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

Takeshi Nakamura, Shinichi Miyagawa, Yoshinao Katsu, Hajime Watanabe, Takeshi Mizutani, Tomomi Sato, Ken-Ichirou Morohashi, Takashi Takeuchi, Taisen Iguchi, Yasuhiko Ohta

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 (E2), 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 estrogen 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 appears 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 is 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; Vainio 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 extra-cellular 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; Huelsen and Birchmeier, 2001; van Noort and Clevers, 2002), the canonical Wnt β-catenin pathway has been well studied.

The Wnt signaling is highly responsive to variable hormone concentration and location (Weber-Hall et al., 1994). It is well known that Wnt signaling 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, we 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 (Miyagawa et al., 2004b). In the present study, we report that neonatal exposure of DES and E2 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, we 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.

### 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′′-tetra[(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).

<table>
<thead>
<tr>
<th>Gene</th>
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</table>

* F, forward; R, reverse.

#### 2.2. Animals and treatments

C57BL/6J mice and 129Sv/Ei mice were purchased from CLEA Japan (Tokyo, Japan); Wnt4 mutant mice (129Sv 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/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 12,500 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, we 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 1. PCR conditions were as follows: 94 °C for 10 min, and 32 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, 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 s and 60 °C for 1 min in 15 μl volumes. Relative RNA equivalents
Table 2
Microarray data of Wnt genes in vaginas of adult mice (10-week old) exposed neonatally to DES.

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The values shown in bold indicate statistically significant change compared to controls.

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 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 deparaffinized were incubated with 0.3% H2O2 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°C. The sections were visualized with LSAB™ 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 were 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 2). Surprisingly, only Wnt4 and Wnt7b showed higher (3 to 6-fold) to moderate (1.97 to 2.42-fold) spikes in the neonatally DES-exposed mouse vagina than controls. On the other hand, Wnt11 showed a decrease (0.21 to 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, we 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 1–10) 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. 1A and D). By contrast, neonatally oil-treated control mice had atrophied vaginal epithelium after ovariectomy (Fig. 1C and 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. 1E and F). To investigate the transcriptional regulation of Wnt4 and Wnt11 mRNA by exogenous estrogen, we administered 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 neonatally to DES, were significantly decreased (Fig. 1B, E, and 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, we 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. 2). Wnt4 staining was observed in the basal and middle layers of epithelial cells in vagina of mice exposed neonatally to DES (Fig. 2A), but no Wnt4 staining was observed in oil-treated control mouse vagina (Fig. 2C). This suggests that Wnt4 might be associated with epithelial cell proliferation and further keratinization. We 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, we 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 ovarioctomized mice exhibited epithelial cell proliferation, stratification and superficial keratinization (Fig. 3A–D). Wnt4 expression was found to increase after neonatal DES or PPT treatment (Fig. 3E). A simultaneous decrease in Wnt11 expression was also observed in DES- or PPT-treated vagina (Fig. 3F). However, DPN treatment neither changed the Wnt4 and Wnt11 expression nor epithelial cell proliferation. Vaginal epithelium of ovarioctomized mice treated neonatally with oil (Fig. 3A) or 25 μg DPN (Fig. 3D) 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).

To clarify the role of Wnt4 in vaginal histological modulation by estrogen, we used Wnt4 hetero (Wnt4+/−) mice, since
Wnt4<sup>−/−</sup> mice exhibit fetal lethality (Stark et al., 1994; Vainio et al., 1999). We thought that Wnt4 expression levels in the vagina of wild type (Wnt4<sup>+/+</sup>) mice were higher than Wnt4<sup>−/−</sup> mice. All Wnt4<sup>+/+</sup> and Wnt4<sup>−/−</sup> mice treated neonatally with oil, vaginal epithelia were composed of 2–3 layers of cuboidal cells (Table 3). While, all neonatally DES-exposed Wnt4<sup>+/+</sup> and Wnt4<sup>−/−</sup> mice exhibited vaginal epithelial stratification or stratification with superficial keratinization (Table 3). Wnt4 expression levels and histology in vaginae between Wnt4<sup>+/+</sup> and Wnt4<sup>−/−</sup> mice were not different. Wnt4 was highly expressed in neonatally DES-exposed mice both in Wnt4<sup>+/+</sup> and in Wnt4<sup>−/−</sup> mice (Fig. 4A), showing epithelial stratification with superficial keratinization (Fig. 4B and C). The vagina of Wnt4<sup>+/+</sup> and Wnt4<sup>−/−</sup> mice exposed neonatally to DES having only epithelial stratification show no up-regulation of Wnt4 expression (Fig. 4B and C) suggesting that Wnt4 plays a role in epithelial keratinization in the vagina.

4. Discussion

In the present study, we intended to clarify the mechanism of ovary-independent proliferation of vaginal epithelial cells. First, we 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, we 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, Wnt5α, were abundant in the adult female reproductive tract, but become relatively scarce during gestation. In addition to Wnt4, Wnt5α and Wnt7α 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, we confirmed the expressions of several Wnt family genes, i.e., Wnt4, Wnt5α, Wnt5β, Wnt7β and Wnt11 mRNA in the neonatally DES-exposed or oil control mouse vagina using DNA microarray analysis. Although we recorded an elevated expression for Wnt4 and Wnt7β, and reduced expression of Wnt11, but Wnt5α and Wnt5β remain unchanged. Therefore, we 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 (Connoly and Schnitt, 1993; Stark et al., 1994; Kispert et al., 1998; Brisken et al., 2000; Smalley and Dale, 2001; Jeays-Ward et al., 2004; Yu et al., 2006). Wnt11 is a non-canonical Wnt family, regulates ureteric branching (Majumdar et al., 1998) and is expressed in the female reproductive tract and prostate (Brisken et al., 2000; Jilka et al., 1999). The expression in the embryonic male reproductive tract suggests a role in testicular development (Jilka et al., 1999).

Table 3

<table>
<thead>
<tr>
<th>Treatments</th>
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</table>
proliferation and persistent however, Fig. 18 the that (Maye 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 cERKO 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, we used Wnt4+/- mutant mice to study the function of Wnt4 in the estrogen-induced vaginal epithelial stratification and keratinization, since Wnt4+/- mice 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.

In conclusion, we suggested that Wnt4 mRNA is permanently up-regulated, and Wnt11 mRNA is permanently down-regulated in the vagina exposed neonatally to DES or ERα specific ligand, PPT. Wnt4 might be participated in the irreversible superficial keratinization in the mouse vagina. However, the ER-independent repressive role of Wnt11 in vaginal keratinization, need to address more critically in the near future.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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