Retinoic acid signaling determines the fate of uterine stroma in the mouse Müllerian duct

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The Müllerian duct develops into the oviduct, uterus, and vagina, all of which are quite distinct in their morphology and function. The epithelial fate of these female reproductive organs in developing mice is determined by factors secreted from the stroma; however, how stromal differentiation occurs in the female reproductive organs derived from the Müllerian duct is still unclear. In the present study, roles of retinoic acid (RA) signaling in developing female reproductive tracts were investigated. Retinoaldehyde dehydrogenase 10 (RDH10) and aldehyde dehydrogenase family 1 subfamily A2 (ALDH1A2) mRNAs and proteins and transactivation activity of endogenous RA were found in the stroma of proximal Müllerian ducts and gradually decreased from the proximal to caudal regions in fetal mice. In organ-cultured Müllerian ducts, retinaldehyde or RA treatment induced uterine epithelial differentiation, defined as a layer of columnar epithelial cells negative for oviductal and vaginal epithelial markers. In contrast, inhibition of RA receptor (RAR) signaling induced vaginal epithelial differentiation, characterized as vaginal epithelial marker genes–positive stratified epithelium. Grafting experiments of the organ-cultured Müllerian duct revealed irreversible epithelial fate determination. Although RAR did not directly bind to the homeobox A10 (Hoxa10) promoter region, RA–RAR signaling stimulated Hoxa10 expression. Thus, RA–RAR signaling in the Müllerian duct determines the fate of stroma to form the future uterus and vagina.

Müllerian duct | oviduct | uterus | vagina | retinoic acid

In female mice, Müllerian ducts remain and differentiate into oviducts, uterus, and vagina; however, they are morphologically and functionally distinct. The oviducal epithelium consists of ciliated cells and secretory cells (1), whereas the uterine epithelium is composed of simple columnar luminal and glandular epithelia (2). The vaginal epithelium develops into a stratified cuboidal epithelium (3, 4). Tissue recombination experiments between the epithelium and stroma have been performed previously to investigate the mechanisms underlying differentiation of the epithelia from the Müllerian ducts (3–5). Data from these studies indicate that factors secreted from the stroma determine the fate, differentiation, and growth of the overlying epithelia into the oviduct, uterus, and vagina of developing mice. The epithelial fate-determining factors have been identified in the female reproductive tracts (4, 6–9), and posterior Hoxa genes, which are differentially expressed along the A–P axis, are important for development of the female reproductive tracts (10); however, earlier steps in the stromal differentiation of the female reproductive tracts from the Müllerian ducts remain unclear.

DNA microarray analysis (11, 12) revealed that the expression of several genes involved in retinoic acid (RA) signaling is higher in the uterus of neonatal mice than that in the vagina. In the first step of RA synthesis, retinol is oxidized to retinaldehyde by alcohol dehydrogenases (ADHs) and retinol dehydrogenases (RDHs), mainly including RDH11. 10 and dehydrogenase/reductase (SDR family) member 9 (DHRS9) (13–15). RA oxidized from retinaldehyde by aldehyde dehydrogenase family 1 subfamily A (ALDH1A1s) binds to retinoic acid receptors (RARs). RARs form obligate heterodimers with the retinoid X receptors (RXRs). The RAR/RXR heterodimer binds to RA-responsive elements (RARE) and regulates transcription of the target genes (16). Degradation of RA is promoted by cytochrome P450 26A1 (CYP26A1), CYP26B1, and CYP26C1 (17–19). In adult mice, Dhrs9, Aldh1a1, Cyp26, and Aldh1a2 are expressed in the uterus (20–22), whereas Aldh1a1, Aldh1a2, and Cyp26 are not detected in the vagina (20). These data suggest that RA signaling may act in the adult uterus.

The necessity of vitamin A and its derivatives in Müllerian duct development was first observed when fetuses from pregnant rat dams reared on vitamin A-deficient diets exhibited agenesis or incomplete development of the Müllerian ducts (23). Müllerian ducts in Rara−/− or Rarb−/− or Rara−/−/Rarb−/− mice and the caudal Müllerian ducts of Rara−/−/Rarb−/− mice were absent at embryonic day 12.5 (E12.5) and E13.5 (24, 25), suggesting that RA signaling through RARs, particularly RARα, is essential for Müllerian duct development. Feeding vitamin A-deficient diets for 10 or 14 wk from birth causes squamous metaplasia in the uterine epithelium (26–28), suggesting a role for RA signaling in maintaining the uterine epithelium. However, the role of RA signaling in the differentiation of the female reproductive tracts from the Müllerian duct has not been elucidated.

In the present study, we investigated the role of RA signaling in the developing mouse female reproductive tracts. First, ontogenic mRNA and protein expression of the RA-signaling pathway components together with the transactivation activity of endogenous RA in the female reproductive tracts from embryonic to adult stages were examined. To study the roles of RA signaling in developing female reproductive tracts, effects of RA or RAR antagonists on organ-cultured female reproductive tracts were investigated. Finally, RAR binding to Hoxa gene promoter was examined to determine whether Hoxa genes were candidates for downstream genes of RA signaling.

Results

Developmental mRNA and Protein Expression of the RA-Synthesizing Enzymes, RARs, and RA-Metabolizing Enzymes in Female Reproductive Tracts. Expression of components of the RA-signaling pathway components in the neonatal uterus and vagina was investigated by RT-PCR. Rdh10 was expressed in the uterus and vagina at 14.5 days postcoitum (dpc), whereas Rdh10 was not expressed in the vagina at 14.5 dpc. However, Rdh10 was expressed in the vagina at 16.5 dpc. In adult mice, Rdh10 was expressed in the uterus and vagina at 8 weeks postcoitum (wpc), whereas Rdh10 was not expressed in the vagina at 8 wpc. In adult mice, Rdh10 was expressed in the uterus and vagina at 12 weeks postcoitum (wpc), whereas Rdh10 was not expressed in the vagina at 12 wpc. In adult mice, Rdh10 was expressed in the uterus and vagina at 16 weeks postcoitum (wpc), whereas Rdh10 was not expressed in the vagina at 16 wpc. In adult mice, Rdh10 was expressed in the uterus and vagina at 20 weeks postcoitum (wpc), whereas Rdh10 was not expressed in the vagina at 20 wpc.

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Significance

Oviduct, uterus, and vagina are all derived from the Müllerian duct. Epithelial fate of these female reproductive organs in developing mice is determined by factors secreted from the stroma; however, how the preceding stromal differentiation of female reproductive tracts from the Müllerian duct occurs is still unclear. This study showed that retinoic acid (RA) signaling was activated in the proximal Müllerian duct, which develops into oviduct and uterus. Furthermore, RA treatment induced uterine stromal differentiation, whereas inhibition of RA receptor signaling induced vaginal stromal differentiation. Therefore, we concluded that RA establishes a border between the stroma of uterus and vagina.

Author contributions: T.N. and T.S. designed research; T.N. performed research; T.N. and T.S. analyzed data; and T.N., T.I., and T.S. wrote the paper.

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postnatal day 2 (P2) and P15, and Dhrs9 was localized in the uterine and vaginal epithelium at P2 and P15, and in the vaginal stroma at P15. Rdh10 and Rdh7 were not detected (Fig. 1A); therefore, the primary producers of retinaldehyde in the uterus and vagina are probably RDH10 and DHR9. Aldh1a1 was not expressed at P2 and mainly expressed in the uterine epithelium at P15. Aldh1a2 was expressed in the uterine stroma, vaginal epithelium, and vaginal stroma at P2 but only in the uterine stroma at P15. Aldh1a3 was not detected. Rara, Rarg, Ruen, and Rurb were expressed in the uterus and vagina at P2 and P15, but Rarb and Rorg were not detected. Cyp26a1 was localized in the uterine epithelium at P15, whereas Cyp26b1 was not detected in the uterus and vagina. RDH10 protein expression in the uterus at P2 was higher than that in the vagina and in the uterus at P15 and P90 (Fig. 1B). ALDH1A2 expression was similar between the uterus and vagina at P2 and P15. A single injection of 17β-estradiol (E2) increased expression of ALDH1A2, but not RDH10 in the uterus and vagina at P90. We used quantitative PCR (qPCR) to characterize temporal changes in gene expression of the RA-synthesizing enzymes, RARs and RA-metabolizing enzymes in female reproductive tracts after birth. The mRNA expression in the uterine epithelia (UtE), uterine stroma (UtS), vaginal epithelia (VgE), and vaginal stroma (VgS) of P2 and P15 mice was analyzed by RT-PCR (A). The expression of RDH10 and ALDH1A2 proteins in the uterus and vagina of P2 and P15 mice and oil- or E2-treated P90 mice was analyzed by Western blot (B).

**Fig. 1.** mRNA and protein expression and localization of the RA-synthesizing enzymes, RARs and RA-metabolizing enzymes in female reproductive tracts after birth. The mRNA expression in the uterine epithelia (UtE), uterine stroma (UtS), vaginal epithelia (VgE), and vaginal stroma (VgS) of P2 and P15 mice was analyzed by RT-PCR (A). The expression of RDH10 and ALDH1A2 proteins in the uterus and vagina of P2 and P15 mice and oil- or E2-treated P90 mice was analyzed by Western blot (B).

expression in the uterus and vagina did not show a marked change with age (Fig. S1). Rarb expression in the uterus and vagina decreased with age. Thus, Rara and Rarg were expressed in both the uterus and vagina throughout the ages examined. Because expression of Rdh10 and Aldh1a2 was high in the middle Müllerian ducts at E17.5 (Fig. 2), the expression pattern in the Müllerian ducts at E14.5 and E17.5 was examined (Fig. 3A). Rdh10 expression was high in the proximal and middle Müllerian ducts, and Cyp26a1 was high in the proximal region at E17.5. Dhrs9, Aldh1a1, Aldh1a2, Rara, Rarb, and Rang expression was similar, from the proximal to caudal regions. By immunohistochemistry, slight ALDH1A2 protein expression was detected as brown staining in the stroma of proximal Müllerian duct at E14.5, but not in the middle and caudal Müllerian duct (Fig. 3B–D). RDH10 protein was not detected in the female reproductive tracts from E14.5 to P90 by immunohistochemistry (Fig. S2). These results indicated that RDH10 and ALDH1A2 mRNAs and proteins were expressed in the stroma of proximal Müllerian duct and decreased from the proximal to the caudal Müllerian duct.
Effects of RA or RAR Antagonists on Stromal Differentiation in the Organ-Cultured Müllerian Ducts. Because no marker gene for stromal differentiation in Müllerian ducts has yet been identified, expression of stroma-induced epithelial markers (4, 5) was taken as an indication of stromal differentiation. Organ-cultured E14.5 Müllerian ducts, including proximal and middle regions treated with RA, displayed no overt changes, whereas epithelia treated with AGN differentiated into a vagina-like stratified epithelium, accompanied by expression of the vaginal epithelial marker transformation related protein 63 (TRP63) (Fig. S5 K and L). However, the differentiated vaginal epithelial marker, keratin 14 (KRT14), was not detected in the AGN-treated epithelia, suggesting that differentiation into a vaginal epithelium was incomplete (Fig. S5 M–P). At E17.5, the middle Müllerian duct treated with retinaldehyde or RA did not show apparent changes (Fig. 5 B and F and Fig. S7 B and F), whereas caudal epithelium treated with retinaldehyde or RA differentiated into TRP63+ and KRT14+ vagina-like stratified epithelia (Fig. 5 N and R and Fig. S7 N and R). AGN treatment induced TRP63+ and KRT14+ vagina-like stratified epithelia in middle Müllerian ducts, and caudal epithelia treated with AGN expressed KRT14.

Effects of RA or an RAR Antagonist on Activation of RA Signaling in Organ-Cultured Müllerian Ducts. To investigate the role of RA signaling in the Müllerian duct stroma, RA and/or RAR antagonists were added to the medium of organ-cultured Müllerian ducts of RARE-lacZ mice at E14.5 and E17.5. Absence of androgen receptor (AR)-expressing Wolffian ducts (Fig. S4 A and B) and paired box 2 (PAX2)− epithelium of the urogenital sinus in the organ-cultured Müllerian ducts (Fig. S4 C–N) were confirmed by immunohistochemistry, except for PAX2+ partially stratified epithelium of caudal Müllerian duct (Fig. S4 K and N). As expected, RA treatment stimulated RA signaling in the stroma of E14.5 and E17.5 Müllerian ducts organ cultured for 8 d (Figs. S5 A–J and S6). In contrast, treatment with the pan-RAR antagonist, AGN193109 (AGN), inhibited RA signaling in the stroma of the Müllerian duct in the presence or absence of RA, whereas AGN treatment alone partially increased RA signaling in the epithelium of caudal Müllerian ducts (Fig. S6P).

Transactivation Activity of Endogenous RA in the Female Reproductive Tracts. Endogenous levels of RA have not previously been measured in female reproductive tracts. Expression of well-known RA-responsive genes was examined in the uterus and vagina (Cyp26a1 in Fig. 2; Rarb in Fig. S1; others in Fig. S3); however, the expression of those genes was inconsistent. Thus, we used a reporter mouse expressing lacZ under the control of a strong RARE (29) to investigate the temporal and spatial distribution of active RA signaling in the Müllerian duct. The β-galactosidase activity was histochemically detected from E14.5 to P15 (Fig. 4). In the stroma of proximal and middle Müllerian duct, β-galactosidase activity was highly detected from E14.5 to P0, slightly detected at P2, and not detected by P15 (Fig. 4 A–E). From E17.5 to P2, β-galactosidase activity in the proximal Müllerian duct (Fig. 4 G–I) was higher than that in the middle portion (Fig. 4 K–M). In the caudal Müllerian duct and vagina, β-galactosidase activity was not detected (Fig. 4 N–Q). Thus, the levels of biologically active, endogenous RA were highest in the stroma of proximal Müllerian ducts and gradually decreased from the proximal to caudal regions.

Fig. 3. mRNA and protein expression and localization of the RA-synthesizing enzymes, RARs, and RA-metabolizing enzymes in female reproductive tracts of fetal mice. The mRNA expression in the proximal and middle Müllerian duct of E14.5 mice, and the proximal, middle, and caudal Müllerian duct of E17.5 mice was analyzed by qPCR (A). Data were expressed relative to mRNA expression of whole Müllerian ducts of E14.5 mice (n = 10). * P < 0.05. The protein localization of ALDH1A2 in the proximal (B), middle (C), and caudal (D) Müllerian ducts of E14.5 mice and in the uterus of P90 mice as a positive control (E) was analyzed by immunohistochemistry (n = 5). (Scale bar, 50 μm.) Dashed line, basement membrane; e, epithelium; s, stroma.

Fig. 4. Transactivation activity of endogenous RA in the female reproductive tracts. β-Galactosidase activity in whole female reproductive tracts (A–E) and the sagittal section of female reproductive tracts (F–Q) of E14.5 (A, F, J, and N), E17.5 (B, G, K, and O), P0 (C, H, L, and P), P2 (D, I, M and Q), and P15 (E) RARE-lacZ mice was analyzed by X-gal staining (n = 3). (Scale bar, 1 mm (A and B), 2 mm (C and D), 3 mm (E), and 50 μm (F–Q). Solid arrows, predicted area of oviducts at E14.5 and E17.5 or oviducts from P0 to P15; dashed arrows, predicted area of uteri at E14.5 and E17.5 or uteri from P0 to P15; arrowheads, vaginae; dashed line, basement membrane; e, epithelium; s, stroma.)
Müllerian ducts had oviduct-like epithelia consisting of β-tubulin (TUBB) ciliated cells or oviductal glycoprotein 1 (OVGP1) secretory cells (Fig. 6 E–G and Fig. S10 I and J) and uterus-like epithelia (Fig. 6 F). AGN treatment irreversibly induced TRP63+ vagina-like epithelia in host mice, irrespective of E2 treatment (Fig. 6 C and D). KRT14 was expressed in the vagina-like epithelium in E2-treated host mice (Fig. S10 B), but not in oil-treated host mice (Fig. S10 A). PGR was not detected in the vagina-like epithelium in the oil-treated host mice (Fig. S10 E), indicating that the PGR expression pattern was similar to vaginal epithelium (2). These data suggested that AGN treatment irreversibly induced vaginal epithelium.

In E2-treated host mice, grafted AGN-treated Müllerian ducts did not have oviduct-like epithelia (Fig. 6 F and Fig. S10 D). Cotreatment with RA + AGN canceled the effects of AGN on induction of vaginal epithelium (Fig. 6 C and D and Fig. S10 B) and inhibition of oviductal epithelium (Fig. 6 F). RA treatment appeared to decrease the size of the smooth muscle actin (SMA) expression domain in both oil- and E2-treated animals and this effect was blocked by AGN (Fig. S10 K and L). Grafted, organ-cultured E17.5 middle Müllerian ducts at day 30 postgrafting had only uterus-like epithelia (Fig. 6 L). AGN treatment irreversibly induced TRP63+ vagina-like epithelia in the presence or absence of E2 (Fig. 6 J and K). In the vagina-like epithelia, E2 treatment strongly (Fig. 5 D, H, and T). In middle Müllerian ducts, BMS195614 (BMS; RARα antagonist) treatments induced only a few TRP63+ epithelial cells (Fig. S7 C, arrows). In contrast, treatments with LEI35 (LE; RARβ antagonist) or MM11253 (MM; RARγ antagonist) did not appear to affect morphology (Fig. S8). Cotreatment with RA + AGN (Fig. 5 C and G) or RA + BMS (Fig. S7 D) canceled the effects of AGN or BMS, and AGN canceled the effects of RA (Fig. 5 O, S, and W). The organ-cultured P15 uterus treated with RA or AGN showed no morphological changes (Fig. S9 B and G). RA treatment decreased KRT14 expression in the vaginal epithelium, but it did not affect TRP63 expression (Fig. S9 D and J). AGN treatment increased the number of epithelial layers in the vagina, but it did not affect TRP63 and KRT14 expression (Fig. S9 E and J). These data suggested that retinaldehyde or RA treatment induced uterine epithelium in the Müllerian duct via activation of RA signaling in the stroma at the developing stage, whereas inhibition of RAR signaling induced vaginal epithelium.

**Modulating RA Signaling Irreversibly Alters Stromal Differentiation in the Female Reproductive Tracts.** To investigate the role of RA signaling in the differentiation of female reproductive tracts, RA and/or RAR antagonists were added to the medium in organ-cultured Müllerian ducts at E14.5 or E17.5, and those tissues were then grafted under the renal capsules of host mice. Epithelia of the grafted Müllerian ducts expressed estrogen receptor α (ESR1) and progesterone receptor (PGR) (Figs. S10 C–F and S11 C–F and M–P) and showed cell proliferation in response to E2 treatment (Figs. S10 G and H and S11 G, H, Q, and R), suggesting that the grafted Müllerian ducts retained characteristics of the female reproductive tracts. At day 30 postgrafting, the grafted E14.5 Müllerian ducts had oviduct-like epithelia consisting of β-tubulin (TUBB)+ ciliated cells or oviductal glycoprotein 1 (OVGP1)+ secretory cells (Fig. 6 E–G and Fig. S10 I and J) and uterus-like epithelia (Fig. 6 F). AGN treatment irreversibly induced TRP63+ vagina-like epithelia in host mice, irrespective of E2 treatment (Fig. 6 C and D). KRT14 was expressed in the vagina-like epithelium in E2-treated host mice (Fig. S10 B). PGR was not detected in the vagina-like epithelium in the oil-treated host mice (Fig. S10 E), indicating that the PGR expression pattern was similar to vaginal epithelium. These data suggested that AGN treatment irreversibly induced vaginal epithelium. In E2-treated host mice, grafted AGN-treated Müllerian ducts did not have oviduct-like epithelia (Fig. 6 F and Fig. S10 D). Cotreatment with RA + AGN canceled the effects of AGN on induction of vaginal epithelium (Fig. 6 C and D and Fig. S10 B) and inhibition of oviductal epithelium (Fig. 6 F). RA treatment appeared to decrease the size of the smooth muscle actin (SMA) expression domain in both oil- and E2-treated animals and this effect was blocked by AGN (Fig. S10 K and L). Grafted, organ-cultured E17.5 middle Müllerian ducts at day 30 postgrafting had only uterus-like epithelia (Fig. 6 L). AGN treatment irreversibly induced TRP63+ vagina-like epithelia in the presence or absence of E2 (Fig. 6 J and K). In the vagina-like epithelia, E2 treatment
induced KRT14 expression (Fig. S11B), whereas PGR was not detected in the oil-treated host mice similar to those in vaginal epithelia (Fig. S11E). Grafted, organ-cultured E17.5 caudal Müllerian ducts had TRP63+ and KRT14+ vagina-like epithelia (Fig. 6 O and P and Fig. S11 K and L). RA treatment permanently induced uterus-like epithelia, but it did not induce TUBB3+ or OVGP1+ oviduct-like epithelia (Fig. 6Q). These results indicated that RA–RAR signaling permanently induced the uterine epithelium in the Müllerian ducts. Because RA treatment did not induce oviductal differentiation in the organ-cultured caudal Müllerian duct, even after grafting, we hypothesized that the fate of oviductal stroma had already been determined by E14.5. To investigate this hypothesis, the intact proximal and middle E14.5 Müllerian ducts had already been determined by E14.5. To investigate this hypothesis, the intact proximal and middle E14.5 Müllerian ducts were grafted. At day 30 postgrafting, the proximal duct grafts had only TUBB3+ or OVGP1+ oviductal epithelia, whereas middle Müllerian ducts had only uterine epithelia (Fig. S12 A–E). Furthermore, homeobox A10 (Hoxa10) expression in the middle Müllerian duct at E14.5 was already higher than that in the proximal Müllerian duct (Fig. S12F).

**Indirect Transcriptional Regulation of Hoxa Genes by RA Signaling in the Müllerian Duct.** Expression of Hoxa genes as candidate downstream effectors of RA signaling was investigated in organ-cultured E14.5 Müllerian duct. Hoxa10 and Hoxa11 expression in RA-treated Müllerian ducts was slightly higher than that in vehicle-treated ducts (Fig. S13A). AGN otreatment abrogated the effects of RA treatment. Hoxa9 and Hoxa13 expression was not changed by RA treatment. The Hoxa10 promoter region from -2,000 to 1,000 bp contains a putative RARE by bioinformatical search (RARE score matrix based on ref. 30). To investigate the ability of this putative Hoxa10 RARE to recruit RAR, we used ChIP-qPCR. RNA polymerase II bound to Hoxa10 transcription start site (TSS) both in the proximal or middle Müllerian ducts at E14.5 (Fig. S13F). We did not detect binding of RAR to the putative Hoxa10 RARE, the Hoxa10 TSS, or a nonspecific region located at -3,000 kb from putative Hoxa10 RARE (Fig. S13C). Thus, we infer that RAR did not bind to TSS or the predicted RARE of Hoxa10 in the Müllerian duct.

**Discussion**

Results from ontogenic mRNA and protein expression of genes in the RA signaling pathway together with the activity of RA signaling in the female reproductive tracts showed that RA was synthesized and acted in the stroma of proximal and middle Müllerian duct. Addition of RA or RAR antagonists in organ-cultured Müllerian ducts and the grafting experiment revealed that RA–RAR signaling determines the fate of the uterine stroma and that inhibition of this signaling is essential for the fate determination of the vaginal stroma.
activates a heterodimer of RARα/RXRα to stimulate Hoxa10 promoter activity (37). Therefore, in the middle Müllerian ducts, RAR may bind to the upstream 5′ promoter region of Hoxa10 because RA also increased Hoxa11 (located in the 5′ region of Hoxa10) expression. However, the 3′ region of Hoxa10 because the downstream DNA sequence is also important for Hoxa genes (38), and a predicted RARE is located at +2,869. On the other hand, Hoxa10 expression has already been associated with determining the border between oviduct and uterus (39); therefore, other factors can also mediate the effects of RA signaling on determination of the borderline between uterine and vaginal stroma.

In conclusion, the presence of RA–RAR signaling in the Müllerian duct determined the border between uterine and vaginal stroma in prenatal stages (Fig. 7). The fate-determined strong RA signal from distal factors induces their respective epithelia. In female mouse embryos, RA inhibits inhibin βA expression in the urogenital sinus (40), and activin A induced by the inhibin βA is important for vaginal epithelial differentiation (7, 8). These data support a model in which key factors regulated by RA–RAR signaling can play critical roles in fate determination in epithelium and stroma of the female reproductive tracts.

Materials and Methods

Animals. C57BL/6J and transgenic mice, which have three RAREs linked to the Hoxa10 promoter and lacZ gene (RARE-lacZ mice purchased by The Jackson Laboratory) (29) were maintained in accordance with the NIH’s Guide for the Care and Use of Laboratory Animals (41). At P0, uteri and vaginas were collected at the diestrus or estrus as determined by vaginal smear. All experiments were approved by the animal care committee in Yokohama City University.

Organ Culture System and Grafting. Müllerian duct organ culture was performed as described previously (42). E14.5 Müllerian duct including proximal and middle regions (caudal regions could not be separated), middle and caudal E17.5 Müllerian duct, and P0 or P15 uterus and vagina were cultured in a collagen gel for 8 or 5 d, respectively with daily medium changes. A total of 10 μM all-trans retinoic acid (Sigma), 10 μM all-trans retinylidenehydrogenase (Sigma), 1 μM all-trans RA (Sigma), 2 or 10 μM AGN (Santa Cruz), 10 μM BM5 (Tocris), 10 μM E (Santa Cruz), or 10 μM MM (Tocris) were added to the medium. Four to six organ-cultured Müllerian ducts or intact proximal or middle E14.5 Müllerian ducts were grafted under the renal capsule of C57BL/6J from P50 to P90 as described previously (44). At the time of grafting, all host mice were OVX. At 30 d postgrafting, host mice were given s.c. injections of 0.1 μg/g bw E2 or sesame oil for 3 d, euthanized, and tissues harvested. Two independent grafting experiments were carried out.

Assay for treatment, β-galactosidase activity, RNA isolation, RT-PCR, qPCR, Western blot, immunohistochemistry, CHIP assay, and statistical analysis were performed as described in SI Materials and Methods. Primers were listed in Table S1. Negative control for immunohistochemistry was shown in Fig. S17.

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