Results

Expression of *Hh*, *Ptch* and *Gli* in uterus and vagina at days 2, 15 and 90

In order to understand the expression profiles of genes related to HH signaling in the uterus and vagina at days 2, 15 and 90, real-time RT-PCR was performed. The expression of *Shh* was dramatically higher in the vagina compared with the uterus at day 2 and then significantly decreased in both the uterus and vagina at day 15 (Fig. 1a). In contrast, the expression of *Ihh* drastically increased only in the uterus at day 15. The expression of *Dhh* in the uterus and vagina was low at day 2 and then significantly increased at day 15.

At day 2, the expression of *Ptch1* and *Gli1*, both target genes of HH signaling, was significantly higher in the vagina compared with that in the uterus and then decreased in the vagina but increased in the uterus at day 15 (Fig. 1b).

The expression of the other receptor, *Ptch2*, was higher in the vagina compared with the uterus at day 2 and

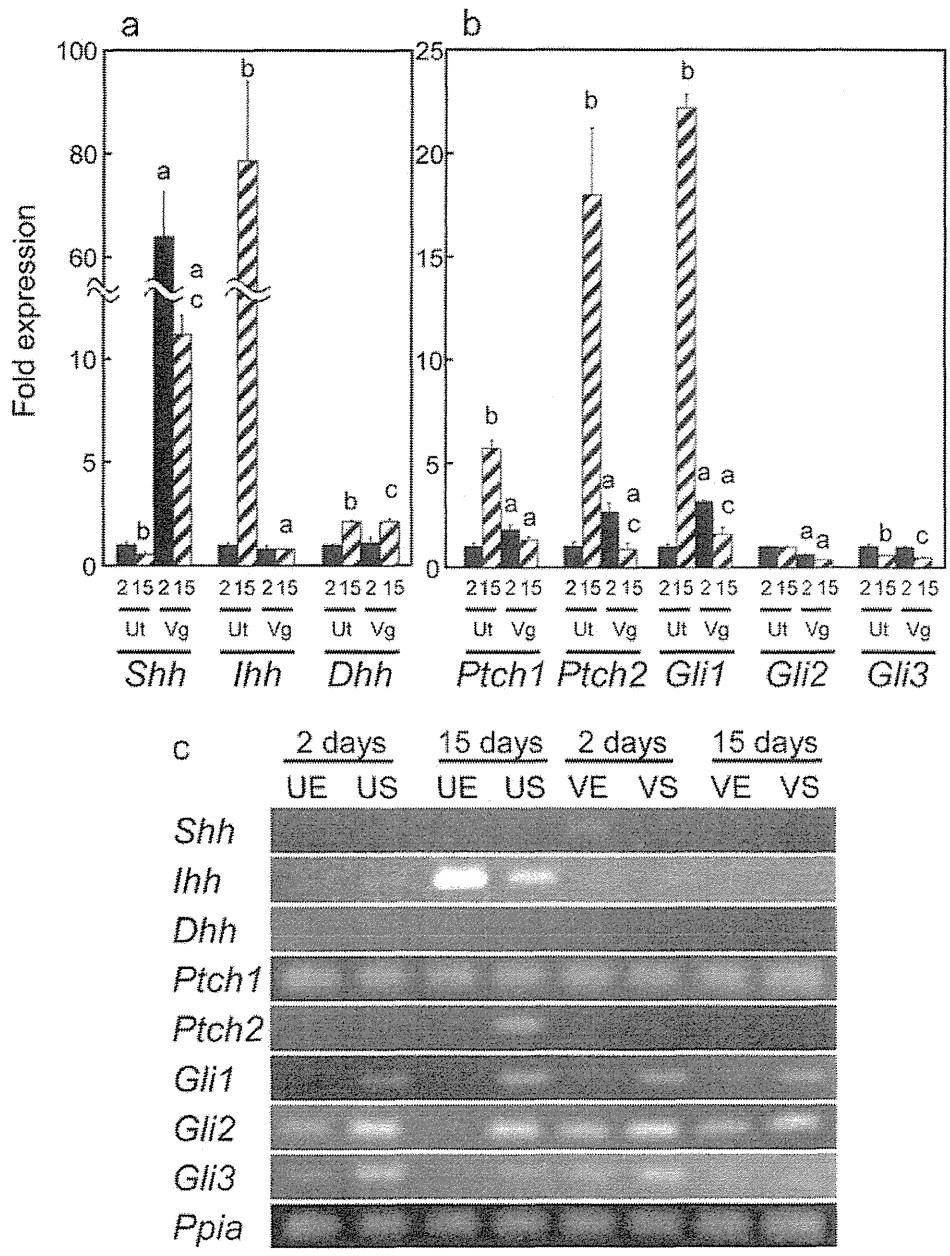

Fig. 1 mRNA expression levels of genes for various Hedgehog types, transmembrane receptors and transcription factors (*Shh*, *Ihh*, *Dhh*, *Ptch1*, *Ptch2*, *Gli1*, *Gli2* and *Gli3*) in the uterus and vagina at days 2 and 15 (**a**, **b**) were analyzed by real-time reverse transcription with the polymerase chain reaction (RT-PCR). Data are expressed relative to mRNA expression in the uterus at day 2; relative expression=1.0 (2: 2 days, 15: 15 days, Ut: uterus, Vg: vagina; $^{a}P<0.05$ compared with the age-matched uteri, $^{b,c}P<0.05$ compared with the uteri or vaginae at day 2). *Shh*, *Ihh*, *Dhh*, *Ptch1*, *Ptch2*, *Gli1*, *Gli2* and *Gli3* mRNA expression in the uterine epithelium (UE), uterine stroma (US), vaginal epithelium (VE) and vaginal stroma (VS) at days 2 and 15 was analyzed by RT-PCR (**c**). Peptidylprolyl isomerase A (*Ppia*) was chosen as an internal standard

subsequently decreased in the vagina and increased in the uterus at day 15 (Fig. 1b). The expression of *Gli2* was significantly lower in the vagina compared with the uterus at days 2 and 15. In both the uterus and vagina, the expression of *Gli3* was significantly higher at day 2 than at day 15. At day 90, the expression of genes related to HH signaling in the vagina was not altered by E2 treatment, except for *Dhh* and *Ptch1*, which were decreased (see Supplemental Figure).

Thus, HH signaling in the vagina was activated during the neonatal period, whereas in the uterus, it was activated at day 15.

Localization of *Hh*, *Ptch* and *Gli* in uterus and vagina at days 2 and 15

The localization of *Hh*, *Ptch* and *Gli* in the uterus and vagina at days 2 and 15 was analyzed by RT-PCR by using the epithelial-stromal separation technique, because of failure to detect those mRNAs by *in situ* hybridization. In order to confirm whether the epithelium and stroma were completely separated, the expression of *Krt8* and *vimentin* was determined by RT-PCR (data not shown). *Shh* expression was observed only in the VE at day 2 (Fig. 1c, Table 1). At day 2, *Ihh* expression was faintly detected in all the tissues but was mainly localized in the stroma. At day 15, *Ihh* expression was observed in the uterus, being mainly localized in the UE. *Dhh* expression was not found in the uterus or vagina at days 2 and 15.

Ptch1 expression was found in the all tissues, whereas *Ptch2* expression was only detected in the US at day 15 (Fig. 1c, Table 1). *Gli1* expression was observed in the US and VS at days 2 and 15. *Gli2* and *Gli3* expression was found in all the tissues, except for UE at day 15 but these genes were mainly localized in the stroma.

Thus, *Shh* and *Ihh* were mainly detected in the uterine and VE, whereas *Gli* was mainly expressed in the US and VS.

Table 1 Localization of *Hedgehog* (*Shh*, *Ihh*, *Dhh*), *Patched* (*Ptch1*, *Ptch2*) and *Glioma-associated oncogene homolog* (*Gli1*, *Gli2*, *Gli3*) mRNAs in the uterine epithelium (UE), uterine stroma (US), vaginal epithelium (VE) and vaginal stroma (VS) at days 2 and 15. − negative, −/+ slightly detectable, + detectable

Gene	2 days		15 days		2 days		15 days	
	UE	US	UE	US	VE	VS	VE	VS
Shh	−	−	−	−	+	−	−	−
Ihh	−/+	−/+	+	+	−/+	−/+	−	−
Dhh	−	−	−	−	−	−	−	−
Ptch1	+	+	+	+	+	+	+	+
Ptch2	−	−	−	+	−	−	−	−
Gli1	−	+	−	+	−/+	+	−/+	+
Gli2	+	+	−	+	+	+	+	+
Gli3	+	+	−	+	+	+	+	+

Effects of cyclopamine on HH signaling in organ-cultured uterus and vagina

In organ-cultured vagina cultured for 2 days, the expression of *Shh* and *Gli1* was higher than that in the organ-cultured uterus, whereas the expression of *Ihh* did not differ between the uterus and vagina (Fig. 2a). Thus, *Shh*, *Ihh* and *Gli1* expression patterns in the organ-cultured uterus and vagina were similar to those in vivo at day 2.

To investigate the role of HH signaling in the uterus and vagina, the HH signaling inhibitor cyclopamine was added to the medium of the organ-cultured uterus and vagina. Addition of cyclopamine did not affect the expression of *Shh* and *Ihh* but reduced the expression of *Gli1* and *Ptch1*, both being target genes of HH signaling, in the organ-cultured uterus and vagina (Fig. 2a). Thus, 10 μM cyclopamine attenuated HH signaling activation in the organ-cultured uterus and vagina.

Effects of HH signaling attenuation on cell proliferation, epithelial differentiation and mRNA expression of growth factors in organ-cultured uterus and vagina

At 2 days of culture, the addition of cyclopamine decreased the BrdU labeling index in the epithelium of organ-cultured uterus. At day 5 of culture, after cyclopamine treatment, the BrdU labeling index in the epithelium of organ-cultured vagina was decreased, whereas it was increased in the stroma of organ-cultured uterus (Fig. 2b). Thus, reduction of HH signaling altered cell proliferation in the neonatal uterus and vagina, suggesting that HH signaling stimulated cell proliferation in the epithelium of organ-cultured uterus and vagina and inhibited this in the stroma of organ-cultured uterus. Because some stromal cells died in organ-cultured vagina cultured for 2 days, the BrdU labeling index could not be examined.

In order to investigate the effects of HH signaling attenuation on epithelial differentiation, the protein expression of TRP63 and KRT14 was examined immunohistochemically in organ-cultured uterus and vagina treated with cyclopamine. Histological analysis of organ-cultured uterus and vagina cultured for 2 and 5 days showed no effect of cyclopamine (data not shown). In all organ-cultured uteri, TRP63 and KRT14 expression was not detected (data not shown). Cyclopamine did not alter TRP63 and KRT14 expression in the epithelium of organ-cultured vagina cultured for 2 and 5 days (Fig. 3a-l) and therefore, reduction of HH signaling did not affect epithelial differentiation.

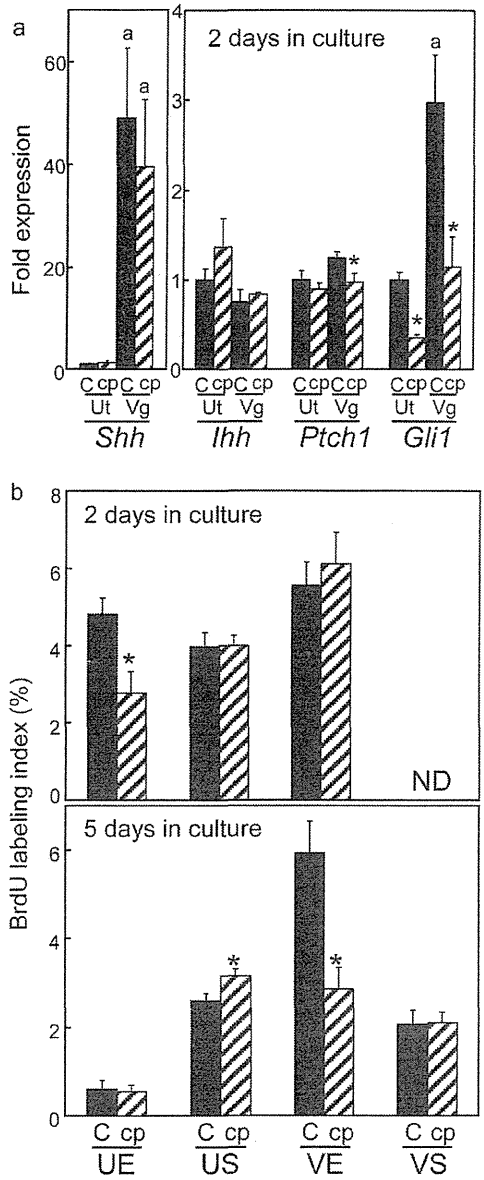

Fig. 2 *Shh*, *Ihh*, *Ptch1* and *Gli1* mRNA expression levels in organ-cultured uterus and vagina cultured for 2 days in control medium (C) or medium containing 10 μM cyclopamine (cp) were analyzed by real-time RT-PCR (**a**). Data are expressed relative to mRNA expression of the organ-cultured uterus cultured in control medium; relative expression=1.0 (Ut: organ-cultured uterus, Vg: organ-cultured vagina; $^{a}P<0.05$ compared with medium-matched uteri, $^{*}P<0.05$ compared with organ-cultured uteri or vaginae cultured with control medium). The BrdU labeling index in organ-cultured uterine epithelium (UE), uterine stroma (US), vaginal epithelium (VE) and vaginal stroma (VS) cultured for 2 days or 5 days in the control medium (C) or in medium containing 10 μM cyclopamine (cp) was analyzed by immunohistochemistry (**b**; *P<0.05 compared with BrdU labeling index in organ-cultured UE, US, VE, or VS with control medium, ND not determined)

The mRNA expression of growth factors regulated by HH signaling was examined by real-time RT-PCR in organ-cultured uterus and vagina. At 2 days in culture, the expression of *Tgf1*, *Bmp4*, *activin βA-* and *βB-subunits*, *Fgf7*, *Fgf10* and *Wnt5a* was not altered by addition of cyclopamine to the organ-cultured uterus and vagina (Fig. 3m).

Expression of *Shh* and *Ihh* in tissue recombinants at days 5 and 7 post-grafting

In order to investigate the regulation of *Shh* and *Ihh* expression by the stroma, *Shh* and *Ihh* expression in the uterine and vaginal tissue recombinants was examined by real-time RT-PCR. At days 5 and 7 post-grafting, the expression of *Shh* in VE+US was significantly higher than that in the other tissue recombinants, whereas it was low in VE+VS (Fig. 4). At day 7 post-grafting, the expression of *Ihh* in UE+US was significantly higher than that in UE+VS. The expression of *Ihh* in tissue recombinants containing US was high at day 7 post-grafting, whereas it did not increase in UE+VS at day 7 post-grafting. The expression of *Ihh* in VE+VS was higher at day 7 post-grafting than at day 5.

These results indicated that *Shh* expression in the epithelium was inhibited by the VS and that *Ihh* expression was stimulated by the US.

Discussion

HH signaling regulates epithelial differentiation and cell proliferation through epithelial-stromal signaling in the prostate, gastrointestinal tract and lung during development (Pepicelli et al. 1998; Ramalho–Santos et al. 2000; Freestone et al. 2003; Wang et al. 2003; Berman et al. 2004; Madison et al. 2005; Doles et al. 2006; White et al. 2006). Our results show that activated HH signaling in the stroma regulates cell proliferation in the neonatal uterus and vagina (Fig. 5).

In neonatal mice, *Shh* is expressed only in the VE and *Ihh* is expressed in the UE, US, VE and VS, whereas all *Gli* genes are mainly expressed in the stroma. The expression of *Gli1* and *Ptch1*, target genes of HH signaling, is high in the vagina at day 2 and therefore, SHH in the VE activates HH signaling in the VS (Fig. 5). Although *Ihh* expression in the uterus at day 2 is low and similar to that in the vagina, the reduction of HH signaling in the neonatal uterus affects cell proliferation, indicating that IHH is also involved in HH signaling activation in the stroma of the neonatal uterus and vagina. At day 15, *Gli1* and *Ptch1* expression decrease in the vagina but increase in the uterus, suggesting that HH signaling in the vagina is activated by abundant *Shh* only at the neonatal stage, whereas in the uterus, it is more highly activated, with growth accompanied by a drastic increase of *Ihh*. Indeed, *Ihh* in the adult UE is highly induced by progesterone during the early stages of pregnancy and is critical for

embryo implantation (Matsumoto et al. 2002; Lee et al. 2006). Moreover, HH signaling might not be involved with E2 in the adult vagina, since most genes related to HH signaling in the adult vagina were not affected by E2 treatment.

In the mammary gland and lung, activated HH signaling in the stroma stimulates epithelial cell proliferation (van Tuyl and Post 2000; Fiaschi et al. 2007; Visbal et al. 2011). In this study, the reduction of HH signaling by cyclopamine decreases the BrdU labeling index in organ-cultured VE, indicating that HH signaling stimulates epithelial cell proliferation in the neonatal vagina (Fig. 5). High expression of *Shh* coincides with the beginning of vaginal epithelial stratification and therefore, *Shh* might be important for cell proliferation in the epithelial stratification process.

In the organ-cultured uterus, the BrdU labeling index in the epithelium decreases after the reduction of HH signaling but increases in the stroma, indicating that HH signaling in cell proliferation acts contrastingly in the epithelium and stroma of neonatal uterus (Fig. 5). In this study, HH signaling inhibits stromal cell proliferation in the neonatal uterus. However, in the adult uterus, IHH stimulates stromal cell proliferation (Matsumoto et al. 2002; Lee et al. 2006; Franco et al. 2010b). In the prostate, HH signaling has age-dependent effects on cell proliferation (Lamm et al. 2002; Freestone et al. 2003; Wang et al. 2003; Berman et al. 2004). In the uterus, constitutive activation of SMO from neonatal day 5 causes an increase in stromal size in adults, whereas constitutive activation of SMO from embryonic days causes a decrease in stromal size in the adult (Franco et al.

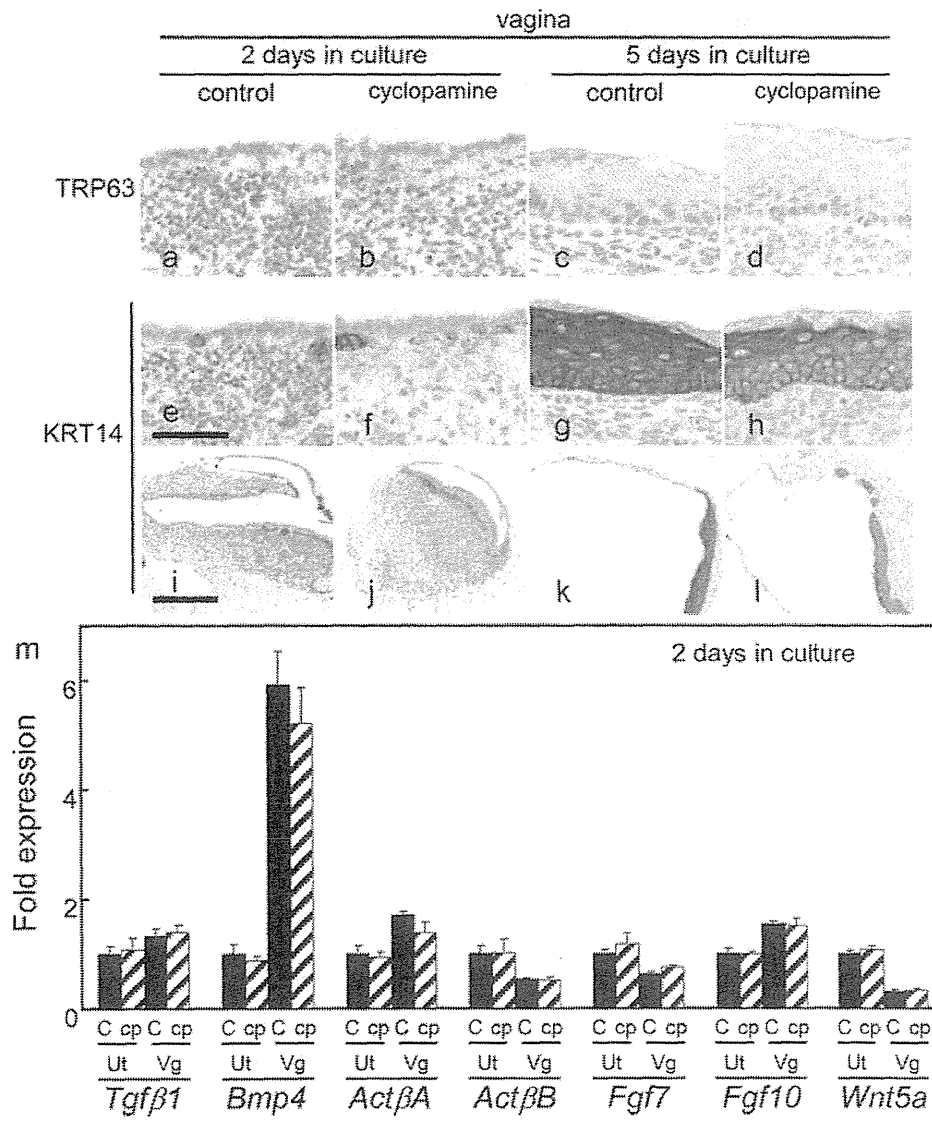

Fig. 3 Expression (*brown*) of TRP63 (**a-d**) and KRT14 (**e-l**) in the epithelium of organ-cultured vagina cultured for 2 days (**a**, **b**, **e**, **f**, **i**, **j**) or 5 days (**c**, **d**, **g**, **h**, **k**, **l**) in control medium (**a**, **c**, **e**, **g**, **i**, **k**) or medium containing 10 μM cyclopamine (**b**, **d**, **f**, **h**, **j**, **l**). *Bar* 50 μm (**a-h**), 200 μm (**i-l**). *Transforming growth factor β1* (*Tgfβ1*), *bone morphogenetic protein 4* (*Bmp4*), *activin βA-subunit* (*ActβA*) or *activin βB-subunit* (*ActβB*), *fibroblast growth factor 7* (*Fgf7*), *fibroblast growth factor 10* (*Fgf10*), or *wingless-related MMTV integration site 5a* (*Wnt5a*) mRNA expression levels in organ-cultured uterus and vagina cultured for 2 days in control medium (C) or medium containing 10 μM cyclopamine (cp) were analyzed by real-time RT-PCR (**m**). Data are expressed relative to mRNA expression of the organ-cultured uterus cultured in control medium; relative expression=1.0 (Ut: organ-cultured uterus, Vg: organ-cultured vagina)

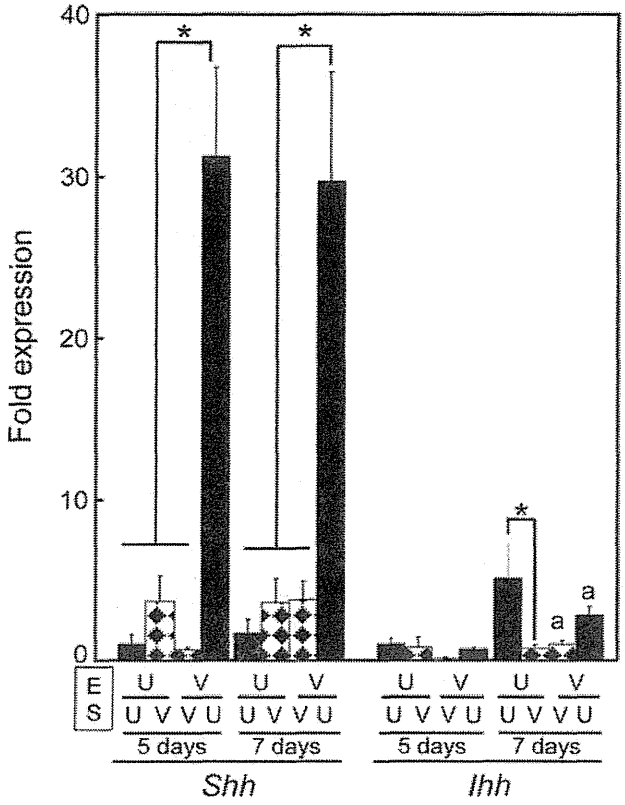

Fig. 4 *Shh* and *Ihh* mRNA expression levels in tissue recombinants at days 5 or 7 post-grafting as analyzed by real-time RT-PCR (E: epithelium, S: stroma, U: uterus, V: vagina). Data are expressed relative to mRNA expression of UE+US at day 5 of post-grafting; relative expression=1.0 (*$P<0.05$, $^{a}P<0.05$ compared with tissue recombinants at day 5 post-grafting)

2010a; Migone et al. 2011). Thus, the role of HH signaling in stromal cell proliferation in the uterus seems to change around day 5. In addition, constitutive activation of SMO reduces the number of uterine glands (Franco et al. 2010a; Migone et al. 2011). In the developing prostate and lung, HH signaling regulates epithelial budding (Podlasek et al. 1999; Lamm et al. 2002; White et al. 2006). Therefore, HH signaling might be involved in uterine gland formation through the regulation of cell proliferation in the UE and US.

In the developing prostate and lung, HH signaling stimulates the expression of several growth factors such as the *Tgf* superfamily and *Fgf* (Pepicelli et al. 1998; Lebeche et al. 1999; Wang et al. 2003; Pu et al. 2004;). In the uterus, the constitutive activation of SMO from embryonic days stimulates *Wnt5a* expression at day 24 (Migone et al. 2011). In this study, the mRNA expression of growth factors is not altered by the addition of cyclopamine, suggesting that HH signaling in the neonatal uterus and vagina is not accompanied by the expression of these genes. HH signaling in the neonatal uterus and vagina might have different target genes from those in adults.

Reduction of HH signaling does not appear to affect epithelial differentiation in the uterus and vagina grown *in vitro*. The structure of the UE is not altered by conditional knockout of *Ihh* in the uterus (Lee et al. 2006; Franco et al. 2010b). However, constitutive activation of SMO in the uterus results in TRP63-positive stratified cells in adults (Franco et al. 2010a; Migone et al. 2011), suggesting that HH signaling promotes epithelial differentiation. Thus, the role of HH signaling in epithelial differentiation of neonatal uterus and vagina is still controversial.

Our experiments on tissue recombinants have shown that epithelial *Shh* expression is inhibited by the VS, whereas *Ihh* expression is stimulated by the US (Fig. 5). At day 15, *Shh* expression is decreased in the vagina, whereas *Ihh* expression is increased in the uterus *in vivo*. Thus, *Shh* or *Ihh* expression might be regulated by the VS or US until day 15. In the adult uterus, progesterone stimulates epithelial *Ihh* expression via progesterone receptors (PRs) in the stroma (Simon et al. 2009). The stromal PR proteins are strongly expressed from day 15 (Hayashi et al. 2011), supporting our hypothesis that *Ihh* expression at day 15 is stimulated by the US through PR signaling.

In conclusion, we have demonstrated the role of HH signaling in cell proliferation in the neonatal uterus and vagina (Fig. 5). In neonatal mice, *Shh* in the VE and *Ihh* in the uterus and vagina activate HH signaling in the stroma. Activated HH signaling stimulates epithelial cell proliferation in the uterus and vagina but inhibits stromal cell proliferation in the uterus. The expression of *Shh* and *Ihh* is regulated by the US or VS. In contrast, attenuated HH signaling does not appear to affect epithelial differentiation in the uterus and vagina grown *in vitro*.

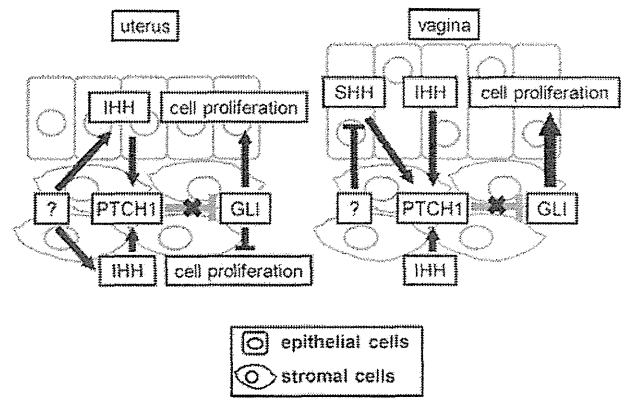

Fig. 5 Representation of the epithelial-stromal regulation of neonatal uterine or vaginal cell proliferation by HH signaling

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FULL PAPER *Laboratory Animal Science*

p21[Q1] and Notch Signalings in the Persistently Altered Vagina Induced by Neonatal Diethylstilbestrol Exposure in Mice

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ABSTRACT. Female reproductive organs show organ-specific morphological changes during estrous cycles. Perinatal exposure to natural and synthetic estrogens including diethylstilbestrol (DES) or estrogenic chemicals induces estrogen-independent persistent proliferation of vaginal epithelium in mice. To understand the underlying mechanism of the estrogen-independent persistent vaginal changes induced by perinatal DES exposure, we examined global gene expressions in the vaginae of ovariectomized adult mice exposed neonatally to DES using a microarray. The cell cycle-related gene, *p21*, a cyclin-dependent kinase inhibitor, showed upregulation in the vagina, and p21 protein was localized in the basal layer of the vaginal epithelium in mice exposed neonatally to DES and an estrogen receptor α agonist, propyl pyrazole triol (PPT). The expressions of Notch receptors and Notch ligands were unchanged; however, the mRNAs of hairy-related basic helix-loop-helix (bHLH) transcription factor genes, *Hes1*, *Hey1* and *Heyl* were persistently downregulated in the vagina, indicating estrogen-independent epithelial cell proliferation in mice exposed neonatally to DES and PPT.
KEY WORDS: diethylstilbestrol, neonatal exposure, p21 and Notch signalings, persistent vaginal changes, vagina.

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Long-term estrogenic stimulation is a known risk factor for carcinogenesis in laboratory animals and humans [25, 26]. In humans, transplacental exposure to a synthetic estrogen, diethylstilbestrol (DES), induced vaginal clear-cell adenocarcinoma in young women [16]. In mice, developmental exposure to estrogens within a critical developmental period elicits various permanent alternations in female reproductive tracts [5, 7, 29, 30, 32–35, 47]. 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 [8, 27, 48].

Previously, we characterized the mRNA expression patterns in the neonatal mouse vagina exposed to DES at different ages and the persistently altered vagina resulting from neonatal DES exposure using a DNA microarray and real-time quantitative RT-PCR [30, 46]. 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 [30]. 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 [29, 30]. The vaginae of mice exposed to DES at different ages showed that 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 [46].

p21 (also called WAF1, CAP20, Cip1 and Sdi1) [6, 10, 38, 56], a founding member of the Cip/Kip family of CKIs including p27 [41, 52] and p57 [21, 52], can bind and inhibit a broad range of cyclin/Cdk complexes, with a preference for those containing Cdk2 [11, 56]. It plays an essential role in growth arrest after DNA damage [1, 2, 4], and its overexpression leads to G_1 and G_2 [37] or S-phase [39] arrest. Moreover, the anti-oncogenic effect of Notch family genes, one of the fundamental signaling pathways that regulate metazoan development and adult tissue homeostasis, appear to be mediated by p21 and by repression of Shh and Wnt signalings [3, 36, 50]. Wnt signaling is suppressed by Notch1 activation in keratinocytes, showing that Notch1 activation downregulates this pathway by suppressing *Wnt-4* expression [3]. p21 mediates this negative regulation; Notch1 activation increases p21 protein levels, which is subsequently associated with E2F1 transcription factors at

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the *Wnt4* promoter, downregulating *Wnt4* expression [3]. On the other hand, p21 is often responsible for stress-induced p53-dependent and p53-independent cell cycle arrest, which permits cells to pause and to repair damage and then to continue cell division [9], and p21 expression has been shown to be regulated largely at the transcriptional level by both p53-dependent and p53-independent mechanisms [9].

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

MATERIALS AND METHODS

Reagents: Diethylstilbestrol (DES) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). An 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, U.S.A.). Sesame oil and dimethyl sulfoxide (DMSO) were obtained from Kanto Chemical (Tokyo, Japan).

Animals and treatments: C57BL/6J mice purchased from CLEA Japan (Tokyo, Japan) were maintained under a 12 hr light/12 hr dark cycle 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 the current study. Newborn female 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 of age 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 of age, were sacrificed at 15 weeks of age, and their vaginae were used for real-time quantitative RT-PCR and immunohistochemistry. Since we have already demonstrated that there is no age difference in histology and gene expression at least between 2–4 months of age in neonatally DES-exposed ovariectomized mice [29, 30, 32, 33], we used preserved mouse vaginae treated with the abovementioned chemicals to reduce animal use.

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, U.S.A.) and purified using an RNeasy mini kit (Qiagen, Chatsworth, CA, U.S.A.). Quality and quantity of total RNA were confirmed with an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA, U.S.A.). cRNA probes were prepared from the purified RNA using an Affymetrix cRNA probe kit (Affymetrix, Santa Clara, CA, U.S.A.) according to the manufacturer's protocol. All preparations met the recommended criteria of Affymetrix for application to 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 [55]. To confirm the estrogen-related changes in gene expression revealed by DNA microarray analysis, we independently repeated the same experiment twice. The expression data were analyzed with GeneSpring software (Agilent) as described previously [53–55].

Real-time quantitative RT-PCR: Total RNA, isolated with an 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 an ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.) and SYBR Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. The 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 with a 15 μl reaction mixture. Relative RNA equivalents for each sample were obtained by standardization of ribosomal protein L8 levels. The sequences of the gene primer sets are given in Supplementary File. 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.

Immunohistochemistry: Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 4 μm. Deparaffinized 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 a Universal LSAB™ 2 kit (Dako, Carpinteria, CA, U.S.A.) according to the manufacturer-supplied protocol. Anti-p21 monoclonal antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). The sections were incubated at 1:50 dilution in PBS containing 1% BSA (Sigma) overnight at 4°C. For negative controls, murine IgG2b (Dako) was used at the same dilution.

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

RESULTS

DNA microarray analysis: The DNA microarray analysis revealed 11,219 of 22,689 Genechip probes in the vaginae of 10-week-old control mice or 2.5 μg DES-exposed mice ovariectomized at 8 weeks of age. Compared with the controls, 423 probes were upregulated and 351 probes were downregulated in the vagina exposed neonatally to DES (data not shown). We further analyzed genes related to the cell cycle. Compared with the oil controls, 26 genes (31 probes) were upregulated (Table 1) and 8 genes (10 probes)

Table 1. Upregulated 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 2. Downregulated 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_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

were downregulated (Table 2) in the DES-exposed vagina.

p21 and p53 mRNA expression: Since the expression of *p21* was specifically upregulated in the cell cycle-related genes compared with the controls, we 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. This was because we previously showed persistent proliferation and keratinization of epithelial cells in the vagina exposed neonatally to DES and PPT [33, 34]. The expression of *p21* was very low in the vaginae of controls and the ovariectomized mice neonatally exposed to 25 μg DPN. In contrast, *p21* was upregulated in the vaginae of mice exposed neonatally to 2.5 μg DES and 25 μg PPT (Fig. 1). The mRNA of p53, unlike that of p21, was unaltered in the DES-, PPT- and DPN-exposed vagina (Fig. 1).

p21 protein localization: We performed immunohisto-

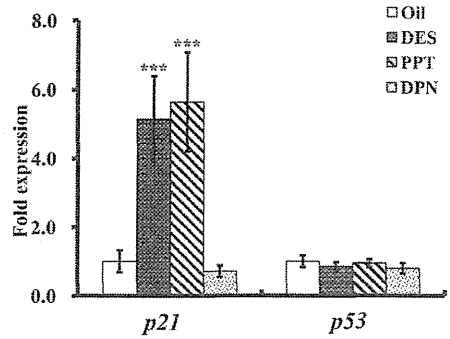

Fig. 1. Expression of *p21* and *p53* mRNA in vaginae of 15-week-old ovariectomized mice exposed neonatally to 2.5 μg DES (n=4), 25 μg PPT (n=4), 25 μg DPN (n=4) or the oil vehicle alone (n=4) for the first 5 days. The expression of each mRNA in the vagina of the oil-treated controls was regarded as the basal level (1.0). ***P<0.001 vs controls (Student's t-test or Welch's t-test followed by F-test).

chemistry to investigate the localization of p21. Fifteen-week-old, ovariectomized mice neonatally exposed to 2.5 μg DES or the oil vehicle alone were stained with p21 antibody (Fig. 2A and 2C) or murine IgG2b as a negative control (Fig. 2B and 2D). p21 was localized in the basal layer of vaginal epithelial cells in mice exposed neonatally to DES (Fig. 2A), but no p21 staining was observed in the oil-treated controls (Fig. 2C), indicating that p21 expression was correlated with proliferation of the vaginal epithelial cells.

Expression of Notch signaling pathway-related genes in the vagina of mice exposed neonatally to DES, PPT or DPN: We analyzed the expression of genes related to the Notch signaling pathway; however, all Notch receptors mRNA (*Notch 1*, *Notch 2*, *Notch 3* and *Notch 4*) (Fig. 3A) and Notch ligands mRNA (*Dll 1*, *Dll 4*, *Jagged 1* and *Jagged 2*) (Fig. 3B) were unaltered in the DES-exposed vagina. On the other hand, the expression of Notch target genes, *Hes1*, *Hey1* and *HeyL*, was significantly decreased in the vagina exposed neonatally to DES or PPT compared with the control and neonatally DPN-treated mice (Fig. 3C).

DISCUSSION

Estrogens tightly regulate cell proliferation and differentiation in the female reproductive tracts [17, 48]. However, perinatal exposure to estrogens, including synthetic estrogen, DES and other estrogenic chemicals, induces persistent anovulation caused by alteration of the hypothalamo-pituitary-gonadal axis, polyovular follicles, uterine abnormalities and persistent vaginal changes in mice [5, 7, 16–18, 34, 35, 45–48]. In paticular, transplacental exposure to DES, which was routinely prescribed to pregnant women for preventing miscarriages from the 1940s to 1970s, reportedly induced vaginal clear-cell adenocarcinoma in young women [16]. 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 [13, 15, 16, 40].

DNA microarrays have 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 [30, 33, 46, 55]. In the present study, we found upregulation of cell cycle-relat-

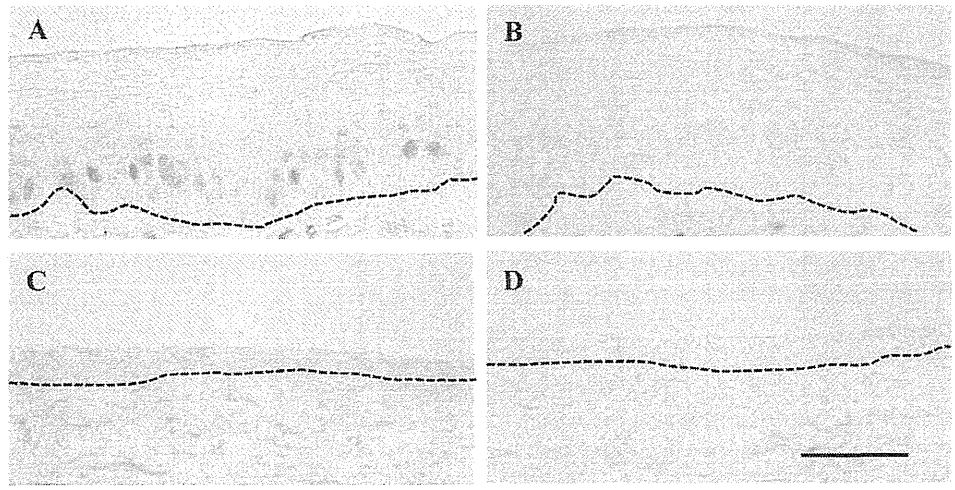

Fig. 2. Immunohistochemistry of p21. Vaginae of 15-week-old ovariectomized mice exposed neonatally to 2.5 μg DES (A, B) or the oil vehicle alone (C, D). p21 was immunostained in the neonatally DES-exposed vagina (A) and the age-matched control mouse vagina (C). As negative controls, sections were incubated with pre-immune serum instead of the primary antibody (B, D). Staining of blood vessels in the stroma is a nonspecific reaction. The boundary between the epithelium and stroma is indicated by a dotted line. Bar, 50 μm.

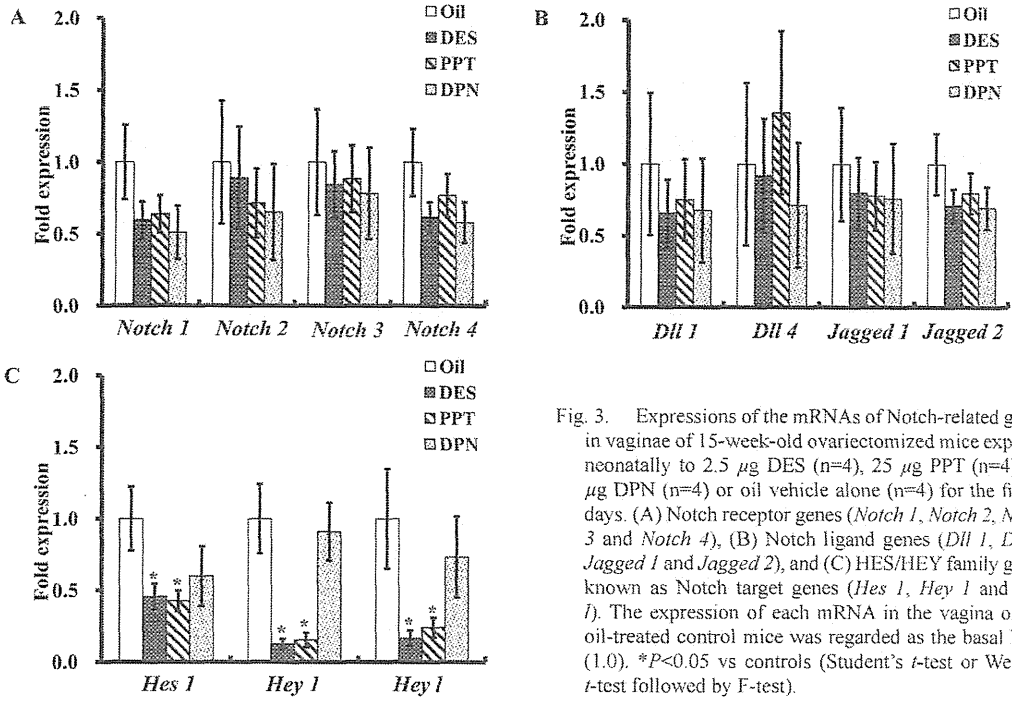

Fig. 3. Expressions of the mRNAs of Notch-related genes in vaginae of 15-week-old ovariectomized mice exposed neonatally to 2.5 μg DES (n=4), 25 μg PPT (n=4), 25 μg DPN (n=4) or oil vehicle alone (n=4) for the first 5 days. (A) Notch receptor genes (*Notch 1*, *Notch 2*, *Notch 3* and *Notch 4*), (B) Notch ligand genes (*Dll 1*, *Dll 4*, *Jagged 1* and *Jagged 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 the 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).

ed genes in the persistently proliferating vaginal epithelial cells of mice neonatally exposed to DES. In particular, *p21* mRNA showed persistent upregulation 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 upregulation of *p21* in various cell types [23, 28]. Moreover, Akt phosphorylated by PI3K leads to the stabilization of p21 and enhanced tumor cell survival [22]. Previously, we found the phosphorylation of Akt and persistent expression of some growth factors in the vagina neonatally exposed to DES [29, 30]. 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 the 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 [19, 20, 42, 51]. The anti-oncogenic effect of Notch1 in murine skin appears to be mediated by p21 induction and by repression of Wnt signaling [3]. In the present study, the mRNA expressions 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 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 expressions of other Notch target genes, i.e., *Hes1* [43], *Hey1* [24] and *Heyl*, were permanently downregulated in the vagina neonatally exposed to DES or PPT. 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 hearing ability by regulating cell migration, cell alignment and polarity [49]. Moreover, overexpression 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 [14]. Irreversible epithelial proliferation in the vagina exposed neonatally to DES might be affected by downregulation of *Hes1*, *Hey1* and *Heyl*. Ström *et al.* [12, 44] have previously shown that the expression of HES-1 is downregulated by 17β-estradiol (E_2) and that forced expression of HES-1 inhibits an E_2-mediated proliferation of breast cancer cells. Moreover, Müller *et al.* [31] have revealed a novel negative estrogen responsive element (ERE) associating with the HES-1 promoter, recruiting nuclear receptor co-regulators to the ERE in response to E_2 and then docking in the HES-1 promoter region. In the present study, downregulation 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 [29, 30].

In conclusion, we demonstrated that the expressions of

both *p21* mRNA and p21 protein are permanently upregulated and the *Hes1*, *Hey1* and *Heyl* mRNAs are permanently downregulated in the vagina exposed neonatally to DES. Additional elucidation of the molecular mechanism of the irreversible proliferation in vaginal epithelial cells is essential in the near future.

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p21[Q1] and Notch Signalings in the Persistently Altered Vagina Induced by Neonatal Diethylstilbestrol Exposure in Mice

Comments

[Q1] P21 → p21

[Q2] Corrected. 1485 → 1495

[Q3] W. → W. S.

[Q4] 650–652 → 639–649

[Q5] K. → K. F.

[Q6] 28 → 275

[Q7] A. R. → A. B.

[Q8] 4822 → 4882

[Q9] Polyyak → Polyyak

[Q10] Exp. Biol. Med.--> Exp. Biol. Med. (Maywood)

[Q11] Watababe → Watanabe

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

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Abstract. *Estrogen regulates morphological changes in reproductive organs, such as the vagina and uterus, during the estrous cycles in mice. Estrogen depletion by ovariectomy in adults results in atrophy accompanied by apoptosis in vaginal and uterine cells, while estrogen treatment following ovariectomy elicits cell proliferation in both organs. Sequential changes in mRNA expression of wingless-related MMTV integration site (Wnt) and Notch signaling genes were analyzed in the vagina and uterus of ovariectomized adult mice after a single injection of 17β-estradiol to provide understanding over the molecular basis of differences in response to estrogen in these organs. We found estrogen-dependent up-regulation of Wnt4, Wnt5a and p21 and down-regulation of Wnt11, hairy/enhancer-of-split related with YRPW motif-1 (Hey1) and delta-like 4 (Dll4) in the vagina, and up-regulation of Wnt4, Wnt5a, Hey1, Heyl, Dll1, p21 and p53 and down-regulation of Wnt11, Hey2 and Dll4 in the uterus. The expression of Wnt4, Hey1, Hey2, Heyl, Dll1 and p53 showed different patterns after the estrogen injection. Expression patterns for Wnt5a, Wnt11, Dll4 and p21 in the vagina and uterus were similar, suggesting that these genes are involved in the proliferation of cells in both those organs in mice.*

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Key Words: Estrogen, mouse, uterus, vagina, p21 and Notch signaling, Wnt family genes.

The rodent vaginal epithelium exhibits cyclical changes in response to cyclical ovarian secretions of sex hormones, displaying an alternating pattern of keratinization and mucification (1). 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 (2-4). Estrogens alter cellular physiology by modulating the transcriptional activity of specific nuclear estrogen receptors (ERs) (5), 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* (6). However, the regulation of vaginal gene expression by estrogens and the molecular mechanisms underlying estrogen-mediated cell proliferation remain unclear.

Previously, we reported on expression patterns of mRNAs in the persistently-altered vagina of neonatal mice exposed to diethylstilbestrol (DES) using DNA microarrays and real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) (7-12). 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 (11). 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 (12) in the neonatally

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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 the functional mechanisms of estrogen regulating morphogenesis and maintenance of the reproductive organs. 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, we 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).

Materials and Methods

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

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.

BrdU immunostaining. Tissues embedded in paraffin were sectioned at 8 μm and stained with hematoxylin and eosin. In addition, de-paraffinized 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 at 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 (7, 8).

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 I. 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.

Immunohistochemistry. Tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin and sectioned at 4 μm. The sections were de-paraffinized and 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™ 2 kit, Universal (Dako, Carpinteria, CA, USA), according to the manufacturer-supplied protocol. Polyclonal antibody against 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°C. For negative controls, a normal goat immunoglobulin (IgG) fraction (Dako) was used at the same dilution. Monoclonal antibody against 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°C. For negative controls, murine IgG2b (Dako) was used at the same dilution.

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

Results

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 E_2 injection are shown in Figures 1A-C. The epithelium exhibited proliferation at 24 h (Figure 1B), and underwent stratification with superficial keratinization at 48 h after the injection (Figure 1C). The basal cells of vaginal epithelium at 24 h after the injection exhibited a high proliferative activity (10.2%), confirmed by BrdU immunostaining (Figure 1D). At 48 h after the injection, proliferative activity declined to 4.0% (Figure 1D).

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 (Figure 2A), and that of keratin 1 had a similar pattern to that of cyclin A2 (Figure 2B). Up-regulation of cyclin A2 and keratin 1 was correlated with epithelial stratification and keratinization in the vagina.

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

Gene	Primer (5'-3')	Product size (bp)	Gene accession no.
Cyclin A2	F: ACAGAGCTGGCCTGAGTCAT R: TTGACTGTTGGGCCATGTTGT	119	NM_009828
Keratin 1	F: AAGAGGACCAACGCAGAGAA R: TTGGCCTGAAGCTCAACTTT	87	NM_008473
Wnt4	F: CATCGAGGAGTGCCAATACCA R: GACAGGGAGGGAGTCCAGTGT	70	NM_009523
Wnt5a	F: GCGTGGCTATGACCAGTTTAAGA R: TTGACATAGCAGCACCAGTGAA	75	NM_009524
Wnt11	F: ATGTGCGGACAACCTCAGCTA R: CGCATCAGTTTATTGGCTTGG	100	NM_009519
Dll1	F: CTGGGTGTCGACTCCTTCAG R: GGAGGGCTTCAATGATCAGA	124	NM_007865
Dll4	F: AGGTGCCACTTCGGTTACAC R: TAGAGTCCCTGGGAGAGCAA	72	NM_019454
Hey1	F: TTTTCCTTCAGCTCCTTCCA R: ATCTCTGTCCCCCAAGGTCT	92	NM_010423
Hey2	F: CAGTCCTCAGCAGACAAGAC R: GGTTGGGCATGTAAGTGTAGTTGT	73	NM_013904
Heyl	F: GGTGACTTCCACCCAGAGAG R: GGGATTGGGACTATGCTCCT	99	NM_013905
p21	F: GTACTTCCTCTGCCCTGCTG R: AGAGTGCAAGACAGCGACAA	70	NM_001111099
p53	F: AAAGGATGCCCATGCTACAG R: TATGGCGGGAAGTAGACTGG	92	NM_011640

F, Forward; R, reverse; Wnt, wingless-related MMTV integration site; Dll, delta-like; Hey, hairy/enhancer-of-split related with YRPW motif.

Localization of Wnt4 and p21 proteins in the 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 24 h after the E_2 injection (Figure 3A). p21 was localized in the basal layer of vaginal epithelium in E_2-treated mice (Figure 3C). No response was visible in the vaginal epithelium stained with normal goat immunoglobulin (IgG) as a negative control (Figure 3B and D). Hence, th Wnt4 and p21 proteins were evident in the proliferating vaginal epithelial cells.

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, we 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 (Figure 4A). 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 (Figure 4B). 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 (Figure 4C). 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.

The 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 3 h in the uterus (Figure 4D). 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 (Figure 4E). Expression of *Heyl* was increased 6-12 h after the administration only in the uterus (Figure 4E and F). However, the expression showed no change in the vagina after E_2 injection (Figure 4F). The expression of *Dll1* was increased in the uterus 6 h after administration, while the expression remained unchanged in the vagina (Figure 4G). The 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 (Figure 4H).

The expression of *p21* and *p53* was increased at 6-12 h after E_2 administration in the uterus (Figure 4I and J). In the vagina, however, E_2 injection only increased *p21* expression at 12 h, with no effect on the expression of *p53* (Figure 4I and J).

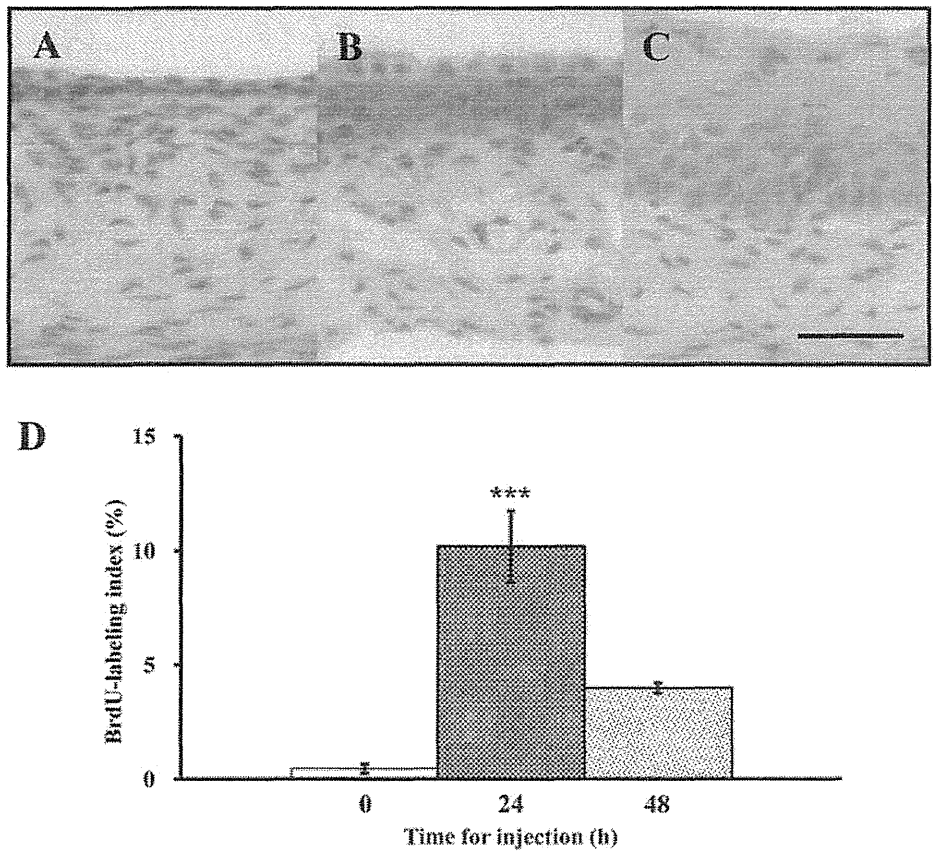

Figure 1. *Histology of vagina of a 10-week-old mouse, ovariectomized at 8 weeks of age, and killed just before (A) or 24 h (B) and 48 h (C) after a single abdominal injection of 50 ng 17β-estradiol (E_2)/g bw dissolved in sesame oil at 10 weeks of the injection. Bar=50 μm. Incidence (%) of BrdU-incorporated 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).*

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 (2-4). In order to understand the underlying mechanisms of estrogen functions in reproductive organs in mice, we have analyzed estrogen-responsive genes in reproductive organs, and found that expression patterns of genes are different between the vagina and uterus (9, 13, 14). Previously, we reported on the expression of Wnt- and Notch-related genes in the vagina and uterus exposed-neonatally to DES (9-12), and the localization of Wnt4 or p21 proteins and quantitation of BrdU-positive cells after E_2 administration in the ovariectomized mouse uterus (15, 16). Regarding the vagina, however, detailed information has not been reported. Therefore, we 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 (13, 17, 18) 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 (17). These findings suggest important roles of Wnt family genes in various reproductive physiologies. In the present study, *Wnt4*, but not *Wnt5a* and *Wnt11*, showed different expression 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 (19). 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, similarly to the BrdU labeling index and the expression of *Wnt4*. These results suggest that Wnt4 might act on epithelial stratification and keratinization in the vagina. In the uterus, *Wnt4* transiently increased 3 h after E_2 treatment; *Wnt4* is expressed in the uterine epithelium at pro-estrus at the time of highest

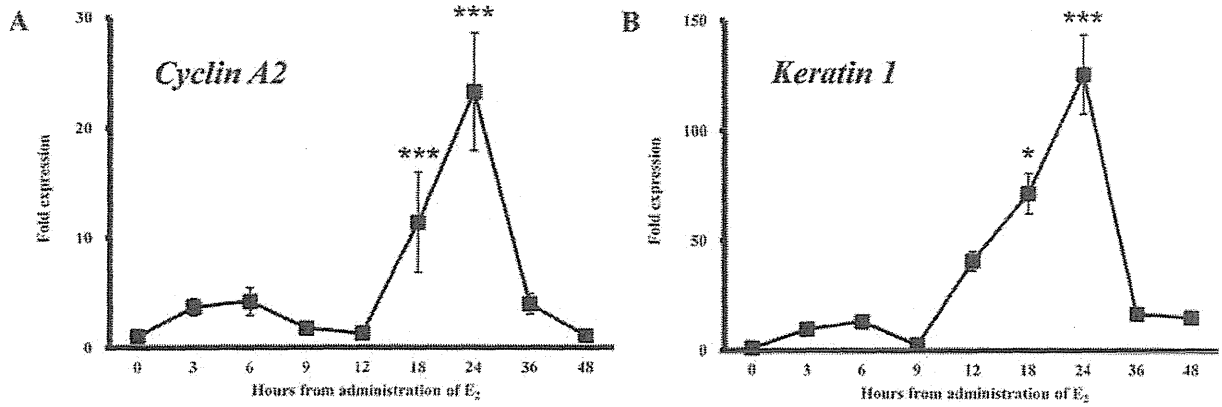

Figure 2. *Fold expression of cyclin A2 (A) and keratin 1 (B) after a single injection of 50 ng 17β-estradiol (E_2)/g bw. Expression of these genes in control mice was regarded as the basal level (1.0). Data are the means±standard error (n=3). *p<0.05 and ***p<0.001 vs. controls (one-way ANOVA).*

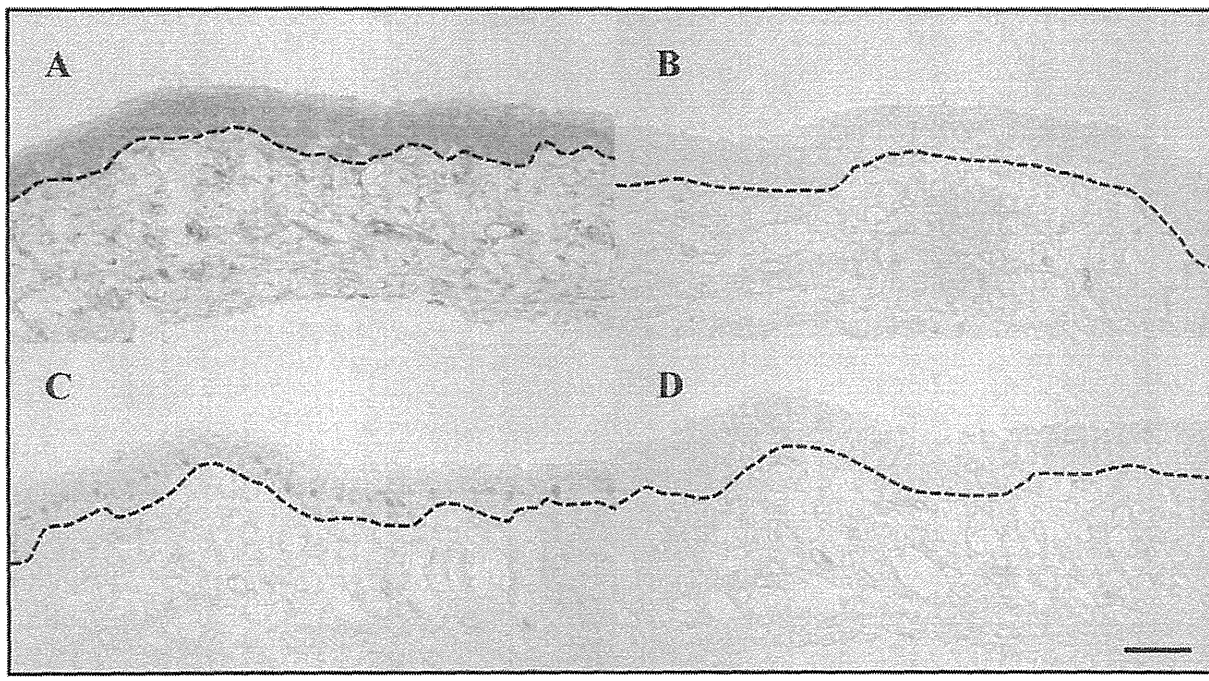

Figure 3. *Immunohistochemistry by wingless-related MMTV integration site-4 (Wnt4) (A) and p21 (C). Vaginae 10-week-old, ovariectomized mice were killed 24 h after a single injection of 17β-estradiol (E_2). For negative controls, sections were incubated with pre-immune serum instead of primary antibody (B, D). Bar=50 μm. The boundary between the epithelium and the stroma is indicated by a dotted line.*

estrogen levels (17, 20), 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 (17), and it is expressed at higher levels at pro-estrus in the vaginal epithelium and stroma (17), suggesting involvement in cell proliferation and keratinization in the vagina. We 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 in the uterine stroma at pro-estrous (17). Zhu *et al.*

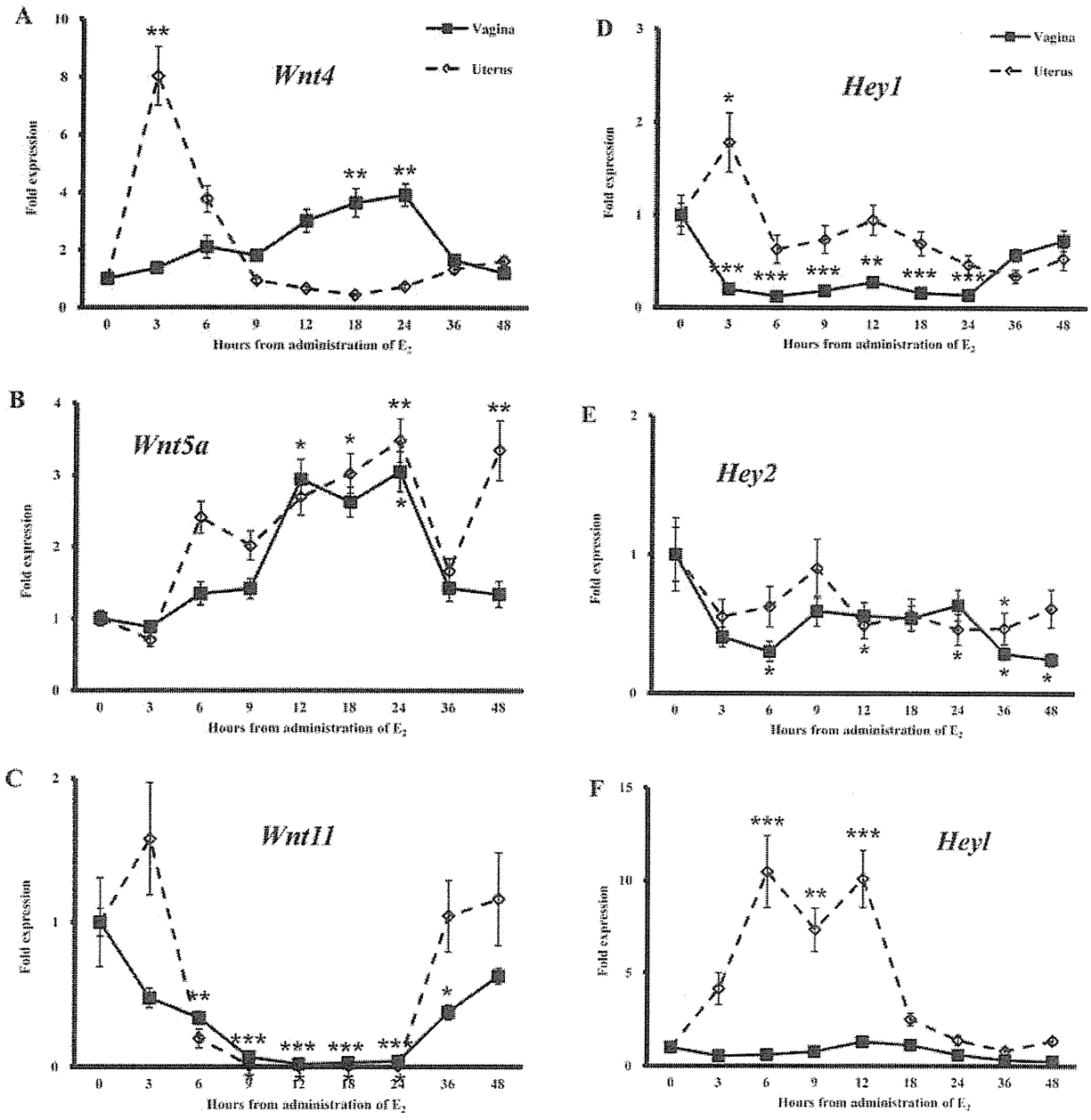

Figure 4. *Continued*

(21) reported that the expression of *Wnt11* is inhibited by androgens. 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 (11).

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 (12). 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