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—Original Article—

Activated Vitamin D_3 and Pro-activated Vitamin D_3 Attenuate Induction of Permanent Changes Caused by Neonatal Estrogen Exposure in the Mouse Vagina

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Abstract. Exposure of mice to a high dose of estrogens including diethylstilbestrol (DES) during the neonatal period modifies the developmental plan of the genital tract, which leads to various permanent changes in physiology, morphology and gene expression. These changes include development of an abnormal vaginal epithelium lined with hyperplastic mucinous cells accompanied by *Tff1* gene expression in mice. Here, the influence of vitamin D on the direct effect of estrogen on the developing mouse vagina was examined. The mid-vagina of neonatal mice was cultured in a serum-free medium containing estradiol-17β (E_2) and various concentrations of 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2$D) *ex vivo* and then was transplanted under the renal capsule of ovariectomized host mice for 35 days. Exposure to E_2 alone caused the vaginal tissue to develop estrogen-independent epithelial hyperplasia and to express TFF1 mRNA, while addition of a low nanomolar amount of 1,25$(OH)_2$D added at the same time as E_2 to the culture medium attenuated the effects of estrogen. Expression of vitamin D receptor was also evident in the neonatal mouse vagina. Interestingly, addition of 25-hydroxyvitamin D_3, a pro-activated form of vitamin D, at the micromolar level was found to be potent in disrupting the developmental effects of E_2, while cholecalciferol was not at least at the dose examined. Correspondingly, expression of Cyp27B1, a kidney-specific 25-hydroxyvitamin D hydroxylase, was evident in the neonatal mouse vagina when examined by RT-PCR. In addition, simultaneous administration of 1,25$(OH)_2$D successfully attenuated DES-induced ovary-independent hyperplasia in the vagina in neonatal mice *in vivo*. Thus, manipulation of vitamin D influenced the harmful effects of estrogens on mouse vaginal development.

Key words: Cyp27B1, Estrogens, Trefoil factor 1, Vaginal development, Vitamin D

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Exposure to exogenous estrogen during the early stages of life causes endocrine disruption and organogenetic abnormalities, which sometimes lead to severe results such as infertility, deformity and carcinogenesis, in laboratory animals and humans [1–4]. Prenatal exposure to diethylstilbestrol (DES), a potent synthetic ligand for estrogen receptors, resulted in various reproductive tract abnormalities including cervix cancer, the so-called DES syndrome or developmental estrogenization syndrome, in humans [e.g., 5, 6]. Similar genital abnormalities have been shown in experimental animals exposed perinatally to estrogens. Neonatal treatment of female mice with physiologically overdoses of estrogenic substances such as estradiol-17β (E_2) and DES, induces both ovary-dependent (via its effects on brain development that leads to continuous secretion of follicle-stimulating hormone and corresponding secretion of estrogen from the ovary) and ovary-independent proliferation and cornification of the vaginal epithelium [7–9]. The latter vaginal changes, induced via direct action of estrogen on the developing vagina, are irreversible and frequently lead to the development of cancerous lesions when the mice become adults. The toxic effects of estrogen appear to be a result of disrupted epithelial-mesenchymal interaction, the cellular and molecular mechanisms of which are largely unknown. Previous experiments, through investigation of recombination of the epithelium and stroma, have suggested that the initial permanent change induced by neonatal estrogens in the perinatal mouse vagina is that on the epithelium but not the stroma [10, 11] and that the permanent changes are linked to the unusual expression of the *Tff1* gene on the vaginal epithelium [11].

The developmental effect of neonatal exposure to overdosed estrogens can be influenced by other bioactive factors such as vitamins and growth factors. In fact, vitamin A (retinol) and a FGF receptor 2 (IIIb) blocker attenuated the estrogen effects on the neonatal mouse vagina in our previous studies, which provided insights on the mechanisms by which estrogens misleads the developmental processes of the genital tracts [12, 13]. In the present study, the influence of vitamin D and its active derivates on the estrogen effects was explored for the first time. Vitamin D is one of hydrophobic vitamins, and can be ingested from a dietary source and/or synthesized by means of UV exposure in the skin from the pro-vitamins synthesized from cholesterol in the liver. Vitamin D_3 (VD_3), also designated cholecalciferol, is the major type of dietary and/or biosynthetic vitamin D in animals. In general, it is readily converted to 25-hydroxyvitamin D_3 (25(OH)D), a pro-active form of vitamin D, by a hepatic enzyme cytochrome

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P450 2R1 (Cyp2r1), and subsequently to 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D$; calcitriol), an active form, by cytochrome p450 27B1 (Cyp27B1) in the renal proximal tubule. The activated vitamin D then interacts with the vitamin D receptor (VDR), a member of the nuclear receptor family of transcription factors, in the target tissues and contributes to various functions including calcium/phosphate homeostasis [14]. The *Cyp27b1* gene is expressed exclusively in the kidney and placenta in mice [15], although it is rather widely expressed in humans [16].

There have been practically no reports concerning the effect of vitamin D on the neonatal mouse vagina so far. In some studies, however, the active form of vitamin D was reported to inhibit the proliferative effect of keratinocyte growth factor (Kgf) in the prostate, a male organ comparable to the female cervicovagina [17, 18]. On the other hand, Kgf is involved in the E_2 effects on the developing vagina in mice [13, 19]. These findings prompted us to explore the possible modifications of DES syndrome in the mouse vagina in response to vitamin D.

Materials and Methods

Animals and chemicals

C3H strain mice purchased from Clea Japan (Tokyo, Japan) were kept at the Laboratory Animal Resource Center of the University of Tsukuba. They were housed in a plastic cage in standard laboratory animal facilities with controlled lighting (14 h/day), the temperature controlled to 25 ± 1 C and food and water provided *ad libitum*. All experimental protocols involving animals conformed to the Tsukuba University Guidelines for Care and Use of Laboratory Animals.

Neonatal administration of estrogen and ovariectomy in adulthood were performed basically as previously described [12] with minor modifications. Briefly, neonatal mice were subcutaneously administrated a daily single shot of E_2 (20 μg), DES (1 μg) or the vehicle (20 μl of sesame oil per head) alone for 4 days starting from day 1, the first day when the pup was identified (at 1000 h). In some cases $1,25(OH)_2D$ was added to the DES solution or the vehicle from concentrated stocks dissolved in ethanol to give the final amount described in the Results. The mice were ovariectomized at day 35, and the vagina was excised at day 42 of age.

All chemicals including vitamin D and estrogen were purchased from Sigma-Aldrich Japan (Tokyo, Japan) unless otherwise noted.

Tissue culture and grafting of the vagina

The middle one-third of the vagina was dissected from neonatal mice at day 1 and cultured in a defined medium *ex vivo* for 4 days as described previously [13]. Briefly, the vaginal tissues were cultured in Waymouth's MB752/1 medium supplemented with penicillin G and streptomycin and with or without combinations of E_2 (5 μg/ml) and vitamin D_3 (the dose was mentioned in Results) at 37 C in a 5% CO_2 atmosphere. The tissues were then grafted under the kidney capsule of ovariectomized adult female hosts and left there for 35 days at which point they were excised for examination of histology or gene expression. Overall, approximately 70% of the grafts were recovered, and more than three specimens were examined in each experimental group to confirm reproducibility. In some cases, the host mice with the vaginal grafts were injected with E_2 (20 μg/100 μl sesame oil, daily) for 2 days just before autopsy.

Bioactive substances were added to the basal culture medium to give the final concentrations described in the Results from concentrated stocks: E_2, cholecalciferol, 25(OH)D and 1, $25(OH)_2D$ were dissolved in ethanol at 2 mM, 10 mM, 1 μM and 10 μM, respectively.

Histology and immunohistochemistry

Immediately after sacrifice of the host mice, the grafted vaginal tissues and/or the host vagina *in situ* were taken out and fixed in PBS-buffered 10% neutralized formalin. The specimens were embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin. Images of the vaginal sections were recorded using a conventional digital microscope camera attached to a standard microscope (DP-12 and BX40; Olympus Optical, Tokyo, Japan).

Some formalin-fixed paraffin-embedded sections of neonatal mouse vagina were reacted with a rat monoclonal antibody against vitamin D receptor (9A7; BIOMOL Research Laboratories, Plymouth Meeting, PA, USA) at 5 μg/ml or control rat Ig after rehydration, and the specific binding was visualized with a mouse-absorbed biotinylated rabbit anti-rat IgG and a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's protocol.

mRNA analysis by RT-PCR

Conventional qualitative reverse transcription and PCR were performed as described previously [11]. Briefly, extraction of total RNA from the neonatal mouse vagina and random hexamer-primed synthesis of the complementary DNA were performed. The cDNAs of the *Tff1*, *Cyp2r1*, *Cyp27b1* and *Gapdh* genes were amplified by conventional PCR with 28 cycles (the former 3 genes) or 18 cycles (the latter one), and visualized with ethidium bromide staining, basically following the methods described previously [11, 12] and using a new set of gene-specific primers for *Cyp2r1* and *Cyp27b1* : mCYP2R1-FW, 5'-tttagatcttggaggcatatcaac-3'; mCYP2R1-RV, 5'-ctaccatatctggaattgagtaagc-3'; mCYP27B1-FW, 5'-ctatgtcactatgc-cacttcaagg-3'; and mCYP27B1-RV, 5'-ctacaaactgtagattgatgctcc-3'.

Results

Permanent neonatal E_2 exposure-induced changes in the vaginal epithelium in C3H mice in vivo and ex vivo

The C3H strain of mice was used in the present study instead of SHN mice, which used in our previous studies, as the latter mice, which were infected with murine mammary tumor virus (MTV), were difficult to keep in our present facility. So, we started by examining effects of exposure to estrogen, E_2 in this case, on the neonatal C3H-MTV$^-$ mouse vagina *in vivo* and *ex vivo*. First, newborn pups were injected with E_2, and the vagina was then examined in adulthood after the ovariectomy. As expected, the vaginal epithelium was a thick stratified squamous one that consisted of several layers of cells and a keratinized luminal surface in neonatally estrogenized mice, while 2-3 layers of cuboidal cells were found in vehicle-treated controls. Thus, a typical permanent effect of neonatal exposure to estrogen on the vaginal development was confirmed in female C3H mice *in vivo* (data not shown).

Next, effects of E_2 on the developing vagina were examined *ex vivo*. After transplantation in the ovariectomized host for 35 days, the

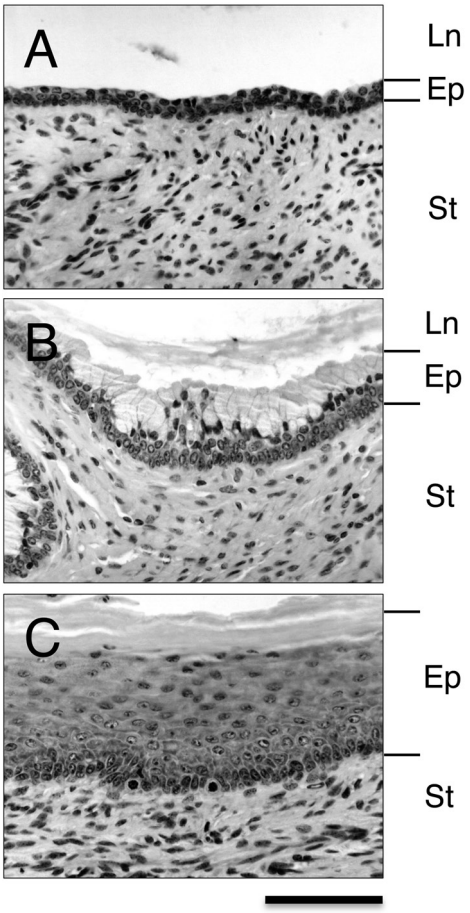

Fig. 1. Effects of E_2 on the development of the neonatal mouse vagina *ex vivo*. The neonatal mouse vagina was exposed to E_2 *ex vivo* and incubated in ovariectomized hosts. Tissue sections were exposed to the basal medium alone (A) or E_2 (B and C) and stained with hematoxylin and eosin. The bar indicates 50 μm. Ln, lumen; Ep, epithelium; St, stroma.

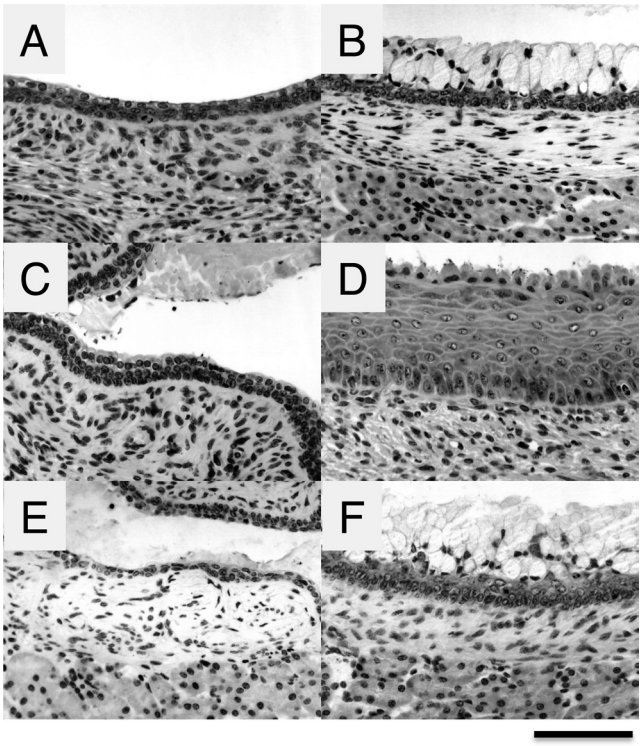

Fig. 2. Influence of VD_3 on the E_2-exposed neonatal vagina *ex vivo*. The neonatal mouse vagina was exposed to E_2 *ex vivo* in the presence of vitamin D and incubated in ovariectomized hosts. Tissue sections were stained with hematoxylin and eosin. The vagina was treated with E_2 with $1,25(OH)_2D$ at 1 nM (A) or at 0.1 nM (B) or with $1,25(OH)_2D$ alone at 100 nM (C), and exogenous E_2 was then injected into the host 2 days before fixation of the transplanted tissue (D). The neonatal mouse vagina was treated with E_2 plus 25(OH)D at 1 μM (E) or cholecalciferol at 10 μM (F). Bar indicates 50 μm.

luminal surface of the vagina was mostly lined with tall mucinous cells (Fig. 1B) and/or a multilayered stratified squamous epithelium (Fig. 1C) in all (7/7; in 7 out 7 cases) of the E_2-exposed vaginal tissues, while a thin epithelium consisted from 2-3 layers of cuboidal cells was observed in the control transplants (4/4) (Fig. 1A). The mucinous cells were also observed preceding estrogen-independent hyperplasia in our previous study on SHN mice [11] and considered to be an earlier symptom of the permanent changes induced by developmental estrogenization in the mouse vagina. In fact, after 70 days of ectopic culture under the renal capsule, a thick stratified squamous epithelium was observed in all specimens examined (3/3).

Influence of VD_3 on the estrogen effect on the neonatal vagina ex vivo

First, $1,25(OH)_2D$ was added together with E_2 to the culture media to see if the activated form of the vitamin has an influence on vaginal development. Interestingly, in the presence of 1 nM or a higher concentration of $1,25(OH)_2D$, E_2 failed to induce any symptom of permanent changes in the vagina, and the vaginal epithelium appeared just like that in the vagina without E_2-exposure (Fig. 2A). At 0.1 nM, on the other hand, $1,25(OH)_2D$ did not affect the E_2 effects, and a thick mucinous epithelium was observed in the ectopic vaginal implant (Fig. 2B). Addition of $1,25(OH)_2D$ alone even at a high dose (100 nM) did not appear to influence vaginal development, and the transplants had a thin layer of epithelium (Fig. 2C). The activated vitamin D-exposed epithelium became thick in response to the exogenous estrogen injected into the host mice just 2 days before excision of the transplanted tissue (Fig. 2D), and therefore responsivity to estrogen in adulthood was not influenced by neonatal exposure to $1,25(OH)_2D$.

Whether or not cholecalciferol and 25(OH)D affect the E_2 action that leads to a permanent change in the developing vagina was also investigated. In the presence of cholecalciferol, at least at the dose examined (up to 10 μM), E_2 altered the fate of the developing vagina and induced ovary-independent hyperplasia of the epithelium (Fig. 2F). Interestingly, on the other hand, a high dose (1 μM) of 25(OH)D did attenuate development of an abnormal vaginal epithelium induced by E_2 (Fig. 2E).

Expression of 25(OH)D 1α-hydroxylase in the neonatal mouse vagina

The fact that 25(OH)D acted like 1,25$(OH)_2$D in the isolated neonatal vagina suggested that activation of the pro-activated vitamin into the activated form occurred in the organ. The 1α-hydroxylation of 25(OH)D is catalyzed by Cyp27b1 which is known as a renal-specific enzyme in mice. So, amplification of *Cyp27b1* gene mRNA by RT-PCR was performed in the neonatal vagina to clarify if the tissue expresses the gene. The results shown in Fig. 3 indicate that the neonatal vagina expresses Cyp27b1 mRNA.

Tff1 expression in the E_2- and/or 1,25$(OH)_2$D-exposed vaginal transplants

Tff1 is a molecular marker for the permanently modified epithelium in the neonatally E_2-exposed mouse vagina [11]. To confirm from the viewpoint of gene expression that 1,25$(OH)_2$D attenuated E_2-action in the developing vagina, expression of Tff1 gene mRNA was examined in the E_2- and/or 1,25$(OH)_2$D-exposed transplants by RT-PCR. As shown in Fig. 4, Tff1 mRNA was detectable in the E_2-exposed vaginal transplants after 35 days of incubation under the kidney capsule in the host mice but not in tissue exposed to both E_2 and 1,25$(OH)_2$D. The *Tff1* gene was not induced in the latter tissue even when the host mice were injected with E_2 so that the vaginal epithelium would proliferate and form thick stratified squamous cell layers.

Distribution of VDR in the neonatal mouse vagina

The results above indicated that vaginal tissue from neonatal mice responded to activated vitamin D_3, which suggests existence of the receptor for the vitamin, VDR. To clarify which type(s) of vaginal cells is responsible for the vitamin D_3 action, the distribution of VDR in the neonatal mouse vagina was examined by immunohistochemistry (Fig. 5). As a result, almost all cell nuclei were found to be immunoreactive for VDR. The signaling in the epithelium appeared to be enhanced by E_2 exposure (Fig. 5C).

Attenuation of the permanent estrogen effect on neonatal vagina in vivo by the activated form of VD_3

The influence of 1,25$(OH)_2$D on the development of DES syndrome was examined in neonatal mice *in vivo*. When 1 pmole of 1,25$(OH)_2$D was injected simultaneously with DES daily for 4 days in the neonatal mice, many (5/8) of the pups died for unidentified reasons within several days after the first injection. The rest of the pups, however, grew up to be young adults without any abnormalities as far as we knew based on our daily conventional observations, such as monitoring of weight and suckling behavior. Their vaginas showed no symptoms of the permanent changes, and the epithelium consisted of 2-3 layers of cuboidal epithelial cells after ovariectomy (Fig. 6C). The lower dose (0.1 pmole) of 1,25$(OH)_2$D showed neither lethal effects on the neonates nor an inhibitory influence on the neonatal E_2 effects (Fig. 6D). Thus, a sublethal level of activated vitamin D_3 attenuated the direct and permanent effects of estrogen on the neonatal mouse vagina.

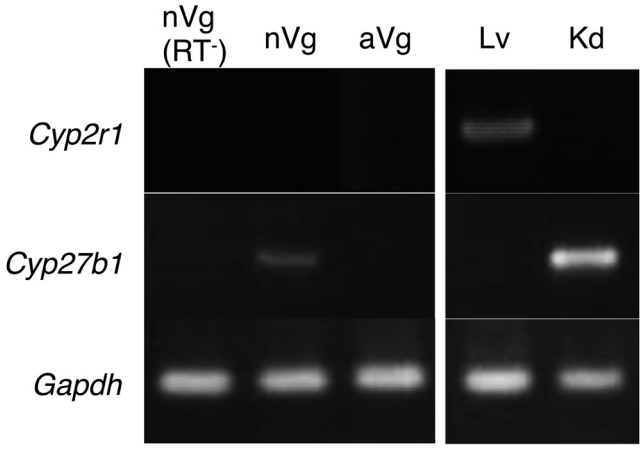

Fig. 3. Expression of vitamin D-activating enzymes in the mouse vagina. Expression of mRNA for the *Cyp2r1* and *Cyp27b1* genes was examined by RT-PCR in the neonatal and adult mouse vagina (nVg and aVg, respectively). The *Gapdh* gene served as the internal control, and the liver (Lv) and kidney (Kd) was served as the positive controls. A sample without reverse transcription (RT^-) was used as the negative control to exclude possible contamination of genomic DNA in the cDNA sample.

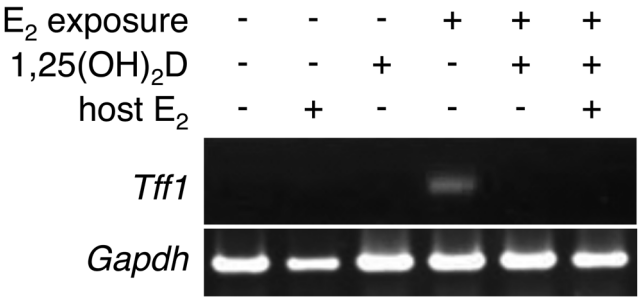

Fig.4. *Tff1* gene expression in the E_2- and/or 1,25$(OH)_2$D-exposed vaginal transplants. Expression of Tff1 gene mRNA was examined in the E_2- and/or 1,25$(OH)_2$D-exposed neonatal mouse vagina by RT-PCR after 35 days of incubation under the kidney capsule in the ovariectomized host mice. Some host mice were treated with E_2 before tissue examination (host E_2). Quite similar results were obtained from three different sets of experiments.

Discussion

The main finding of the present study is that activated vitamin D is an effective modulator of estrogen action and attenuates the E_2 effects that make the neonatal mouse vagina prone to the permanent alterations in the later developmental program of the epithelium. Our preliminary experiments *in vivo* failed to find an appropriate dose of vitamin D to explore its effects on the neonates, and we gave up exploring further the influence of vitamin D on mouse vaginal development (data not shown). The present study suggests that our previous frustration came partly from the narrow range for an effective dose of activated vitamin D between a lethally toxic overdose and a noneffective underdose. Establishing an *ex vivo*

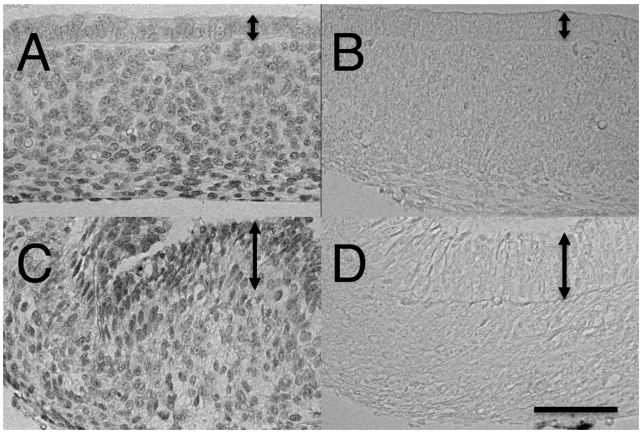

Fig. 5. Distribution of VDR in the neonatal mouse vagina. The localization of VDR protein was examined by immunohistochemistry in the neonatal mouse vagina. The vagina tissue sections from an intact mouse (A, B) and those from an estrogen-treated newborn (C, D) at day 3 postpartum were immunostained for anti-VDR antibody (A, C) or control rat Ig (B, D). Note that cell nuclei in the epithelium and stroma were specifically immunostained. The arrows indicate the epithelium. The bar indicates 50 μm.

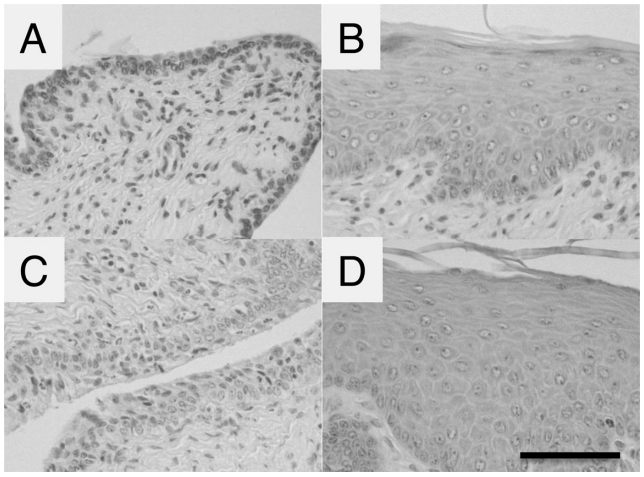

Fig. 6. Influence of $1,25(OH)_2D$ on the neonatal DES-induced changes in vaginal development *in vivo*. $1,25(OH)_2D$ was injected simultaneously with DES daily for 4 days in the neonatal mice, and the vagina was examined at young adulthood after ovariectomy. (A) vehicle-treated control, (B) neonatally DES-treated control, (C) DES with 1 pmole of $1,25(OH)_2D$, (D) DES with 0.1 pmole of $1,25(OH)_2D$. The bar indicates 50 μm.

system, by modifying the method of Kimura *et al.* [20], enabled us to examine the effect of chemicals that are toxic if applied *in vivo*. The effective dose of $1,25(OH)_2D$ *ex vivo* was found to be 1 nM in the present study. The dose was 3–10 times higher than the normal blood levels in mice and humans (approximately 30–100 pg/ml) [21, 22]. Taking account of this information regarding the effective dose, 1 pmole per approximately 1 g of body weight was employed to examine the influence of $1,25(OH)_2D$ *in vivo*, and the dose successfully inhibited the effect of DES that results in permanent changes in the developmental program of the vaginal epithelium. Surprisingly, the dose of $1,25(OH)_2D$ employed in the present study was sublethal to the neonates, although it was much lower than the LD50 reported in the adult rodents (ranging from 0.5 to 5 mg/kg body weight) [21]. By using the present experimental system *ex vivo*, it was revealed that VD_3 directly acted on the developing vagina and attenuated the effects of estrogen. Although the effective dose of activated VD_3 is sublethal *in vivo*, manipulation of local vitamin D signaling may contribute to finding a way of attenuating the developmental effects induced by unexpected exposure to a high dose of estrogens during the perinatal period. In addition, this *ex vivo* system is readily applicable to the examination of effects of the other (sub-)lethal substances such as miscellaneous inhibitors or activators of inter- and intracellular signaling, which is expected to contribute to understanding of the mechanisms by which estrogens mislead the neonatal vagina into abnormal development.

The mechanisms by which activated vitamin D influences the developing mouse vagina might be beyond the scope of the present study. Nevertheless, the distribution of VDR shown in Fig. 5 suggests that vitamin D could act through VDR [23] upon both the epithelium and stroma in the developing mouse vagina. Considering the distribution of estrogen receptors (ERs) in the developing vagina [24] as well, vitamin D/VDR signaling may crosstalk and interfere with estrogen/ERs signaling within a cell or those of different cells through other peripheral cell signaling factors that mediate and/or influence on the estrogen effects in the genital tracts [25–27]. For example, vitamin D/VDR may inhibit KGF/KGFR signaling that is essential for estrogen to evoke permanent changes in the developing vagina [13, 19, 28]. It is an interesting fact that both vitamin A and D attenuated estrogen effects. Both VDR and the retinoic acid receptor (RAR) belong to a subgroup of nuclear receptors that bind with RXR to form "nonpermissive" heterodimers [29]. Specific intranuclear environments governed by these transcriptional factors might be required for overdosed estrogens to act on the developing vaginal epithelium. In any case, further studies are required to explore mechanisms by which VDR signaling influences estrogen receptor (ER) signaling in the developing mouse vagina.

Another interesting finding of the present study is the action of 25(OH)D on the developing vagina and corresponding expression of Cyp27b1 in the organ. This result was surprising because expression of the *Cyp27b1* gene is usually restricted to the kidney and brain in mice, although it is more broadly expressed in humans [30]. Cyp27b1 has been identified as the sole 25(OH)D 1α-hydroxylase in many species, including the mouse and human. Taken together, vagina-borne Cyp27b1 catalyzed 1α hydroxylation of 25(OH)D to produce $1,25(OH)_2D$, which appeared to be responsible for the attenuation of the estrogen effects in the neonatal mouse vagina. On the other hand, cholecalciferol did not have any influence on the E_2 effect, and consistent expression of the *Cyp2r1* gene, a liver- and bone-specific enzyme in mice, was not detectable in the vagina. Although recent analysis of *Cyp2r1* gene-deficient mice has revealed that Cyp2r1 is not an exclusive enzyme responsible for 25(OH)D production [31], it seems clear that the developing vagina does not produce enough $25(OH)VD_3$ to interfere with the exogenous overdosed E_2 effects.

Estrogen exposure during the neonatal period leads to ovary-independent persistent proliferation and cornification in the vaginal epithelium in mice. In the *ex vivo* system used in the present study, development of a hyperplastic stratified squamous epithelium was delayed when compared with the case *in vivo*. Instead, unusual development of mucinous cells accompanied by *Tff1* gene expression was consistently observed, which has previously been identified as a definitive sign of the permanently modified vaginal epithelium induced by estrogen. The delay of the development might come from absence of the ovary *in situ* (as *ex vivo* cultured tissue was transplanted into an ovariectomized host) and therefore absence of estrogen that stimulates vaginal hyperplasia. In neonatal estrogenized mice in particular, changes in the brain lead to ovary-dependent estrogenic stimulation of the vagina after puberty. Ovarian estrogen is not essential for the development of permanent hyperplasia in the neonatally estrogenized vagina but may contribute to the accelerated integration of development of an abnormally hyperplastic vaginal epithelium.

In conclusion, activated vitamin D directly acted on the neonatal mouse vagina and attenuated estrogen-induced permanent changes in the developmental program of the organ.

Acknowledgments

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References

1. **Takasugi N.** Cytological basis for permanent vaginal changes in mice treated neonatally with steroid hormones. *Int Rev Cytol* 1976; **44**: 193–224. [Medline] [CrossRef]
2. **Herbst AL, Bern HA., editors.** Developmental effects of diethylstilbestrol (DES) in pregnancy. New York: Thieme Stratton Inc.; 1981.
3. **Mori T, Nagasawa H., editors.** Toxicity of hormones in perinatal life. Boca Raton FL: CRC Press; 1988.
4. **McLachlan JA.** Environmental signaling: what embryos and evolution teach us about endocrine disrupting chemicals. *Endocr Rev* 2001; **22**: 319–341. [Medline] [CrossRef]
5. **Veurink M, Koster M, Berg LT.** The history of DES, lessons to be learned. *Pharm World Sci* 2005; **27**: 139–143. [Medline] [CrossRef]
6. **Tsutsumi O.** [DES syndrome]. *Nihon Rinsho* 2006; (Suppl 2): 438–441 (In Japanese). [Medline]
7. **Takasugi N, Bern HA, Deome KB.** Persistent vaginal cornification in mice. *Science* 1962; **138**: 438–439. [Medline] [CrossRef]
8. **Boyd J, Takahashi H, Waggoner SE, Jones LA, Hajek RA, Wharton JT, Liu FS, Fujino T, Barrett JC, McLachlan JA.** Molecular genetic analysis of clear cell adenocarcinomas of the vagina and cervix associated and unassociated with diethylstilbestrol exposure in utero. *Cancer* 1996; **77**: 507–513. [Medline] [CrossRef]
9. **Sato T, Ohta Y, Okamura H, Hayashi S, Iguchi T.** Estrogen receptor (ER) and its messenger ribonucleic acid expression in the genital tract of female mice exposed neonatally to tamoxifen and diethylstilbestrol. *Anat Rec* 1996; **244**: 374–385. [Medline] [CrossRef]
10. **Cunha GR, Lung B, Kato K.** Role of the epithelial-stromal interaction during the development and expression of ovary-independent vaginal hyperplasia. *Dev Biol* 1977; **56**: 52–67. [Medline] [CrossRef]
11. **Masui F, Kurosaki K, Mori T, Matsuda M.** Persistent trefoil factor 1 expression imprinted on mouse vaginal epithelium by neonatal estrogenization. *Cell Tissue Res* 2006; **323**: 167–175. [Medline] [CrossRef]
12. **Matsuda M, Masui F, Mori T.** Neonatal estrogenization leads to increased expression of cellular retinol binding protein 2 in the mouse reproductive tract. *Cell Tissue Res* 2004; **316**: 131–139. [Medline] [CrossRef]
13. **Masui F, Matsuda M, Mori T.** Involvement of keratinocyte growth factor (KGF)-KGF receptor signaling in developmental estrogenization syndrome of mouse vagina. *Cell Tissue Res* 2004; **318**: 591–598. [Medline] [CrossRef]
14. **Wang Y, Zhu J, DeLuca HF.** Where is the vitamin D receptor? *Arch Biochem Biophys* 2012; **523**: 123–133. [Medline] [CrossRef]
15. **Vanhooke JL, Prahl JM, Kimmel-Jehan C, Mendelsohn M, Danielson EW, Healy KD, DeLuca HF.** CYP27B1 null mice with LacZreporter gene display no 25-hydroxyvitamin D3-1α-hydroxylase promoter activity in the skin. *Proc Natl Acad Sci USA* 2006; **103**: 75–80. [Medline] [CrossRef]
16. **Adams JS, Hewison M.** Extrarenal expression of the 25-hydroxyvitamin D-1-hydroxylase. *Arch Biochem Biophys* 2012; **523**: 95–102. [Medline] [CrossRef]
17. **Crescioli C, Maggie M, Vannelli GB, Luconi M, Salerno R, Barni T, Gulisano M, Forti G, Serio M.** Effect of a vitamin D3 analogue on keratinocyte growth factor-induced cell proliferation in benign prostate hyperplasia. *J Clin Endocrinol Metab* 2000; **85**: 2576–2583. [Medline]
18. **Marchiani S, Bonaccorsi L, Ferruzzi P, Crescioli C, Muratori M, Adorini L, Forti G, Maggi M, Baldi E.** The vitamin D analogue BXL-628 inhibits growth factor-stimulated proliferation and invasion of DU145 prostate cancer cells. *J Cancer Res Clin Oncol* 2006; **132**: 408–416. [Medline] [CrossRef]
19. **Hom YK, Young P, Thomson AA, Cunha GR.** Keratinocyte growth factor injected into female mouse neonates stimulates uterine and vaginal epithelial growth. *Endocrinology* 1998; **139**: 3772–3779. [Medline]
20. **Kimura T, Basu SL, Nandi S.** Nature of induced persistent vaginal cornification in mice. 3. Effects of estradiol and testosterone on vaginal epithelium in vitro. *J Exp Zool* 1967; **165**: 497–503. [Medline] [CrossRef]
21. **Milne GWA, Delander M.** Vitamin D Handbook: Structure, Synonyms, and Properties. New Jersey: Jhon Wiley & Sons; 2008: 1–235.
22. **Shimada T, Kakitani M, Yamazaki Y, Hasegawa H, Takeuchi Y, Fujita T, Fukumoto S, Tomizuka K, Yamashita T.** Targeted ablation of Fgf23 demonstrates an essential physiological role of FGF23 in phosphate and vitamin D metabolism. *J Clin Invest* 2004; **113**: 561–568. [Medline] [CrossRef]
23. **Abban G, Yildirim NB, Jetten AM.** Regulation of the vitamin D receptor and cornifin beta expression in vaginal epithelium of the rats through vitamin D3. *Eur J Histochem* 2008; **52**: 107–114. [Medline]
24. **Sato T, Okamura H, Ohta Y, Hayashi S, Takamatsu Y, Takasugi N, Iguchi T.** Estrogen receptor expression in the genital tract of female mice treated neonatally with diethylstilbestrol. *In Vivo* 1992; **6**: 151–156. [Medline]
25. **Tsuchiya Y, Saito Y, Taniuchi S, Sakuma A, Maekawa T, Fukamachi H, Takeuchi S, Takahashi S.** Runx3 expression and its roles in mouse endometrial cells. *J Reprod Dev* 2012; **58**: 592–598. [Medline] [CrossRef]
26. **Cunha GR, Cooke PS, Kurita T.** Role of stromal-epithelial interactions in hormonal responses. *Arch Histol Cytol* 2004; **67**: 417–434. [Medline] [CrossRef]
27. **Manabe Y, Tochigi M, Moriwaki A, Takeuchi S, Takahashi S.** Insulin-like growth factor 1 mRNA expression in the uterus of streptozotocin-treated diabetic mice. *J Reprod Dev* 2013; **59**: 398–404. [Medline] [CrossRef]
28. **Masui F, Matsuda M, Mori T.** Vitamin A prevents the irreversible proliferation of vaginal epithelium induced by neonatal injection of keratinocyte growth factor in mice. *Cell Tissue Res* 2003; **311**: 251–258. [Medline]
29. **Pérez E, Bourguet W, Gronemeyer H, de Lera AR.** Modulation of RXR function through ligand design. *Biochim Biophys Acta - Molecular and Cell Biology of Lipids* 2012; 1821: 57–69.
30. **Henry HL.** The 25-hydroxyvitamin D 1α-hydroxylase. In: Feldman D, Pike JW, Glorieux FH (eds.). Vitamin D. 2nd Ed, San Diego, CA: Elsevier Academic Press; 2013: 69–83.
31. **Zhu JG, Ochalek JT, Kaufmann M, Jones G, Deluca HF.** CYP2R1 is a major, but not exclusive, contributor to 25-hydroxyvitamin D production *in vivo*. *Proc Natl Acad Sci USA* 2013; **110**: 15650–15655. [Medline] [CrossRef]