affymetrix, Santa Clara, CA USA) according to the manufacturer's protocol. All preparations met the recommended criteria of Affymetrix for use on their expression array. The amplified cRNA was hybridized to high-density oligonucleotide arrays (Mouse U74A; Affymetrix) containing approximately 12500 genes, and the scanned data were analyzed with GeneChip software (Affymetrix) and processed as described previously (Watanabe et al., 2004).

To confirm the estrogen-related changes in gene expression revealed by DNA microarray analysis, I independently repeated the same experiment twice. The expression data were analyzed with GeneSpring software (Agilent) as described previously (Watanabe et al., 2004).

For the clustering analysis, genes expressed more than 2-fold or less than a half by neonatal DES treatment to controls were selected, and similarities between experiments and expression levels were measured by standard correlation using the GeneSpring program as described previously (Watanabe et al., 2002, 2003, 2004).

2.4. RT-PCR and real-time quantitative RT-PCR

Total RNA, isolated with RNeasy kit (QIAGEN, Chatsworth, CA, USA) from each group of vaginae, was used in RT-PCR or real-time quantitative RT-PCR reactions carried out with SuperScript III reverse transcriptase (Invitrogen). RT-PCR was carried out using AmpliTaq Gold (TAKARA, Ohtsu, Japan). Sequences of gene primer sets are given in Table 4. PCR conditions were as follows: 94˚C for 10 min, and 32 cycles of 94˚C for 30 sec, 60˚C for 30 sec and 72˚C for 30 sec, and 72˚C for 10 min in 25 μl volumes.

Changes in gene expression were confirmed and quantified using ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. PCR conditions were as follows: 50˚C for 2 min, 95˚C for 10 min, and 36 cycles of 95˚C for 15 sec and 60˚C for 1 min in 15 μl volumes. Relative RNA equivalents for each sample were obtained by standardization of ribosomal protein L8 levels. Sequences of gene primer sets are given in Table 4. More than three pools of

samples per group were run in 3-7 groups to determine sample reproducibility, and the average relative RNA equivalents per sample were used for further analysis. Error bars represent the standard error, with all values represented as fold change compared to the control group normalized to an average of 1.0.

2.5. HE staining and immunohistochemistry

Tissues were fixed in neutral buffered 10% formalin, embedded in paraffin and sectioned at 8 μm. Some sections were stained with standard hematoxylin and eosin. Other sections deparafinized were incubated with 0.3% H_2O_2 in methanol for 15 min to eliminate endogenous peroxidase. After washing with PBS, the sections were incubated anti-Wnt4 antibody (R&D Systems, Inc., Minneapolis, MN, USA) at 1:200 dilution in PBS containing 1% BSA (Sigma) overnight at 4℃. The sections were visualized with LSAB^{TM} 2 kit, Universal (Dako, Carpinteria, CA, USA) according to the manufacturer-supplied protocol. For negative controls, normal goat immunoglobulin fraction (Dako) was used at the same dilution.

2.6. Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA), Student's *t*-test or Welch's *t*-test followed by F-test as appropriate. Differences with $P<0.05$ were considered significant.

3. Results

3.1. DNA microarray analysis

Microarray analyses was performed to get an idea about the expression profiles of different Wnt genes, especially, *Wnt4*, *Wnt5a*, *Wnt5b*, *Wnt7b* and *Wnt11* mRNA in the mouse vagina (Table 5). Surprisingly, only *Wnt4* and *Wnt7b* showed higher (3-6 fold) to moderate (1.97-2.42 fold) spikes in the neonatally DES-exposed mouse vagina than controls. On the other hand, *Wnt11* showed a decrease (0.21-0.29 fold) after DES treatment. However, other Wnt genes remained unaffected in vaginal epithelia after neonatal DES treatment. To verify the results of microarray analysis, I examined the expression of *Wnt4* and *Wnt11* mRNA using RT-PCR. Similar to microarray analysis, *Wnt4* or *Wnt11* expression was up- or down-regulated, respectively, in the vaginal epithelium of DES-exposed mice than controls (data not shown). Interestingly, mRNAs of all *Frizzled* family (*Fz 1* to *10*) were detected in the mouse vagina regardless of the neonatal DES exposure (data not shown). Henceforth, further studies were conducted with *Wnt4* and *Wnt11* only.

3.2. Estrogen responsive changes of Wnt genes in mouse vagina

Neonatal DES exposure induced vaginal epithelial stratification with superficial keratinization which was not abolished by ovariectomy (Fig. 5A, D). By contrast, neonatally oil-treated control mice had atrophied vaginal epithelium after ovariectomy (Fig. 5C, D). Expressions of *Wnt4* mRNA was high and *Wnt11* mRNA was low in the vagina of ovariectomized mice exposed neonatally to DES, however, the expression patterns in these genes in the vagina of ovariectomized mice exposed neonatally to oil vehicle alone were reversed (Fig. 5E, F). To investigate the transcriptional regulation of *Wnt4* and *Wnt11* mRNA by exogenous estrogen, I administrated ER antagonist, ICI 182,780, to neonatally 2.5 μg DES-exposed, ovariectomized mice, showing vaginal

epithelial stratification and superficial keratinization. *Wnt4* expression in the neonatally DES-exposed mouse vagina, which treated with ICI 182,780, was significantly decreased, but *Wnt11* expression was not changed by anti-estrogen exposure. Surprisingly, the number of vaginal epithelial cell layers in ICI 182,780-treated mice exposed neonataly to DES, were significantly decreased (Fig. 5B, E, F). This suggested that the DES-responsive changes in Wnt expressions and estrogen responsive epithelial cell proliferation are actually correlated.

To ascertain the role of Wnt genes in vaginal epithelial cell proliferation, I performed immunohistochemistry (IHC) of Wnt4. Ten-week-old ovariectomized mice exposed neonatally to 2.5 μg DES or oil vehicle alone, were used for IHC with anti-Wnt4 antibody (Fig. 6). Wnt4 staining was observed in the basal and middle layers of epithelial cells in vagina of mice exposed neonatally to DES (Fig. 6A), but no Wnt4 staining was observed in oil-treated control mouse vagina (Fig. 6C). This suggests that Wnt4 might be associated with epithelial cell proliferation and further keratinization. I also found that *Wnt4* was expressed in the vagina showing epithelial cell proliferation, while *Wnt11* was restricted to the atrophic vagina having 2-3 epithelial cell layers.

To pinpoint the role of specific estrogen receptor on such transcriptional modulation of Wnt genes and related cell proliferation, I analyzed both *Wnt4* and *Wnt11* mRNA expression and epithelial cell proliferation and keratinization in vagina of 15-week-old ovariectomized mice treated neonatally with 25 μg DPN, 25 μg PPT or 2.5 μg DES. The vaginal epithelium of these ovariectomized mice exhibited epithelial cell proliferation, stratification and superficial keratinization (Fig. 7A-D). *Wnt4* expression was found to increase after neonatal DES or PPT treatment (Fig. 7E). A

simultaneous decrease in *Wnt11* expression was also observed in DES- or PPT-treated vagina (Fig. 7F). However, DPN treatment neither changed the *Wnt4* and *Wnt11* expression nor epithelial cell proliferation. Vaginal epithelia of ovariectomized mice treated neonatally with oil (Fig. 7A) or 25 μg DPN (Fig. 7D) were composed of 2-3 layers of cuboidal cells only. This highlights only *Wnt4*, but not *Wnt11*, is responsible for the persistent vaginal epithelial cell proliferation and persistent activation of ERα (Miyagawa et al., 2004a,b).

To clarify the role of Wnt4 in vaginal histological modulation by estrogen, I used Wnt4 hetero ($Wnt4^{+/-}$) mice, since $Wnt4^{-/-}$ mice exhibit fetal lethality (Stark et al., 1994; Vainio et al., 1999). I thought that Wnt4 expression levels in the vagina of wild type ($Wnt4^{+/+}$) mice were higher than $Wnt4^{+/-}$ mice. All $Wnt4^{+/+}$ and $Wnt4^{+/-}$ mice treated neonatally with oil, vaginal epithelia were composed of 2-3 layers of cuboidal cells (Table 6). While, all neonatally DES-exposed $Wnt4^{+/+}$ and $Wnt4^{+/-}$ mice exhibited vaginal epithelial stratification or stratification with superficial keratinization (Table 6). *Wnt4* expression levels and histology in vaginae between $Wnt4^{+/+}$ and $Wnt4^{+/-}$ mice were not different. *Wnt4* was highly expressed in neonatally DES-exposed mice both in $Wnt4^{+/+}$ and $Wnt4^{+/-}$ mice (Fig. 8A), showing epithelial stratification with superficial keratinization (Fig. 8B, C). The vagina of $Wnt4^{+/+}$ and $Wnt4^{+/-}$ mice exposed neontatally to DES having only epithelial stratification show no up-regulation of Wnt4 expression (Fig. 8B, C) suggesting that Wnt4 plays a role in epithelial keratinization in the vagina.

4. Discussion

In the present study, I intended to clarify the mechanism of ovary-independent proliferation of vaginal epithelial cells. First, I analyzed global gene expression patterns in the DES-exposed mouse vagina. Both microarray analysis and RT-PCR showed differential interplay of Wnt family genes after DES-exposure. Especially, neonatal DES and ERα specific ligand exposure induced persistent up-regulation of *Wnt4* or persistent down-regulation of *Wnt11* in mouse vagina. In addition, I also found that DES induces ER-mediated epithelial stratification and keratinization regulated by *Wnt4*.

During embryonic development, members of the Wnt gene family express in a diverse fashion. Pavlova et al. (1994) have previously noted that murine Wnt gene family, *Wnt5a*, were abundant in the adult female reproductive tract, but become relatively scarce during gestation. In addition to *Wnt4*, *Wnt5a* and *Wnt7a* are also detected at high levels in the murine female reproductive tract and had a specific mesenchymal-epithelial expression pattern (Miller et al., 1998). However, these expressions fluctuate along with estrus cycle progression (Miller et al., 1998). In present study, I confirmed the expressions of several Wnt family genes, i.e., *Wnt4*, *Wnt5a*, *Wnt5b*, *Wnt7b* and *Wnt11* mRNA in the neonatally DES-exposed or oil control mouse vagina using DNA microarray analysis. Although I recorded an elevated expression for *Wnt4* and *Wnt7b*, and reduced expression of *Wnt11*, but *Wnt5a* and *Wnt5b* remain unchanged. Therefore, I decided to focus on *Wnt4* and *Wnt11* genes in the vagina exposed neonatally to DES.

Wnt4 is known to be involved in multiple development processes, such as the formation of kidney, adrenal gland, female reproductive tracts and various cancers (Connony and Schnitt, 1993; Stark et al., 1994; Kispert et al., 1998; Brisken et al.,

2000; Smalley and Dale et al., 2001; Jeays-Ward et al., 2004; Yu et al., 2006). *Wnt11* is a non-canonical Wnt family, regulates ureteric branching (Majumdar et al., 2003), and cardiogenesis (Pandur et al., 2002). In the line of microarray results, our tissue distribution data also suggested similar respective up- and down-regulation of *Wnt4* and *Wnt11* expression after neonatal DES exposure. The reduction in *Wnt11* after DES exposure suggests their repressive role in Wnt pathway (Maye et al., 2004). However, the expression of Fz genes, receptors of *Wnt4* (Lyons et al., 2004), did not change in DES-treated vagina, suggested that *Wnt4* might have other function unrelated to Fzs.

Cellular localization of protein gives an idea about the potential target. Miller et al. (1998) reported the localization of *Wnt4* mRNA in mouse reproductive tract using *in situ* hybridization, however, no information of the localization of Wnt4 protein in the vagina. In the present study, Wnt4 protein was localized in the vaginal epithelium of mice exposed neonatally to DES, especially in the basal epithelial cell layer. Saitoh et al. (1998) reported that Wnt4 protein plays a role in epidermal-dermal (presumably keratinocyte-fibroblast) interactions in the skin. Wnt4 is possibly participating in cell proliferation or keratinization in the mouse vaginal epithelium.

In this regard, our earlier reports suggest that DES-induced persistent proliferation in vagina is actually mediated through ERα (Nakamura et al., 2008). Moreover, in wild-type mice, uterine expression of *Hoxa10*, *Hoxa11* and *Wnt7a* genes exhibited significant decrease shortly after DES treatment (Ma et al., 1998; Kitajewski and Sassoon, 2000; Couse et al., 2001), whereas this effect was not observed in the αERKO mice (Couse et al., 2001). This supports the idea about the obligatory role for ERα in DES-induced alteration of mouse reproductive tract. Interestingly, in the present study, only PPT, but not DPN, induced a similar magnitude of *Wnt4* and *Wnt11*

expression as in DES-exposed vagina. This suggests that the changes in *Wnt4* and *Wnt11* profile are ERα responsive. But anti-estrogen mediated reduction of *Wnt4*, but not *Wnt11*, confirms that *Wnt4* action is regulated by ERα, and *Wnt11* might be regulated by androgen receptor as in prostate cancer (Zhu et al., 2004).

Finally, I used $Wnt4^{+/-}$ mutant mice to study the function of Wnt4 in the estrogen-induced vaginal epithelial stratification and keratinization, since $Wnt4^{-/-}$ mouse show fetal lethality (Vainio et al., 1999; Majumdar et al., 2003). $Wnt4^{+/-}$ mice exposed neonatally to DES showed vaginal epithelial stratification with the superficial keratinization similar to wild-type mouse exposed neonatally to DES. However, *Wnt4* was highly expressed in vagina showing epithelial stratification with the superficial keratinization. Keratins have long and extensively been used as immunohistochemical markers in diagnostic tumor pathology (Moll et al., 2008; Karantza, 2011). Interestingly, *Wnt11* was significantly down-regulated in the vagina of mice showing ovary-independent persistent epithelial proliferation. This confirms that *Wnt4* and *Wnt11* might show the opposite behavior in the mouse vagina. *Wnt4* expression was correlated to the keratinization of vaginal epithelium.

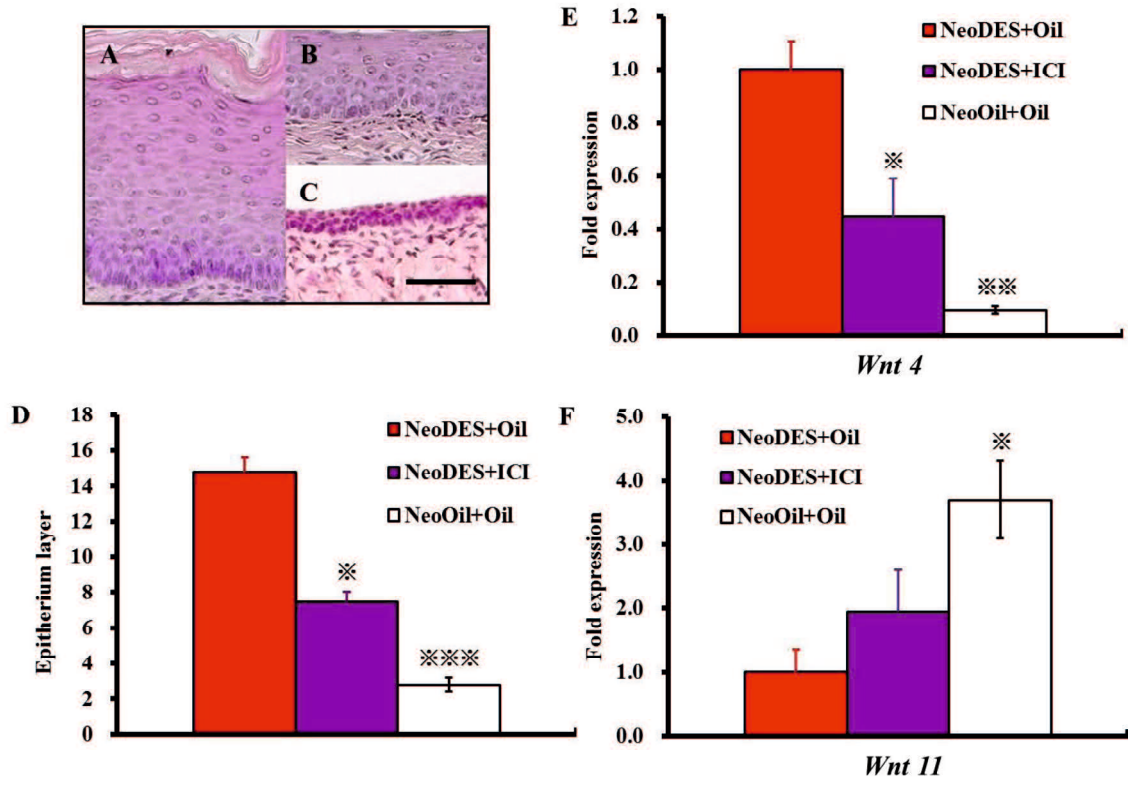


Fig. 5. Administration of anti-estrogen, ICI 182,780, reduced proliferation of vaginal epithelial cells in 10-week-old, ovariectomized mice exposed neonatally to 2.5 μg DES. Vaginal histology of ovariectomized mice exposed neonatally to DES (NeoDES) treated with oil vehicle [NeoDES + Oil (A)] or 5 μg ICI 182,780/g bw [NeoDES + 5 μg/g bw ICI 182,780 (B)] and ovariectomized mice exposed neonatally to oil vehicle alone and oil before sacrifice [NeoOil + Oil (C)] before sacrifice. Sections were stained with hematoxylin and eosin. Number of vaginal epithelial cell layers was significantly decreased in mice treated with ICI 182,780 (D). Expression profiles of Wnt gene mRNA in vagina of NeoDES mice treated with oil or ICI 182,780. *Wnt4* mRNA expression was significantly decreased in mice treated with ICI 182,780 (E), but *Wnt11* mRNA was not changed (F). Bar: 50 μm. *P<0.05 vs NeoDES + Oil, **P<0.01 vs NeoDES + Oil, ***P<0.001 vs NeoDES + Oil (Student's *t*-test or Welch's *t*-test followed by F-test).

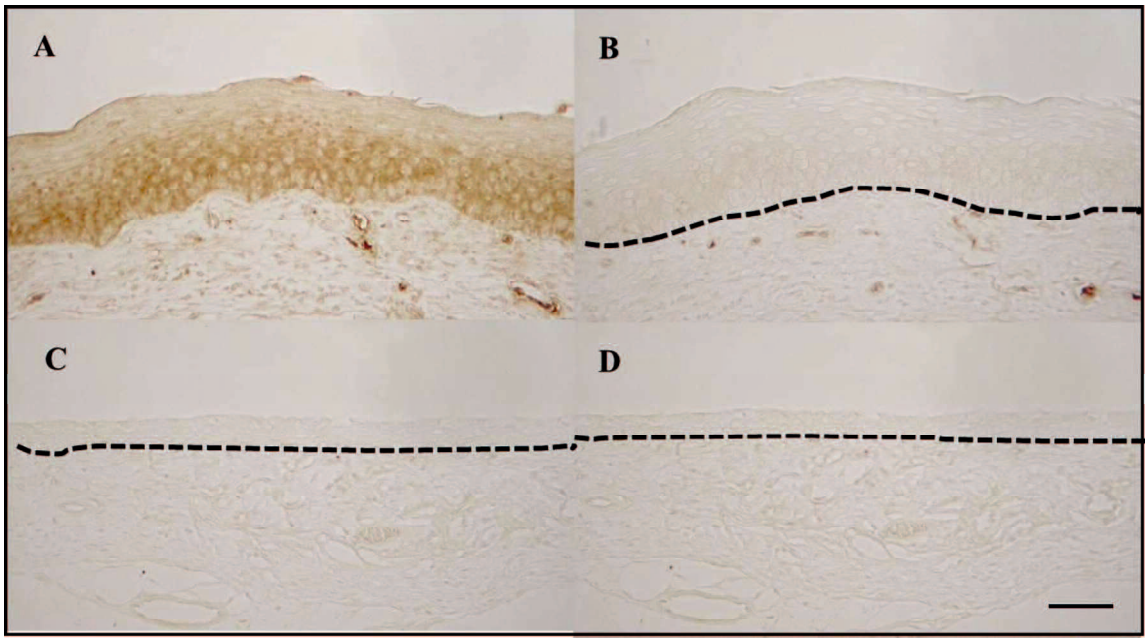


Fig. 6. Immunohistochemistry by Wnt4 antibody. Vaginae of 10-week-old, ovariectomized mice exposed neonatally to 2.5 μg DES (A, B) or oil vehicle alone (C, D). Wnt4 localized in epithelium cells of DES-exposed vagina, especially, in basal layer and middle layers (A). Control mouse vagina was not expressed Wnt4 protein (C). No immunostaining was noted when sections were incubated with preimmune serum instead of primary antibody (B, D). Bar: 50 μm. The boundary between epithelium and stroma is indicated by a dotted line.

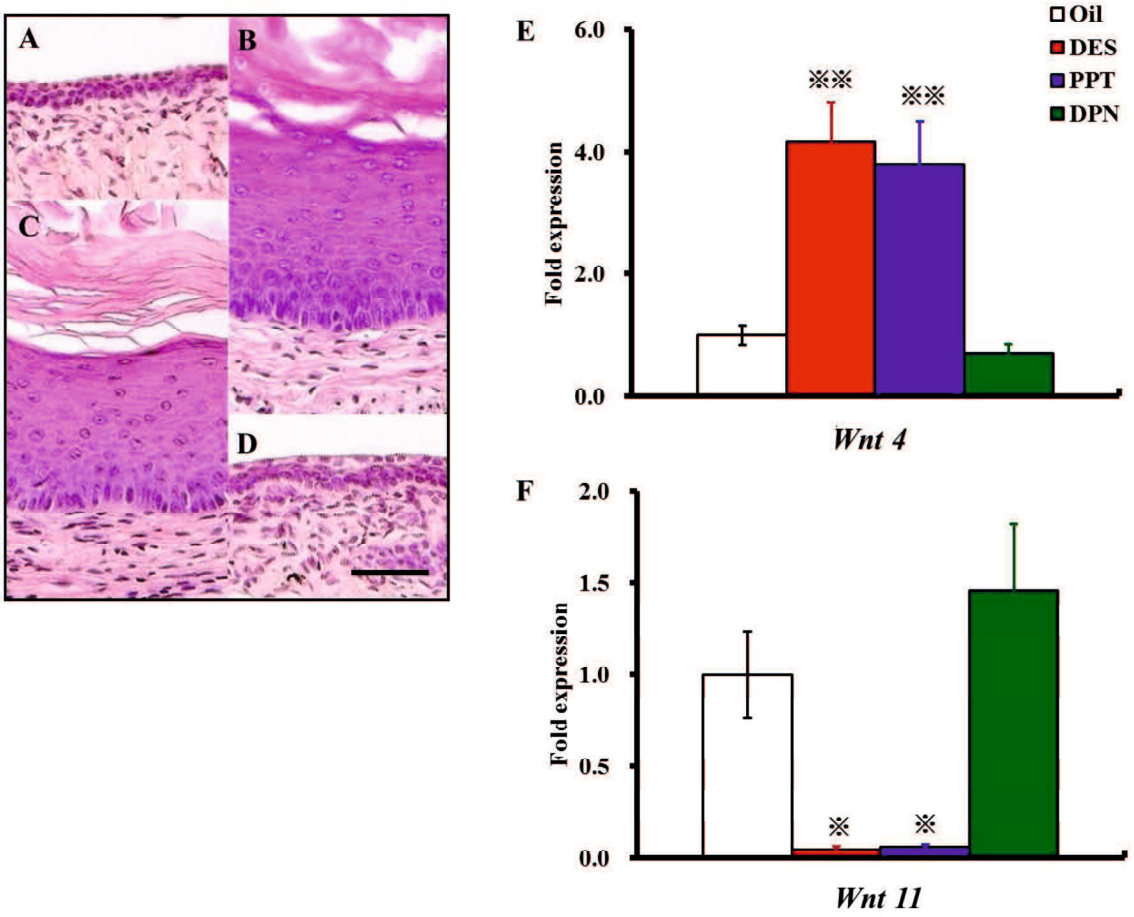


Fig. 7. Histology of vaginae of 15-week-old, ovariectomized mice exposed neonatally to oil (A), 2.5 μg DES (B), 25 μg PPT (C) and 25 μg DPN (D) for the first 5 days. Note ovary-independent persistent proliferation of vaginal epithelium in DES- and PPT-treated mice. Neonatal 2.5 μg DES or 25 μg PPT treatment for the first 5 days induced persistent up-regulation of *Wnt4* mRNA (E), and persistent down-regulation of *Wnt11* mRNA (F) in mouse vagina. The expression of each mRNA in vagina of the oil-treated controls was regarded as the basal level (1.0). Bar: 50 μm. *P<0.05 vs controls, **P<0.01 vs controls (one-way ANOVA).

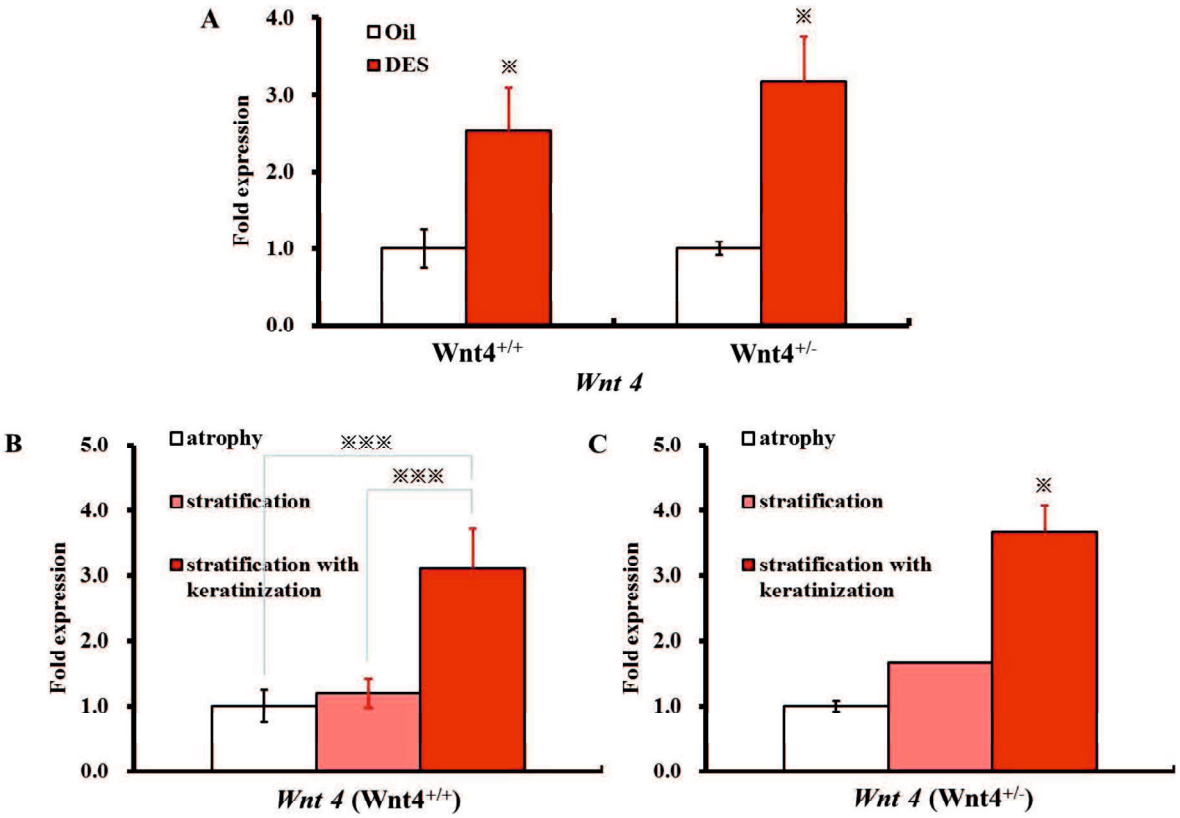


Fig. 8. Vaginae of 10-week-old, ovariectomized $Wnt4^{+/+}$ or $Wnt4^{+/-}$ mice exposed neonatally to 2.5 μg DES or oil vehicle alone. Neonatal exposure to 2.5 μg DES induced persistent up-regulation of *Wnt4* mRNA in vaginae of both $Wnt4^{+/+}$ and $Wnt4^{+/-}$ mice (A). *Wnt4* mRNA expression was significantly correlated to the vaginal epithelial cell proliferation with the superficial keratinization but not for the proliferation only in $Wnt4^{+/+}$ (B) and $Wnt4^{+/-}$ mice (C). Since only one $Wnt4^{+/-}$ mouse showed epithelial stratification only, therefore, statistical analysis could not be done. The number of mice showing vaginal epithelial atrophy, stratification only and stratification with keratinization are correlated to the Table 6. *P<0.05 vs controls, ***P<0.001 vs controls (Student's *t*-test or Welch's *t*-test followed by F-test).

Table 4. Sequences of gene primer sets for real-time quantitative RT-PCR

Gene	Primer (5'-3')[a]	Product size (bp)	Gene accession no.
Wnt4	F: CATCGAGGAGTGCCAATACCA	70	NM_009523
	R: GACAGGGAGGGAGTCCAGTGT		
Wnt11	F: ATGTGCGGACAACCTCAGCTA	100	NM_009519
	R: CGCATCAGTTTATTGGCTTGG		

[a]F, forward; R, reverse.

Table 5. Microarray date of Wnt genes in vaginas of adult mice (10 week-ages) exposed neonatally to DES

Gene accession no.	Name	Fold change			Prove set ID
		0.025	0.25	2.5	
NM_021279	wingless-related MMTV integration site 1	NC	NC	NC	1425377_at
NM_023653	wingless-related MMTV integration site 2	NC	NC	NC	1449425_at
NM_009520	wingless-related MMTV integration site 2b	NC	NC	NC	1421465_at
NM_009521	wingless-related MMTV integration site 3	NC	NC	NC	1450763_x_at
NM_009522	wingless-related MMTV integration site 3A	NC	NC	NC	1422093_at
NM_009523	wingless-related MMTV integration site 4	**4.15**	**5.95**	**3.23**	1450782_at
NM_009524	wingless-related MMTV integration site 5A	0.82	0.91	1.33	1436791_at
NM_009524	wingless-related MMTV integration site 5A	1.29	1.13	1.03	1448818_at
NM_009525	wingless-related MMTV integration site 5B	NC	NC	NC	1422602_a_at
NM_009525	wingless-related MMTV integration site 5B	NC	NC	0.80	1439373_x_at
NM_009526	wingless-related MMTV integration site 6	NC	NC	NC	1419708_at
NM_009527	wingless-related MMTV integration site 7A	NC	NC	NC	1423367_at
NM_001163634	wingless-related MMTV integration site 7B	NC	**2.42**	1.97	1420891_at
NM_001163634	wingless-related MMTV integration site 7B	NC	NC	NC	1420892_at
NM_009290	wingless-related MMTV integration site 8A	NC	NC	NC	1422228_at
NM_011720	wingless-related MMTV integration site 8b	NC	NC	NC	1421439_at
NM_011720	wingless-related MMTV integration site 8b	NC	NC	NC	1421440_at
NM_139298	wingless-type MMTV integration site 9A	NC	NC	NC	1425889_at
NM_011719	wingless-type MMTV integration site 9B	NC	NC	NC	1451711_at
NM_009518	wingless-related MMTV integration site 10a	NC	NC	NC	1460657_at
NM_011718	wingless-related MMTV integration site 10b	NC	NC	NC	1426091_a_at
NM_009519	wingless-related MMTV integration site 11	**0.21**	**0.21**	**0.29**	1450772_at
NM_053116	wingless-related MMTV integration site 16	NC	NC	NC	1422941_at

Table 6. Effect of neonatal treatment of DES on vaginae of $Wnt4^{+/-}$ and $Wnt4^{+/+}$ mice ovariectomized 2 weeks before sacrifice

Treatments	Genotypes	No. of. mice used	No. of mice showing vaginal epitherial		
			atrophy	stratification	stratification with keratinization
Oil	Wnt4 +/+	5	5	0	0
	Wnt4 +/-	5	5	0	0
2.5 μg/g bw DES	Wnt4 +/+	10	0	3	7
	Wnt4 +/-	4	0	1	3

IV. Chapter 3

p21 and Notch Signalings in the Persistently Altered Vagina Induced by Neonatal Diethylstilbestrol Exposure in Mice

1. Introduction

Long-term estrogenic stimulation is a known risk factor for carcinogenesis in laboratory animals and humans (Marselos and Tomatis, 1992a,b). In humans, transplacental exposure to a synthetic estrogen, diethylstilbestrol (DES), induced vaginal clear-cell adenocarcinoma in young women (Herbst et al., 1971). In mice, developmental exposure to estrogens within a critical developmental period elicits various permanent alternations in female reproductive tracts (Takasugi and Bern, 1964; Forsberg, 1969; Newbold and McLachlan, 1982; Newbold et al., 1985; el-Deiry et al., 1993; Miyagawa et al., 2004a,b; Nakamura et al., 2008, 2012). For example, neonatal estrogen administration induces persistent vaginal epithelial cell proliferation and keratinization even after ovariectomy, resulting in hyperplastic lesions and vaginal cancers later in life (Takasugi et al., 1962; McLanhlan et al., 1980; Gartel and Tyner, 1999).

Previously, Miyagawa et al. (2004a,b) and Suzuki et al. (2006) characterized the mRNAs expression patterns in the neonatal mouse vagina exposed to DES at different ages and the persistently altered vagina by neonatal DES exposure using DNA microarray and real-time quantitative RT-PCR. In the vagina of mice exposed neonatally to DES, expressions of various genes were modulated, and interleukin-I (IL-I) and insulin-like growth factor-I (IGF-I) signalings were activated without estrogen stimulation (Miyagawa et al., 2004b). 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 mouse vagina (Miyagawa et al., 2004a,b). In the vagina of mice exposed to DES at different ages showed genes related to keratinocyte differentiation and cell cycle-related genes, such as *Gadd45a*, *14-3-3 sigma*, *Sprr2f* (*small proline-rich protein 2f*), *Klf4* (*Kruppel-like factor 4*) and *p21*, were induced by DES (Suzuki et al., 2006).

p21 (also called WAF1, CAP20, Cip1 and Sdi1) (Forsberg, 1969; Xiong et al., 1993; Noda et al., 1994; Harper et al., 1995), founding member of the Cip/Kip family of CKIs including p27 (Polyak et al., 1994; Toyoshima and Hunter, 1994) and p57 (Toyoshima et al., 1994; Lee et al., 1995) can bind and inhibit a broad range of cyclin/Cdk complexes, with a preference for those containing Cdk2 (Xiong et al., 1993; Hartman et al., 2004). p21 plays an essential role in growth arrest after DNA damage (Dunn et al., 1963; Deng et al., 1995; Devgan et al., 2005), and its over-expression leads to G_1 and G_2 (Niculescu et al., 1998) or S-phase (Ogryzko et al., 1997) arrest. Moreover, the anti-oncogenic effect of Notch family gene, which is one of the fundamental signaling pathway that regulate metazoan development and adult tissue homeostasis, appears to be mediated by p21 and by repression of Shh and Wnt signalings (Dulic et al., 1994; Thelu et al., 2002; Nicolas et al., 2003). Wnt signaling suppressed by Notch1 activation in keratinocytes, showing that Notch1 activation down-regulates this pathway by suppressing *Wnt-4* expression (Dulic et al., 1994). p21 mediates this negative regulation; Notch1 activation increased p21 protein levels, which subsequently associates with E2F1 transcription factors at the *Wnt4* promoter, down-regulating *Wnt4*

expression (Dulic et al., 1994). On the other hand, p21 is often responsible for stress-induced p53-dpendent and p53-independent cell cycle arrest, which permits cells to pause and to repair damage and then to continue cell division (Harper et al., 1993), and p21 expression has been shown to be regulated largely at the transcriptional level by both p53-dependent and -independent mechanisms (Harper et al., 1993).

I, therefore, focused on the p21, p53 and Notch signaling in order to understand the molecular mechanisms underlying the persistently altered vagina by neonatal DES exposure in mice.

2. Materials and Methods

2.1. Reagents

Diethylstilbestrol (DES) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Estrogen receptor α (ERα) specific ligand, 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (propyl pyrazole triol, PPT) and ERβ specific ligand, 2,3-bis(4-hydroxyphenyl)-propionitrile (diarylpropionitrile, DPN), were obtained from Tocris Bioscience (Ellisville, MO, USA). Sesame oil and dimethyl sulfoxide (DMSO) were obtained from Kanto Chemical (Tokyo, Japan).

2.2. Animals and treatments

C57BL/6J mice purchased from CLEA Japan (Tokyo, Japan) were maintained under 12 h light/12 h dark at 23-25°C and fed laboratory chow (CE-2, CLEA) and tap

water *ad libitum*. All procedures and protocols were approved by the Institutional Animal Care and Use Committee at the National Institute for Basic Biology, National Institutes of Natural Sciences.

The middle part of the vagina, Müllerian duct origin, was used for current study. Female newborn mice were given 5 daily subcutaneous (s.c.) injections of 2.5 μg DES/g body weight (bw) (n=6) dissolved in sesame oil or the oil vehicle alone (n=6) beginning from day 0 (the day of birth). These mice were ovariectomized at 8 weeks and sacrificed at 10 weeks of age (n=6, in each experimental group). These mice were used for DNA microarray analysis.

Newborn females were given 5 daily s.c. injections of 2.5 μg DES/g bw (n=4), 25 μg/g bw PPT (n=4) or DPN (n=4) dissolved in 5.6% DMSO or the vehicle alone (n=4) beginning from day 0. These mice, ovariectomized at 13 weeks, were sacrificed at 15 weeks of age and vaginae were used for real-time quantitative RT-PCR and immunohistochemistry. Since I have already demonstrated that there is no age difference in histology and gene expression at least between 2-4 months in neonatally DES exposed ovariectomized mice (Miyagawa et al., 2004a,b; Nakamura et al., 2008, 2012). I used preserved mouse vaginae treated abovementioned chemicals to save animal use.

2.3. DNA microarray analysis

Total RNA from vaginae exposed neonatally to 2.5 μg DES/g bw or oil vehicle alone were extracted using TRIZOL (Invitrogen, Carsbad, CA) and purified using an RNeasy mini kit (Qiagen, Chatsworth, CA, USA). Quality and quantity of total RNA were confirmed by the Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, USA). cRNA probes were prepared from the purified RNA using an Affymetrix cRNA probe kit (Affimetrix, Santa Clara, CA, USA) according to the manufacturer's protocol. All preparations met the recommended criteria of Affymetrix were applied for their expression array. The amplified cRNA was hybridized to high-density oligonucleotide arrays (Mouse U74A; Affymetrix) and the scanned data were analyzed with GeneChip software (Affymetrix) and processed as described previously (Watanabe et al., 2004). To confirm the estrogen-related changes in gene expression revealed by DNA microarray analysis, I independently repeated the same experiment twice. The expression data were analyzed with GeneSpring software (Agilent) as described previously (Watanabe et al., 2002, 2003, 2004).

2.4. Real-time quantitative RT-PCR

Total RNA, isolated with RNeasy kit (Qiagen) from vaginae of each group, was used for real-time quantitative RT-PCR reactions carried out with SuperScript III reverse transcriptase (Invitrogen).

Changes in gene expression were confirmed and quantified using the ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and

SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. PCR conditions were as follows: 50˚C for 2 min, 95˚C for 10 min, and 36 cycles of 95˚C for 15 sec and 60˚C for 1 min in 15 μl. Relative RNA equivalents for each sample were obtained by standardization of ribosomal protein L8 levels. Sequences of gene primer sets are given in Table 7. More than three 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.

2.5. Immunohistochemistry

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 4 μm. Deparafinized sections were incubated with 0.3% H_2O_2 in methanol for 15 min to eliminate endogenous peroxidase. After washing with PBS, the sections were stained with LSAB^{TM} 2 kit, Universal (Dako, Carpinteria, CA, USA) according to the manufacturer-supplied protocol. Anti-p21 monoclonal antibody was obtained from Santa Cruz, Inc. (Minneapolis, MN, USA). The sections were incubated at 1:50 dilution in PBS containing 1% BSA (Sigma) overnight at 4℃. For negative controls, murine IgG2b (Dako) was used at the same dilution.

2.6. Statistical analysis

Statistical analyses were performed using Student's *t*-test or Welch's *t*-test followed by F-test as appropriate. Differences with $P<0.05$ were considered significant.

3. Result

3.1. DNA microarray analysis

11,219 of 22,689 in Genechip probes were detected in vaginae of 10-week-old control or 2.5 μg DES-exposed mice, ovariectomized at 8 weeks of age. 423 probes were up-regulated and 351 probes were down-regulated as compared to the controls in the vagina exposed neonatally to DES (data not shown). I further analyzed genes related to cell cycle. 26 genes (31 probes) were up-regulated (Table 8) and 8 genes (10 probes) were down-regulated (Table 9) in DES-exposed vagina as compared to oil controls.

3.2. p21 and p53 mRNA expression

Since the expression of *p21* was specifically up-regulated in the cell cycle-related genes compared to controls, I analyzed *p21* mRNA expression in vaginae of 15-week-old ovariectomized mice exposed neonatally to 25 μg DPN, 25 μg PPT and 2.5 μg DES. Because we previously showed the persistent proliferation and keratinization of epithelial cells in the vagina exposed neonatally to DES and PPT (Nakamura et al., 2008, 2012). *p21* expression was very low in vagina of controls and neonatally 25 μg DPN-exposed ovariectomized mice. In contrast, *p21* was up-regulated in the vagina of mice exposed neonatally to 2.5 μg DES and 25 μg PPT (Fig. 9). p53 mRNA, unlike p21 mRNA, was unaltered in the DES-, PPT- and DPN-exposed vagina (Fig. 9).

3.3. p21 protein localization

I performed immunohistochemistry to investigate the localization of p21. Fifteen-week-old, ovariectomized mice given neonatal exposure to 2.5 μg DES or the oil vehicle alone were stained with p21 antibody (Fig. 10A, C) or murine IgG2b as a negative control (Fig. 10B, D). p21 was localized in the basal layer of vaginal epithelial cells in mice exposed neonatally to DES (Fig. 10A), but no p21 staining was observed in the oil-treated controls (Fig. 10C) showing correlation of p21 expression and proliferation of the vaginal epithelial cells.

3.4. The expression of Notch signal pathway related genes in the vagina of mice exposed neonatally to DES, PPT or DPN

I analyzed the expression of genes related to Notch signal pathway, however, all Notch receptors mRNA (*Notch 1*, *Notch 2*, *Notch 3* and *Notch 4*) (Fig. 11A) and Notch ligands mRNA (*Dll 1*, *Dll 4*, *Jagged 1* and *Jagged 2*) (Fig. 11B) were unaltered in the DES-exposed vagina. Whereas, the expression of Notch target genes, *Hes1*, *Hey1* and *Heyl*, was significantly decreased in the vagina exposed neonatally to DES or PPT compared to the control and neonatally DPN-treated mice (Fig. 11C).

4. Discussion

Estrogens tightly regulate cell proliferation and differentiation in the female reproductive tracts (Takasugi et al., 1962; Iguchi, 1992). However, perinatal exposure of estrogens including synthetic estrogen, DES and other estrogenic chemicals, induces persistent anovulation caused by alteration of hypothalamo-pituitary-gonadal axis, polyovular follicles, uterine abnormalities and persistent vaginal changes in mice (Takasugi et al., 1962; Takasugi and Bern, 1964; Herbst et al., 1971; Forsberg, 1979; Newbold and McLachlan, 1982; Newbold et al., 1985; Iguchi et al., 1986; Iguchi, 1992; el-Deiry et al., 1993; Suzuki et al., 2002, 2006). Especially, transplacental exposure to DES, which was routinely prescribed to pregnant women for preventing miscarriages in 1940's-1970's, induced vaginal clear-cell adenocarcinoma in young women (Herbst et al., 1971). It has been hypothesized that *in utero* DES exposure influences the incidence of breast cancer, squamous neoplasia of the cervix and vagina, and vaginal clear-cell adenocarcinoma later in life (Herbst et al., 1971; Herbst, 2000; Hatch et al., 2001; Palmer et al., 2002).

DNA microarray has been successfully used to analyze estrogen-responsive genes in the mouse vagina, and genes possibly related to persistent vaginal epithelial cell proliferation induced by neonatal DES-exposure (Miyagawa et al., 2004b; Watanabe et al., 2004; Suzuki et al., 2006; Nakamura et al., 2012a). In the present study, I found up-regulation of cell cycle-related genes in the persistently proliferating vaginal epithelial cells of neonatally DES-exposed mice. Especially, *p21* mRNA showed persistent up-regulation in the vagina with irreversible proliferation, and p21 was

localized in the basal layer of vaginal epithelial cells. However, the expression of *p53* mRNA did not changed in the DES- and PPT-exposed mouse vagina. A number of previous studies suggested that *p21* was regulated by p53-independent mechanisms. Notably, many of these studies suggested that serum and other growth factors, e.g. epidermal growth factor (EGF), might be involved in the up-regulation of *p21* in various cell types (Michieli et al., 1994; Macleod et al., 1995). Moreover, Akt phosphorylated by PI3K leads to the stabilization of p21 and enhanced tumor cell survival (Li et al., 2002). Previously, Miyagawa et al. (2004a,b) found the phosphorylation of Akt, and persistent expression of some growth factors in the neonatally DES-exposed vagina. In the present study, *p21* might be regulated by p53-independent mechanisms, and the expression of *p21* might be involved in the persistent epithelial proliferation in mouse vagina exposed neonatally to DES or PPT.

Notch family genes are evolutionarily conserved and participate in a variety of cellular processes; for example, cell fate decision (including proliferation, differentiation and apoptosis), cardiovascular development, endocrine development and cancer (Lathia et al., 2008; Kopan et al., 2009; Tien et al., 2009; Radtke et al., 2010). Anti-oncogenic effect of Notch1 in murine skin appears to be mediated by p21 induction and by repression of Wnt signaling (Dulic et al., 1994). In the present study, mRNA expression of Notch receptors (*Notch 1*, *Notch 2*, *Notch 3* and *Notch 4*) and Notch ligands (*Dll 1*, *Dll 4*, *Jagged 1* and *Jagged 2*) were not changed in the vagina exposed neonatally to DES and PPT, suggesting that these signalings are not regulated

by p21-dependent mechanisms, and not be involved in the persistent vaginal changes induced by neonatal DES or PPT exposure. Further studies are needed to clarify this phenomenon.

On the other hand, the expression of other Notch target genes, i.e. *Hes1* (Sasai et al., 1992), *Hey1* (Maier and Gessler, 2000) and *Heyl*, was permanently down-regulated in the neonatally DES- or PPT-exposed vagina. Hes/Hey gene dosage is essential not only for generation of appropriate numbers of hair cells and supporting cells by controlling cell proliferation, but also for the hearing ability by regulating the cell migration, cell alignment and polarity (Tateya et al., 2011). Moreover, over-expression of HEY-1 inhibits migration and proliferation, whereas, inhibition of HEY-1 expression disrupts the processes of alignment and tube formation and re-establishment of the mature vessel phenotype (Brugarolas et al., 1995). Irreversible epithelial proliferation in the vagina exposed neonatally to DES might be affected with down-regulation of *Hes1*, *Hey1* and *Heyl*. Ström et al. (2000) and Hatch et al (2001) have previously shown that the expression of HES-1 is down-regulated by 17β-estradiol (E_2), and forced expression of HES-1 inhibits an E_2-mediated proliferation of breast cancer cells. Moreover, Müller et al. (2009) have revealed that novel negative ERE associating with the HES-1 promoter and recruiting nuclear receptor co-regulators to the ERE in response to E_2, then docking in the HES-1 promoter region. In the present study, down-regulation of Hes family genes in the vagina exposed neonatally to DES or PPT, might be responsible,

at least in part, for the irreversible epithelial proliferation caused by permanent activation of ERα (Miyagawa et al., 2004a,b).

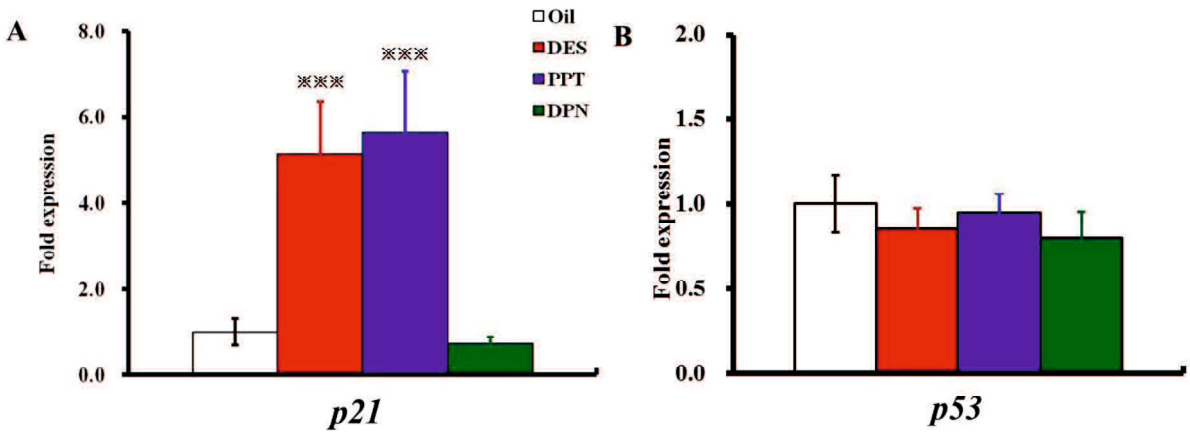


Fig. 9. Expression of *p21* (A) and *p53* (B) mRNA in vaginae of 15-week-old ovariectomized mice exposed neonatally to 2.5 μg DES (n=4), 25 μg PPT (n=4), 25 μg DPN (n=4) or the oil vehicle alone (n=4) for the first 5 days. The expression of each mRNA in vagina of the oil-treated controls was regarded as the basal level (1.0).
***P<0.001 vs controls (Student's *t*-test or Welch's *t*-test followed by F-test).

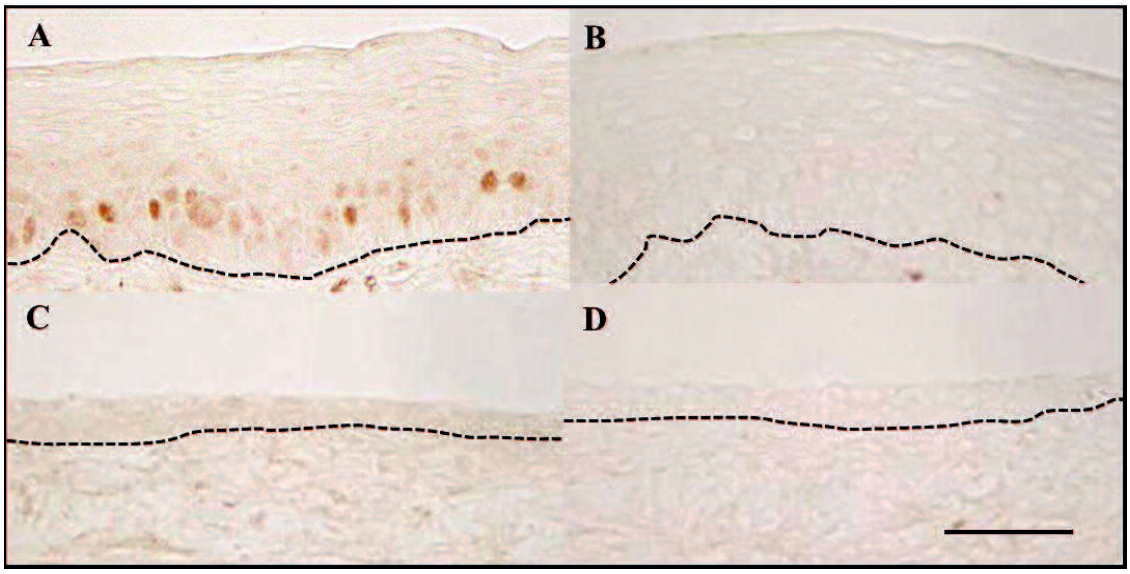


Fig. 10. Immunohistochemistry of p21. Vaginae of 15-week-old ovariectomized mice exposed neonatally to 2.5 μg DES (A, B) or the oil vehicle alone (C, D). p21 was immunostained in the neonatally DES-exposed vagina (A) and the age-matched control mouse vagina (C). As negative controls, sections were incubated with pre-immune serum instead of primary antibody (B, D). Staining of blood vessels in the stroma is no-specific reaction. The boundary between the epithelium and stroma is indicated by a dotted line. Bar: 50 μm.

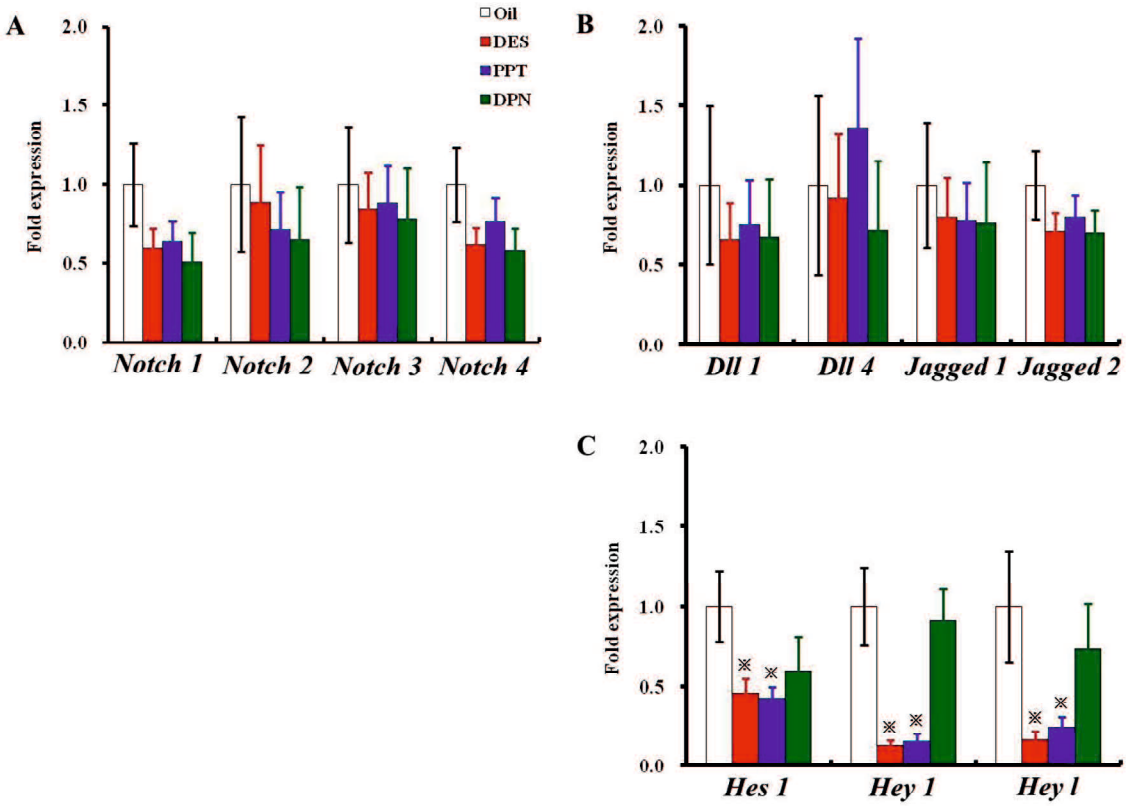


Fig. 11. mRNA expressions of Notch-related genes in vaginae of 15-week-old ovariectomized mice exposed neonatally to 2.5 μg DES (n=4), 25 μg PPT (n=4), 25 μg DPN (n=4) or oil vehicle alone (n=4) for the first 5 days. (A) Notch receptor genes (*Notch 1*, *Notch 2*, *Notch 3* and *Notch 4*), (B) Notch ligand genes (*Dll 1*, *Dll 4*, *Jagged 1* and J*agged 2*), and (C) HES/HEY family genes known as Notch target genes (*Hes 1*, *Hey 1* and *Hey l*). The expression of each mRNA in vagina of the oil-treated control mice was regarded as the basal level (1.0). *P<0.05 vs controls (Student's *t*-test or Welch's *t*-test followed by F-test).

Table 7. Sequences of gene primer sets for real-time quantitative RT-PCR

Gene	Primer (5'-3')[a]	Product size (bp)	Gene accession no.
p21	F: GTACTTCCTCTGCCCTGCTG	70	NM_001111099
	R: AGAGTGCAAGACAGCGACAA		
p53	F: AAAGGATGCCCATGCTACAG	92	NM_011640
	R: TATGGCGGGAAGTAGACTGG		
Notch 1	F: ACCCACTCTGTCTCCCACAC	123	NM_008714
	R: GCTTCCTTGCTACCACAAGC		
Notch 2	F: GAGTGTCTGAAGGGCTACGC	66	NM_010928
	R: GGGGTCTGAATGACACTCGT		
Notch 3	F: CTCTGTGGTGATGCTGGAGA	109	NM_008716
	R:AATCAAGTCGCTCCACTGCT		
Notch 4	F: CAGAACGTGGATCCCCTCAAGTTGC	70	NM_010929
	R: AGGCAGAGAGAGGGCAAGGAGTCAT		
Dll 1	F: CTGGGTGTCGACTCCTTCAG	124	NM_007865
	R: GGAGGGCTTCAATGATCAGA		
Dll 4	F: AGGTGCCACTTCGGTTACAC	72	NM_019454
	R: TAGAGTCCCTGGGAGAGCAA		
Jagged 1	F: GGAAGTGGAGGAGGATGACA	95	NM_013822
	R: GGGCCTTCTCCTCTCTGTCT		
Jagged 2	F: TTGTTATGGGTGGCTCTTCC	80	NM_010588
	R: AGCCACAGCACACTGAACAC		
Hes 1	F: ACACCGGACAAACCAAAGAC	148	NM_008235
	R: ATGCCGGGAGCTATCTTTCT		
Hey 1	F: TTTTCCTTCAGCTCCTTCCA	92	NM_010423
	R: ATCTCTGTCCCCCAAGGTCT		
Hey l	F: GGTGACTTCCACCCAGAGAG	99	NM_013905
	R: GGGATTGGGACTATGCTCCT		

[a]F, forward; R, reverse.

Table 8. Up-regulated cell cycle-related genes in the vagina of ovariectomized 10-week-old mice exposed neonatally to 2.5 μg DES

Gene accession no.	Name	Fold change	Prove set ID
NM_028390	anillin, actin binding protein	3.9	1433543_at
NM_027106	arginine vasopressin-induced 1	4.6	1423122_at
NM_011497	aurora kinase A	3.2	1424511_at
NM_025415	CDC28 protein kinase regulatory subunit 2	3.2	1417457_at
NM_025415	CDC28 protein kinase regulatory subunit 2	3.1	1417458_s_at
NM_007659	cell division cycle 2 homolog A (*S. pombe*)	3.7	1448314_at
NM_023223	cell division cycle 20 homolog (S. cerevisiae)	4.2	1416664_at
NM_023223	cell division cycle associated 20 homolog (*S. cerevisiae*)	4.5	1439377_x_at
NM_026410	cell division cycle associated 5	3.8	1416802_a_at
NM_001164362	centrosomal protein 55	2.3	1452242_at
NM_024190	chromatin modifying protein 1B	2.1	1418816_at
NM_009828	cyclinA2	3.3	1417910_at
NM_009828	cyclinA2	3.6	1417911_at
NM_001111099	cyclin-dependent kinase inhibitor 1A (P21)	7.1	1424638_at
NM_013726	DBF4 homolog (*S. cerevisiae*)	2.2	1418334_at
NM_010288	gap junction protein, alpha 1	6.3	1415801_at
NM_001001999	glycoprotein 1b beta, polypeptide septin 5	4.6	1452357_at
NM_001130443	Harvey rat sarcoma virus oncogene 1	2.4	1422407_s_at
NM_001130443	Harvey rat sarcoma virus oncogene 1	2.2	1424132_at
NM_016692	inner centromere protein	2.3	1439436_x_at
NM_019499	MAD2 mitotic arrest deficient-like 1 (yeast)	2.3	1422460_at
NM_015806	mitogen-activated protein kinase 6	2.9	1419169_at
NM_010937	neuroblastoma ras oncogene Nras	2.0	1422688_a_at
NM_152804	polo-like kinase 2 (*Drosophila*)	2.2	1427005_at

NM_145150	protein regular of cytokinesis 1	2.9	1423775_s_at
NM_012025	Rac GTPase-activating protein 1	2.7	1421546_a_at
NM_033144	septin 8	2.9	1426801_at
NM_033144	septin 8	3.2	1426802_at
NM_018754	stratifin	8.5	1448612_at
NM_026785	ubiquitin-conjugating enzyme E2c	7.6	1452954_at
NM_021284	*v*-ki-ras2 kirsten rat sacrona viral oncogene homolog	2.0	1451979_at

Table 9. Down-regulated cell cycle-related genes in the vaina of ovariectomized 10-week-old mice exposed neonatally to 2.5 μg DES

Gene accession no.	Name	Fold change	Prove set ID
NM_007631	cyclinD1	0.3	1448698_at
NM_007631	cyclinD1	0.4	1417420_at
NM_001161624	cyclin-dependent kinase inhibitor 1C (P57)	0.4	1417649_at
NM_008321	inhibitor of DNA binding 3	0.5	1416630_at
NM_011317	KH domain containing, RNA binding, signal transduction associated 2	0.5	1438462_x_at
NM_019946	microsomal glutathione S-transferase 1	0.3	1415897_a_at
NM_013871	mitogen-activated protein kinase 12	0.4	1449283_a_at
NM_011250	retinoblastoma-like 2	0.5	1418146_a_at
NM_001009935	thioredoxin interacting protein	0.5	1415996_at
NM_001009935	thioredoxin interacting protein	0.5	1415997_at

V. Chapter 4

Sequential Changes in Expression of Wnt- and Notch-Related Genes in Vagina and Uterus of Ovariectomized Mice after Estrogen Exposure

1. Introduction.

The rodent vaginal epithelium exhibits cyclical changes in response to cyclical ovarian secretions of sex hormones, displaying an alternating pattern of keratinization and mucification (Long and Evans, 1922). The mouse vagina is a good model to study epithelial cell proliferation and keratinization. In ovariectomiced mice, estrogens induce proliferation of the vaginal epithelial cells together with superficial keratinization. In response to estrogens, basal epithelial cells proliferate rapidly, leading to the formation of a highly stratified epithelium (Evans et al., 1990; Suzuki et al., 1996; Sato et al., 2003). Estrogens alter cellular physiology by modulating the transcriptional activity of specific nuclear estrogen receptors (ERs) (Cooke et al., 1998), which are believed to stimulate primary response genes, initiating a cascade of transcriptional events, the products of which participate in physiological responses known to be estrogen-dependent events in the target organs *in vivo* (Mangelsdorf et al., 1995). However, the regulation of vaginal gene expression by estrogens and the molecular mechanisms underlying estrogen-mediated cell proliferation remain unclear.

Previously, we reported expression patterns of mRNAs in the persistently altered vagina of neonatal mice exposed to diethylstilbestrol (DES) using DNA microarray and real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) (Miyagawa et al., 2004a,b; Suzuki et al., 2007b; Nakamura et al., 2008, 2012a,b). In the vagina exposed neonatally to DES, we found that wingless-related MMTV integration site 4 (*Wnt4)* was up-regulated without estrogen stimulation and

was correlated to the keratinization of the vaginal epithelium (Nakamura et al., 2012a). Moreover, we identified up-regulation of *p21* (also called Waf1, Cap20, Cip1 and Sdi1) and down-regulation of hairy and enhancer of split 1 (*Hes1)*, hairy/enhancer-of-split related with YRPW motif 1 (*Hey1*) and *Heyl* which are target genes of Notch signaling pathway (Nakamura et al., 2012b) in the neonatally DES-exposed mouse vagina. These genes likely lead to persistent cell proliferation and differentiation in the murine vagina due to neonatal DES exposure.

The estrogen-responsive genes mentioned above in the vagina identified by gene profiling provide an important foundation for understanding functional mechanisms of estrogen regulating morphogenesis and maintenance of the reproductive organ. However, no comprehensive studies have been conducted on mRNA expression of these genes in the vagina and uterus in mice after estrogen exposure. In the present study, I analyzed sequential changes of these gene expressions in the vagina, in comparison to the uterus, of ovariectomized mice given a single injection of 17β-Estradiol (E_2).

2. Materials and Methods

2.1. Reagents

E_2 and sesame oil were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Kanto Chemical (Tokyo, Japan), respectively.

2.2. Animals and treatments

C57BL/6J mice purchased from CLEA Japan (Tokyo, Japan) were maintained under 12 h light/12 h dark at 23-25°C and fed laboratory chow (CE-2; CLEAJapan) and tap water *ad libitum*. All procedures and protocols were approved by the Institutional Animal Care and Use Committee at the National Institute for Basic Biology, National Institutes of Natural Sciences.

C57BL female mice ovariectomized at 8 weeks of age were given a single abdominal injection of 50 ng E_2/g body weight (bw) dissolved in sesame oil at 10 weeks of age, and sacrificed at 3, 6, 9, 12, 18, 24, 36 or 48 h after the injection, or just before injection (0). Vaginae and uteri of these mice were used for real-time quantitative RT-PCR. Three mice were used for each time point.

In addition, C57BL mice ovariectomized at 8 weeks were given a single abdominal injection of 50 ng E_2/g bw (n=3) or the oil vehicle alone (n=3) at 10 weeks of age. These mice were given a single injection of 50 μg of bromodeoxyuridine (BrdU)/g bw 22 or 46 h after the E_2 injection, and were then sacrificed 2 h after the BrdU injection. These mice were used for histology and immunohistochemistry.

2.3. BrdU immunostaining

Tissues embedded in paraffin were sectioned at 8 μm and stained with hematoxylin and eosin. In addition, deparaffinized sections were incubated in 0.3% H_2O_2 in methanol for 30 min, then immersed in 2 N HCl for 20 min in order to denature the

genomic DNA. After washing with phosphate-buffered saline (PBS) in 0.5% Tween, the sections were incubated with anti-BrdU antibody (Boehringer Mannheim, Mannheim, Germany) diluted 1:20 in PBS containing 1% bovine serum albumin (BSA) (SigmaChemical, Co.) overnight at 4˚C. The sections were subsequently incubated with 3,3-diaminobenzidine tetrahydrochloride containing hydrogen peroxide. BrdU-labeling index (percentage) was estimated by counting the number of BrdU-incorporated cells per h in the basal layer of vaginal epithelium as described previously (Miyagawa et al., 2004a,b).

2.4. *Real-time quantitative RT-PCR*

Total RNA, isolated with RNeasy kit (Qiagen, Chatsworth, CA, USA) from each group of vaginae, was used for real-time quantitative RT-PCR reactions carried out with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA).

Changes in gene expression were confirmed and quantified using the ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. PCR conditions were as follows: 50˚C for 2 min, 95˚C for 10 min, and 36 cycles of 95˚C for 15 s and 60˚C for 1 min in 15 μl volumes. Relative RNA equivalents for each sample were obtained by standardization of L8 ribosomal protein levels. Sequences of gene primer sets are given in Table 9. Three 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. Error bars represent the standard error, with all values represented as fold change compared to the control treatment group normalized to an average of 1.0.

2.5. Immunohistochemistry

Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 4 μm. The sections deparaffinized were incubated with 0.3% H_2O_2 in methanol for 15 min to eliminate endogenous peroxidase. After washing with PBS, the sections were stained with LSAB^{TM} 2 kit, Universal (Dako, Carpinteria, CA, USA) according to the manufacturer-supplied protocol. Polyclonal antibody to Wnt4 was obtained from R&D Systems, Inc. (Minneapolis, MN, USA). The tissue sections were incubated at 1:200 dilution in PBS containing 1% BSA overnight at 4℃. For negative controls, normal goat immunoglobulin (IgG) fraction (Dako) was used at the same dilution. Monoclonal antibody to p21 was obtained from Santa Cruz, Inc. (Minneapolis, MN, USA). The sections were incubated at 1:50 dilution in PBS containing 1% BSA overnight at 4℃. For negative controls, murine IgG2b (Dako) was used at the same dilution.

2.6. Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA). Differences or $p<0.05$ were considered significant.

3. Results

3.1. Morphological changes and differential expression of cell cycle-related genes in vaginae of ovariectomized mice given a single injection of E_2

Histological changes in the vagina following the E_2 injection are shown in Fig. 12A-C. The epithelium exhibited proliferation at 24 h (Fig. 12B), and underwent stratification with superficial keratinization at 48 h after the injection (Fig. 12C). The basal cells of vaginal epithelium at 24 h after the injection exhibited a high proliferative activity (10.2%), confirmed by BrdU immunostaining (Fig. 12D). At 48 h after the injection, proliferative activity declined to 4.0% (Fig. 12D).

Since cyclin A2 is expressed from S phase to M phase during cell division, sequential changes in gene expression levels of cyclin A2 in addition to keratin 1 were examined after E_2 treatment. The gene expression of cyclin A2 was increased 18-24 h after E_2 treatment (Fig. 13A), and that of keratin 1 had a similar pattern to that of cyclin A2 (Fig. 13B). Up-regulation of cyclin A2 and keratin 1 was correlated with epithelial stratification and keratinization in the vagina.

3.2. Localization of Wnt4 and p21 proteins in mouse vagina

The localization of Wnt4 and p21 protein in the proliferating epithelial cells of vaginae was examined by immunohistochemistry (IHC). Wnt4 staining was observed in the basal and middle layers of vaginal epithelium at 24 h after the E_2 injection (Fig.

14A). p21 was localized in the basal layer of vaginal epithelium in E_2-treated mice (Fig. 3C). No response was visible in the vaginal epithelium stained with normal goat immunoglobulin (IgG) as a negative control (Fig. 14B and D). Hence, Wnt4 and p21 proteins were evident in the proliferating vaginal epithelial cells.

3.3. Differential expression of Wnt genes and Notch signal pathway-related genes in the vagina and uterus of ovariectomized mice given a single injection of E_2

On the basis of the morphological findings in the vagina of ovariectomized mice given E_2, I compared the expression patterns of Wnt genes and Notch signal pathway-related genes in the vagina and uterus after treatment with E_2. In the vagina, expression of *Wnt4* was increased at 18 and 24 h after the E_2 injection, while in the uterus, the expression was transiently increased only at 3 h (Fig. 15A). Expression of *Wnt5a* was increased at 12 and 24 h after E_2 injection in the vagina and at 18, 24 and 36 h in the uterus, respectively (Fig. 15B). Expression of *Wnt11* was decreased at 6-36 h after E_2 administration in the vagina and at 9-24 h in the uterus, respectively (Fig. 15C). The expression of *Wnt4* was different between the vagina and uterus, while the pattern for *Wnt5a* and *Wnt11* revealed a similar trend in both organs.

Expression of *Hey1* was decreased at 3-24 h after the E_2 injection in the vagina. On the other hand, the expression increased transiently at 3 h in the uterus (Fig. 15D). Expression of *Hey2* was decreased at 12, 24 and 36 h in the uterus, while in the vagina, the expression was decreased at 6, 36 and 48 h (Fig. 15E). Expression of *Heyl* was

increased at 6-12 h after the administration only in the uterus (Fig. 15E and F). However, the expression showed no change in the vagina after the E_2 injection (Fig. 15F). Expression of *Dll1* was increased in the uterus at 6 h after administration, while the expression remained unchanged in the vagina (Fig. 15G). Expression of *Dll4* was decreased in the vagina at 3, 9, 18-48 h after E_2 administration and in the uterus at 3-24 h (Fig. 15H).

The expression of *p21* and *p53* was increased at 6-12 h after E_2 administration in the uterus (Fig. 15I and J). In vagina, however, E_2 injection only increased *p21* expression at 12 h, with no effect on the expression of *p53* (Fig. 15I and J).

4. Discussion

Estrogens induce epithelial cell proliferation and differentiation, whereas estrogen depletion results in atrophy accompanied by apoptosis in adult female mouse reproductive organs, such as the vagina and uterus (Evans et al., 1990; Suzuki et al., 1996; Sato et al., 2003)**.** In order to understand the underlying mechanisms of estrogen functions in reproductive organs in mice, Kitajewski et al. (2000) and Suzuki et al. (2007a,b) have analyzed estrogen-responsive genes in reproductive organs, and found that expression patterns of genes are different between the vagina and uterus. Previously, we reported the expression of Wnt- and Notch-related genes in the vagina and uterus exposed neonatally to DES (Suzuki et al., 2007b; Nakamura et al., 2008; Nakamura et al., 2012a,b), and the localization of Wnt4 or p21 proteins and quantitation of

BrdU-positive cells after E_2 administration in the ovariectomized mouse uterus (Lai et al., 2002; Hou et al., 2004). In the vagina, however, detailed information has not been reported. Therefore, I investigated gene expression of Wnt- and Notch-related genes in the vagina and uterus after a single injection of E_2 in mice.

Sassoon's group (Miller et al., 1998; Kitajewski and Sossoon, 2000; Mericskay et al., 2004) reported that *Wnt4, Wnt7a,* and *Wnt5a* are required for Müllerian duct formation, subsequent differentiation, and posterior growth, respectively, and the expression of these genes in the uterus changes during the estrous cycle (Miller et al., 1998). These findings suggest important roles of Wnt family genes in various reproductive physiologies. In the present study, *Wnt4*, but not *Wnt5a* and *Wnt11*, were differently expressed pattern in the vagina and uterus.

Cyclin A2 is expressed from S phase to M phase, so the cell cycle phase after E_2 treatment can be determined by analyzing changes in cyclin expression (Katsu and Iguchi, 2006). In the present study, expression of cyclin A2 and keratin 1 was increased 24 h after a single injection of E_2 in the vagina, being similar to the BrdU labeling index and expression of *Wnt4*. These results suggest that Wnt4 might act on epithelial stratification and keratinization in the vagina. In the uterus, *Wnt4* was transiently increased at 3 h after E_2 treatment; *Wnt4* is expressed in the uterine epithelium at proestrus at the time of highest estrogen levels (Miller et al., 1998; Rugh, 1990), suggesting that estrogen regulates *Wnt4* expression in the uterus. On the other hand, *Wnt5a* and *Wnt11* had similar expression patterns in the vagina and uterus. *Wnt5a*

expression has been reported to change in both organs during the estrous cycle (Miller et al., 1998), and it is expressed at higher levels at proestrus in the vaginal epithelium and stroma (Miller et al., 1998), suggesting involvement in cell proliferation and keratinization in the vagina. I confirmed the similarity of the expression patterns among *Wnt5a*, cyclin A2 and keratin 1 in the vagina in the present study. In the uterus, *Wnt5a* has been detected in both the epithelium and the stroma at estrus, but only uterine stroma at proestrous (Miller et al., 1998). Zhu *et al.* (2004) reported that the expression of *Wnt11* is inhibited by androgen. The present data indicate that *Wnt11* is down-regulated in both organs by E_2 treatment, showing negative correlation with cell proliferation in both organs, as in the vagina of neonatally DES-exposed mice (Nakamura et al., 2012a).

Previously, we reported that *Hey1* and *Heyl*, Notch target genes, are persistently down-regulated in the vagina exhibiting estrogen-independent epithelial cell proliferation in the neonatally DES-exposed mice (Nakamura et al., 2012a). In this study, *Hey1*, *Hey2* and *Heyl* had differential expression patterns between vagina and uterus in response to E_2 stimulation. *Hey1* was down-regulated at 3-24 h after E_2 treatment in the vagina, suggesting the opposite behavior in proliferation of vaginal cells to that of uterine cells. *Hey1* and *Heyl* may have roles in maintaining undifferentiated quiescent cells, as in satellite muscle cells (Fukada et al., 2011).

We reported that Notch ligand genes, *Dll1* and *Dll4*, are unaltered in the vagina exhibiting estrogen-independent epithelial cell proliferation in the neonatally

DES-exposed mice (Nakamura et al., 2012b). *Dll1* and *Dll4* genes had a differential expression pattern between the vagina and uterus after E_2 treatment in this study. Expression of *Dll1* was transiently increased 6 h after E_2 stimulation but it showed no change in the vagina. Dll4 is reported to be involved in tumor angiogenesis (Noguera-Troise et al., 2006; Ridgway et al., 2006), and is expressed in the arterial endothelium (Shutter et al., 2000). *Dll4*, as well as *Wnt11*, exhibited down-regulation by E_2 in both organs, suggesting a negative correlation with cell proliferation.

Finally, I confirmed the expression of *p21* and *p53*. *p21* is a direct target of p53 tumor suppressor, and mediates p53-dependent cell cycle arrest in response to DNA damage (el-Deiry et al., 1993). In this study, I found the expression patterns of p21 and p53 in the uterus were similar.

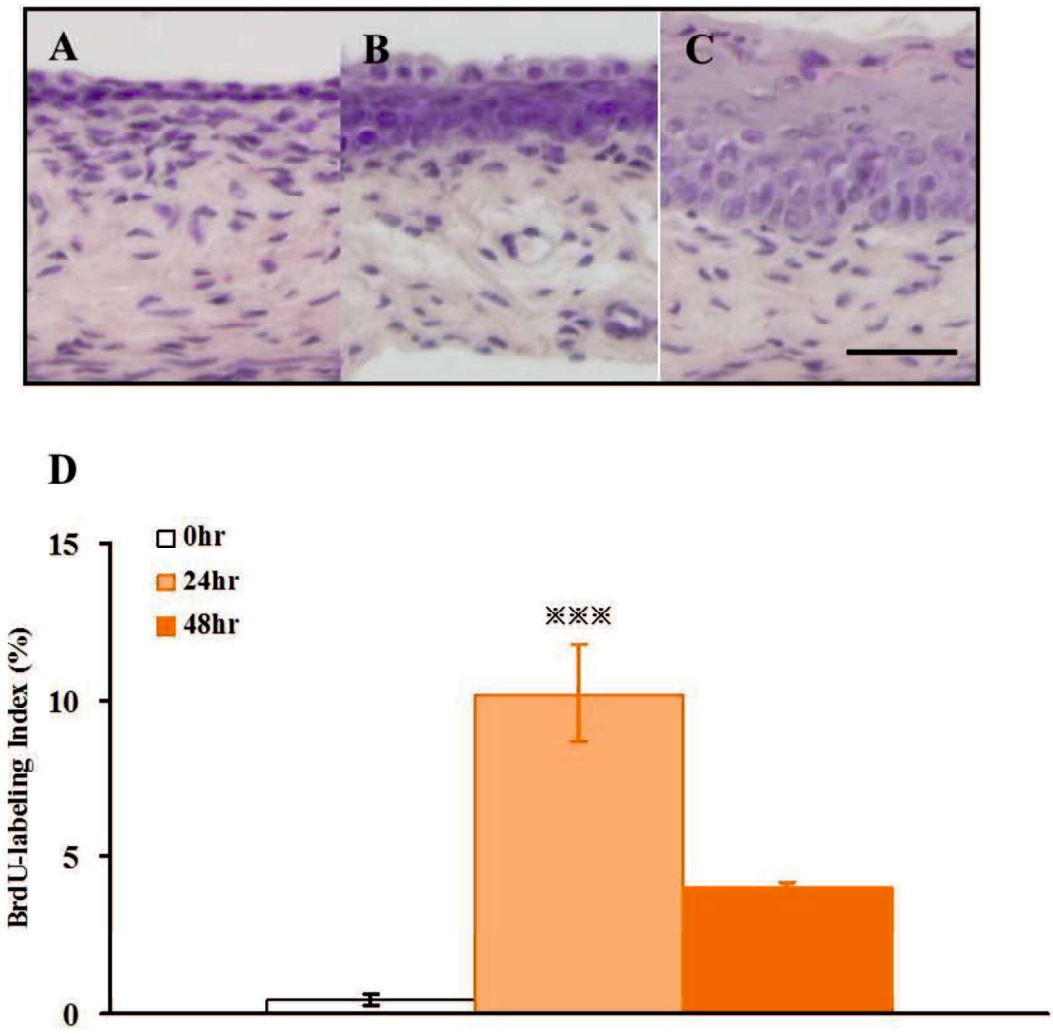


Fig. 12. Histology of vagina of a 10-week-old, mouse ovariectomized at 8 weeks of age, and killed just before (A) or 24 h (B) and 48 h (C) after a single abdominal injection of 50 ng 17β-Estradiol (E_2)/g bw dissolved in sesame oil at 10 weeks of the injection. Bar=50 μm. Incidence (%) of BrdU-incorporated basal cells in the vaginal epithelium of mice sacrificed before injection, and 24 and 48 h after a single E_2 injection (D). Data are the means±standard error (n=3). ***p<0.001 vs. controls (one-way ANOVA).

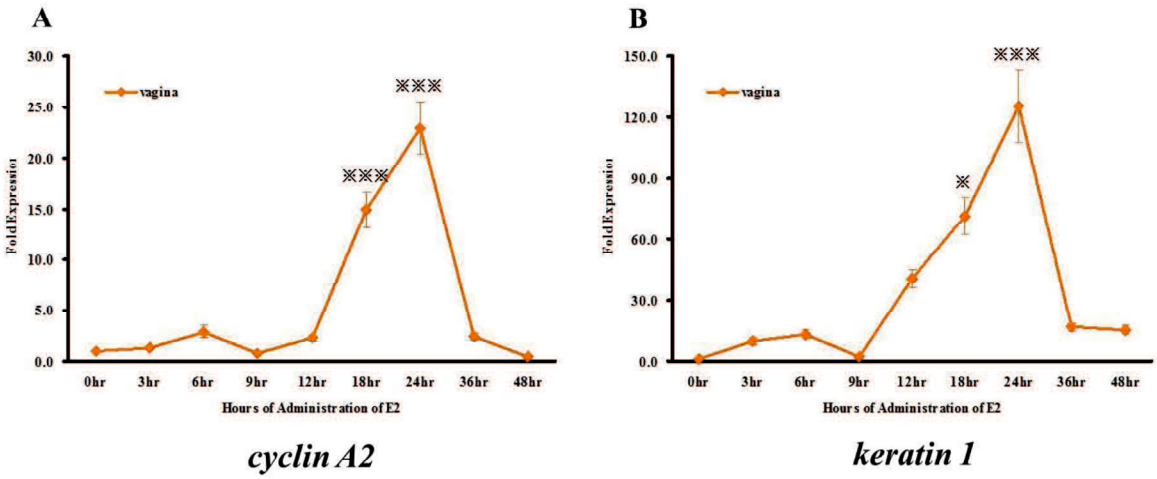


Fig. 13. Fold expression of cyclin A2 (A) and keratin 1 (B) after a single injection of 50 ng 17β-Estradiol (E_2)/g bw. Expression of these genes in control mice was regarded as the basal level (1.0). Data are the means ± standard error (n=3). *p<0.05 and ***p<0.001 vs. controls (one-way ANOVA).

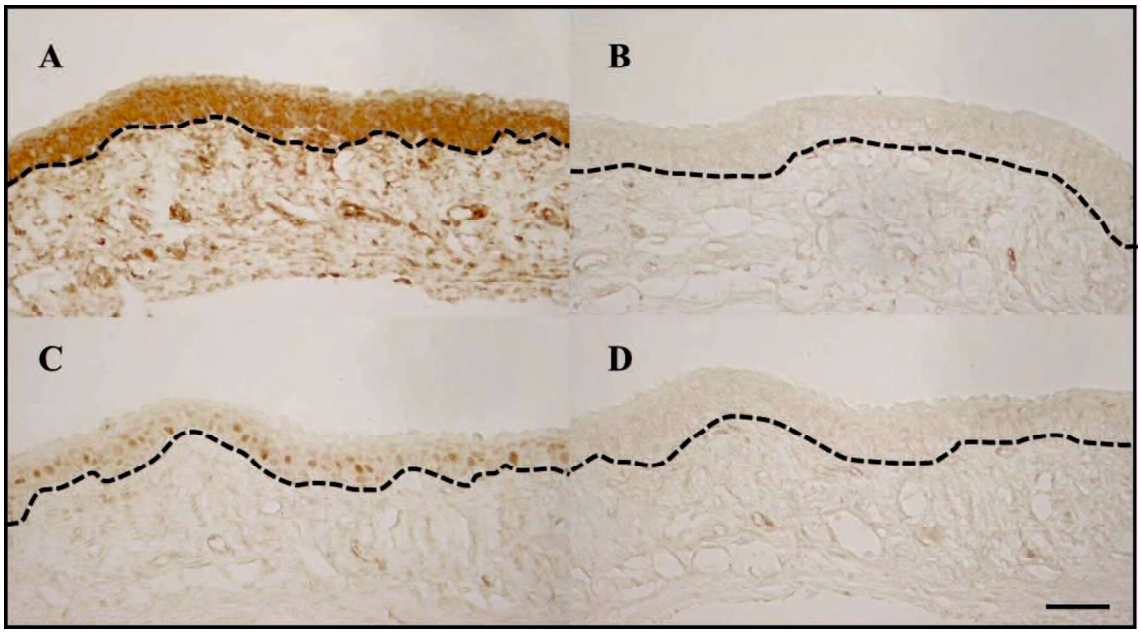
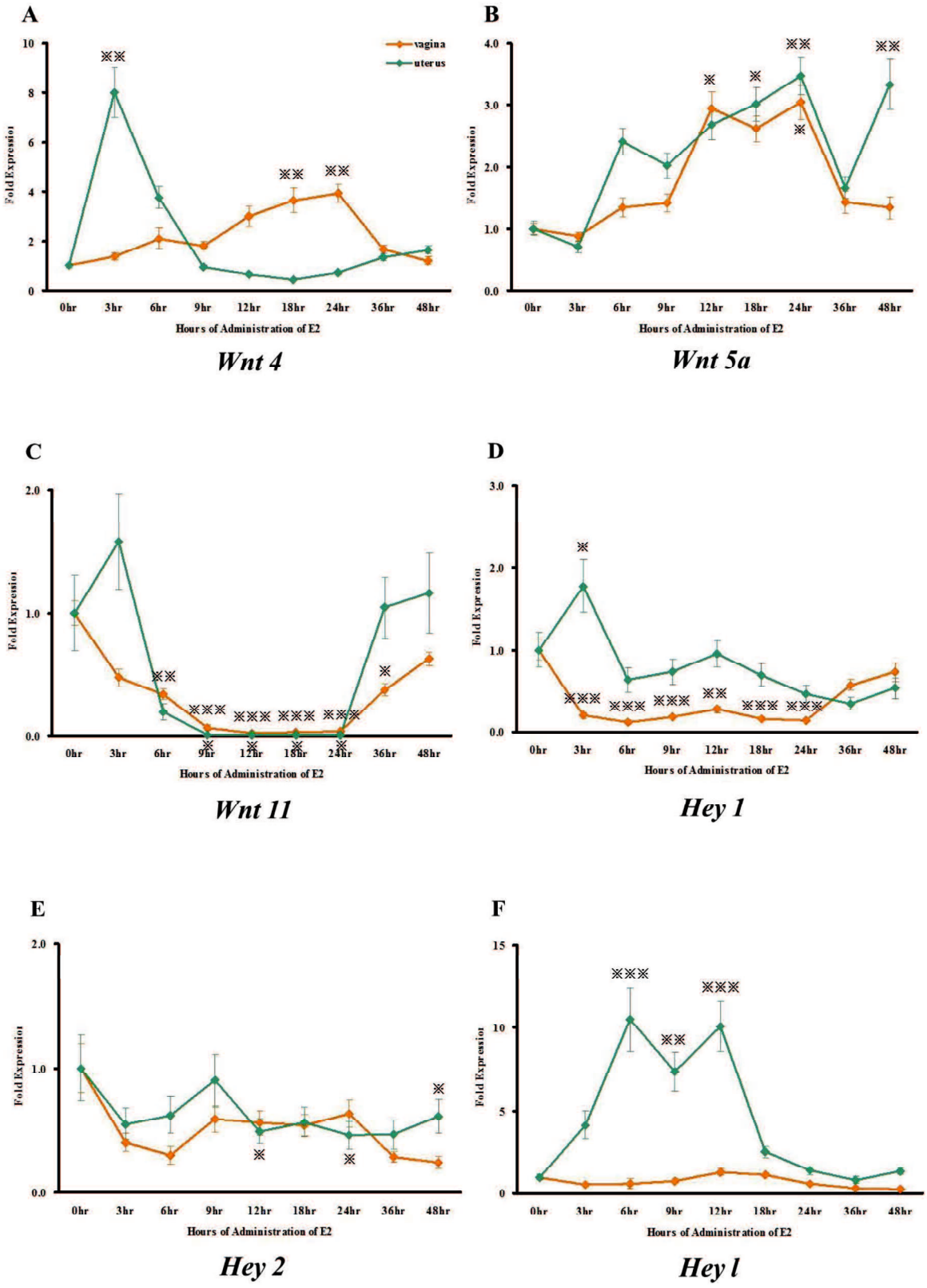


Fig. 14. Immunohistochemistry by wingless-related MMTV integration site 4 (Wnt4) (A) and p21 (C). Vaginae of 10-week-old, ovariectomized mice were killed at 24 h after a single injection of 17β-Estradiol (E_2). For negative controls, sections were incubated with preimmune serum instead of primary antibody (B, D). Bar=50 μm. The boundary between the epithelium and stroma is indicated by a dotted line.


A
vagina
uterus
Fold Expression
Hours of Administration of E2
0hr 3hr 6hr 9hr 12hr 18hr 24hr 36hr 48hr
Wnt 4
B
Fold Expression
Hours of Administration of E2
Wnt 5a
C
Fold Expression
Hours of Administration of E2
Wnt 11
D
Fold Expression
Hours of Administration of E2
Hey 1
E
Fold Expression
Hours of Administration of E2
Hey 2
F
Fold Expression
Hours of Administration of E2
Hey l

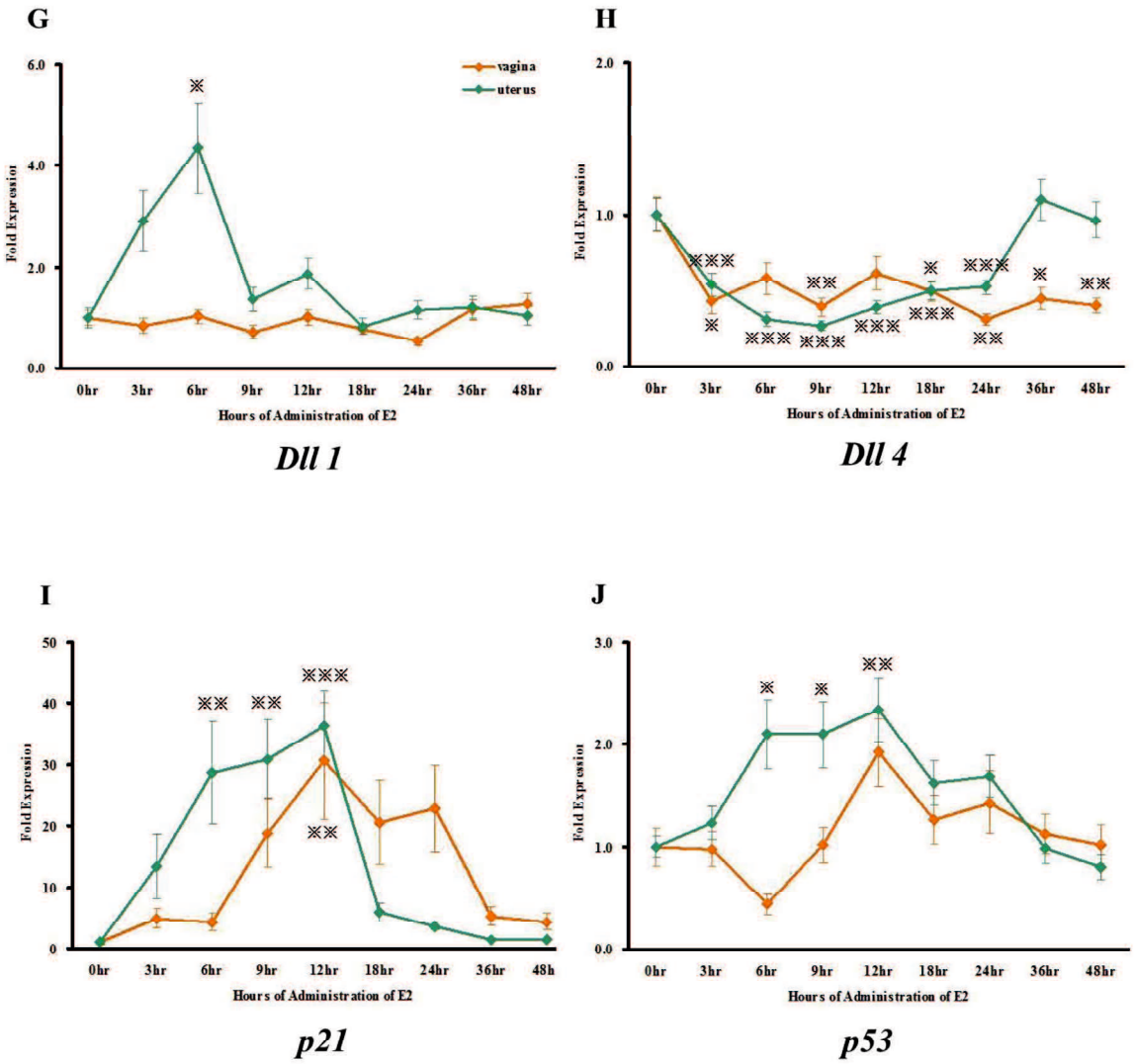


Fig. 15. Fold expression of wingless-related MMTV integration site 4 (Wnt4) (A), Wnt5a (B), Wnt 11 (C), hairy/enhancer-of-split related with YRPW motif 1 (Hey1) (D), Hey2 (E), Heyl (F), delta-like 1 (Dll1) (G), Dll4 (H), p21 (I) and p53 (J) in the vagina and uterus after a single injection of 50 ng 17β-Estradiol (E_2)/g bw. Expression of these genes in control mice before E_2 injection (0 h) was regarded as the basal level (1.0). Data are the means±standard error (n=3). *p<0.05, **P<0.01 and ***P<0.001 vs. controls (one-way ANOVA).

Table 10. Sequences of gene primer sets for real-time quantitative RT-PCR

Gene	Primer (5'-3')[a]	Product size (bp)	Gene accession no.
Cyclin A2	F: ACAGAGCTGGCCTGAGTCAT	119	NM_009828
	R: TTGACTGTTGGGCCATGTTGT		
Keratin 1	F: AAGAGGACCAACGCAGAGAA	87	NM_008473
	R: TTGGCCTGAAGCTCAACTTT		
Wnt4	F: CATCGAGGAGTGCCAATACCA	70	NM_009523
	R: GACAGGGAGGGAGTCCAGTGT		
Wnt 5a	F: GCGTGGCTATGACCAGTTTAAGA	75	NM_009524
	R: TTGACATAGCAGCACCAGTGAA		
Wnt11	F: ATGTGCGGACAACCTCAGCTA	100	NM_009519
	R: CGCATCAGTTTATTGGCTTGG		
Dll 1	F: CTGGGTGTCGACTCCTTCAG	124	NM_007865
	R: GGAGGGCTTCAATGATCAGA		
Dll 4	F: AGGTGCCACTTCGGTTACAC	72	NM_019454
	R: TAGAGTCCCTGGGAGAGCAA		
Hey 1	F: TTTTCCTTCAGCTCCTTCCA	92	NM_010423
	R: ATCTCTGTCCCCCAAGGTCT		
Hey 2	F: CAGTCCTCAGCAGACAAGAC	73	NM_013904
	R: GGTTGGGCATGTAAGTGTAGTTGT		
Hey l	F: GGTGACTTCCACCCAGAGAG	99	NM_013905
	R: GGGATTGGGACTATGCTCCT		
p21	F: GTACTTCCTCTGCCCTGCTG	70	NM_001111099
	R: AGAGTGCAAGACAGCGACAA		
p53	F: AAAGGATGCCCATGCTACAG	92	NM_011640
	R: TATGGCGGGAAGTAGACTGG		

VI. Summary and Conclusion

Proliferation and differentiation of cells in the female reproductive organs, oviduct, uterus and vagina, are regulated by endogenous estrogens. *In utero* exposure to a synthetic estrogen, diethylstilbestrol (DES), induces vaginal clear-cell adenocarcinoma in humans (Herbst et al., 1971; Herbst and Bern, 1981). In mice, perinatal exposure to DES and estrogenic chemicals, results in abnormalities such as in the hypothalamo-pituitary-gonadal axis, polyovular follicles, uterine circular muscle disorganization and persistent vaginal epithelial cell proliferation. Activities of estrogenic chemicals are mediated through estrogen receptors α (ERα) and/or ERβ. However, little is known about the relative contribution of the individual ER subtypes in the induction of abnormalities.

I therefore tested the effects of neonatal exposure to ER selective ligands and DES on the female mouse vagina and uterus. Transactivation assays using mouse ERα and ERβ showed that 10^{-10} M DES activated both ER subtypes and that the ERα agonist (propyl pyrazole triol, PPT) and the ERβ agonist (diarylpropionitrile, DPN) selectively activated their respective ER subtypes at 10^{-9} M. Female mice injected neonatally with DES, PPT or DPN were examined at 13 and 15 weeks of age. Persistent estrus and anovulation were induced in all mice by 0.025-2.5 μg DES and 2.5-25 μg PPT, but not by DPN, suggesting that the observed anovulation was primarily mediated through ERα. Disorganization of uterine musculature and ovary-independent vaginal epithelial cell proliferation accompanied by persistent expression of EGF-related genes and

interleukin-1-related genes were also mediated through ERα. In contrast, polyovular follicles were induced by neonatal treatment with both ERα and ERβ ligands, suggesting that ovarian abnormalities are mediated through both ER subtypes (Chapter 1).

I examined global gene expressions in the vagina of ovariectomized adult mice exposed neonatally to DES using microarray to understand the underlying mechanism of the estrogen-independent persistent vaginal changes induced by neonatal DES exposure. I found persistent up-regulation of *Wnt4* and persistent down-regulation of *Wnt11* in the vaginae of mice exposed neonatally to DES and PPT. Also, the expression of Wn4 protein in vagina is correlated to the stratification of epithelial cells with the superficial keratinization of vaginae, but not epithelial cell stratification alone (Chapter 2).

Moreover, the expression of cell cycle-related genes, p21, cyclin-dependent kinase inhibitor, showed up-regulation in the vagina, and p21 protein was localized in the basal layer of the vaginal epithelium in mice exposed neonatally to DES and PPT. The expression of Notch receptors and Notch ligands were unchanged, however, hairy-related basic helix-loop-helix (bHLH) transcription factor genes, *Hes1*, *Hey1* and *Heyl* were persistently down-regulated in the vaginae showing estrogen-independent epithelial cell proliferation in mice exposed neonatally to DES and PPT (Chapter 3).

Female reproductive organs show organ-specific morphological changes during estrous cycles. Estrogen depletion by ovariectomy in adults results in atrophy

accompanied by apoptosis in vaginal and uterine cells, while estrogen treatment following the ovariectomy elicits cell proliferation in both organs. Sequential changes in mRNA expression of Wnt and Notch signalling genes were analysed in the vaginae and uteri of ovariectomized adult mice after a single injection of 17β-estradiol (E_2) to understand molecular basis of differences in response to estrogen in these organs. I found estrogen-dependent up-regulation of *Wnt4*, *Wnt5a* and *p21* and down-regulation of *Wnt11*, *Hey1* and *Dll4* in the vaginae, and up-regulation of *Wnt4*, *Wnt5a*, *Hey1*, *Heyl*, *Dll1*, *p21* and *p53* and down-regulation of *Wnt11*, *Hey2* and *Dll4* in the uteri. The expression of *Wnt4*, *Hey1*, *Hey2*, *Heyl*, *Dll1* and *p53* showed different expression patterns after estrogen injection. Whereas, *Wnt5a*, *Wnt11*, *Dll4* and *p21* showed similar expression patterns between the vaginae and uteri, suggesting that these genes are involved in proliferation of cells in both organs in mice (Chapter 4).

In conclusion, neonatal treatment with both ERα and ERβ selective agonists, induced polyovular follicles. Therefore, both ER subtypes are involved in induction of ovarian abnormalities. In contrast, anovulatory sterility, disorganization of uterine circular muscle and persistent proliferation of vaginae were induced by neonatal treatment with DES or an ERα-selective ligand, PPT, but not an ERβ-selective ligand, DPN. I conclude that ERα action is essential for induction of abnormalities in the hypothalamo-pituitary-ovarian axis, and uterine and vaginal changes during the critical developmental period in mice. Furthermore, in the vaginae exposed neonatally to DES or PPT, I found that permanent up-regulation of *Wnt4* mRNA, and permanent

down-regulation of *Wnt11* mRNA, suggesting that *Wnt4* participates in the irreversible superficial keratinization in the mouse vagina, however, *Wnt11* participates in an ER-independent repressive role in vaginal keratinization. In addition, I demonstrated that expression of both *p21* mRNA and p21 protein are permanently up-regulated, and *Hes1*, *Hey1* and *Heyl* mRNAs are permanently down-regulated in the vaginae exposed neonatally to DES. I examined the global expression of mRNA, focusing on growth factors in the vagina of mice exposed neonatally to DES, showing persistent hyperplasia and the superficial keratinization. I also performed comprehensive analyses on the expression of Wnt- and Notch-related genes in the vaginae exposed neonatally to DES. Wnt family genes or Notch family genes are one of the fundamental signalling pathways that regulate metazoan development and adult tissue homeostasis. I confirmed persistent up-regulation of *Wnt4* and *p21*, and persistent down-regulation of *Wnt11*, *Hes1*, *Hey1* and *Heyl*, and localization of Wnt4 and p21 protein in the vaginae exposed neonatally to DES. I also demonstrated the difference in expression patterns of *Wnt4*, *Wnt5a*, *Wnt11*, *Hey1*, *Hey2*, *Heyl*, *Dll1*, *Dll4*, *p21* and *p53* between the vaginae and uteri after E_2 stimulation. Moreover, I identified the localization of Wnt4 and p21 protein in the ovariectomized adult mouse vagina exhibiting epithelial stratification after a single injection of E_2. Especially, *Wnt4*, *Wnt11*, *p21* and *Hey1* showed similar trends in the vagina exposed neonatally to DES. These data might provide clues to the biological function of genes involved in the irreversible proliferation in vaginal

epithelial cells. Additional elucidation of the molecular mechanism of cell proliferation in the vagina and uterus is essential in the near future.

VII. Reference

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