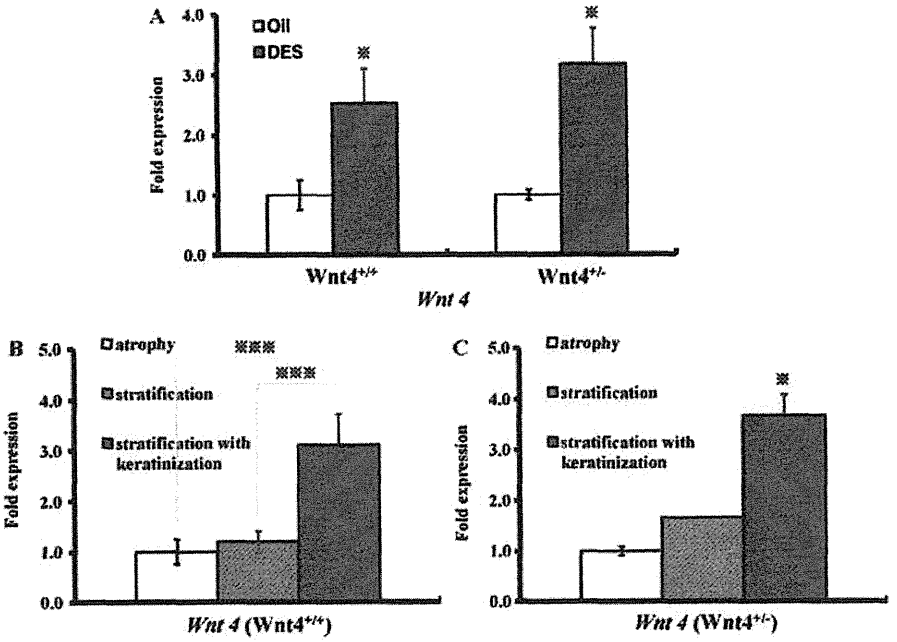

Fig. 4. Vaginae of 10-week-old, ovariectomized $Wnt4^{+/+}$ or $Wnt4^{+/-}$ mice exposed neonatally to 2.5 μg DES or oil vehicle alone. Neonatal exposure to 2.5 μg DES induced persistent up-regulation of *Wnt4* mRNA in vaginae of both $Wnt4^{+/+}$ and $Wnt4^{+/-}$ mice (A). *Wnt4* mRNA expression was significantly correlated to the vaginal epithelial cell proliferation with the superficial keratinization but not for the proliferation only in $Wnt4^{+/+}$ (B) and $Wnt4^{+/-}$ mice (C). Since only one $Wnt4^{+/-}$ mouse showed epithelial stratification only, therefore, statistical analysis could not be done. The number of mice showing vaginal epithelial atrophy, stratification only and stratification with keratinization are correlated to Table 3. $^{*}P < 0.05$ vs. controls, $^{***}P < 0.001$ vs. controls (Student's *t*-test or Welch's *t*-test followed by *F*-test).

et al., 2003), and cardiogenesis (Pandur et al., 2002). In the line of microarray results, our tissue distribution data also suggested similar respective up- and down-regulation of *Wnt4* and *Wnt11* expression after neonatal DES exposure. The reduction in *Wnt11* after DES exposure suggests their repressive role in Wnt pathway (Maye et al., 2004). However, the expression of Fz genes, receptors of *Wnt4* (Lyons et al., 2004), did not change in DES-treated vagina, suggested that *Wnt4* might have other function unrelated to Fzs.

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

In this regard, our earlier reports suggest that DES-induced persistent proliferation in vagina is actually mediated through ERα (Nakamura et al., 2008). Moreover, in wild-type mice, uterine expression of *Hoxa10*, *Hoxa11* and *Wnt7a* genes exhibited significant decrease shortly after DES treatment (Ma et al., 1998; Kitajewski and Sassoon, 2000; Couse et al., 2001), whereas this effect was not observed in the αERKO mice (Couse et al., 2001). This supports the idea about the obligatory role for ERα in DES-induced alteration of mouse reproductive tract. Interestingly, in the present study, only PPT, but not DPN, induced a similar magnitude of *Wnt4* and *Wnt11* expression as in DES-exposed vagina. This suggests that the changes in *Wnt4* and *Wnt11* profile are ERα responsive. But anti-estrogen mediated reduction of *Wnt4*, but not *Wnt11*, confirms that *Wnt4* action is regulated by ERα, and *Wnt11* might be regulated by androgen receptor as in prostate cancer (Zhu et al., 2004).

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

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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2012.02.010.

References

Beato, M., Herrlich, P., Schutz, G., 1995. Steroid hormone receptors: many actors in search of a plot. Cell 83, 851–857.

Brisken, C., Heineman, A., Chavarria, T., Elenbaas, B., Tan, J., Dey, S.K., McMahon, A.P., Weinberg, R.A., 2000. Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. Genes Dev. 14, 650–654.

Cadigan, K.M., Nusse, R., 1997. Wnt signaling: a common theme in animal development. Genes Dev. 11, 3286–3305.

Connoly, J.L., Schnitt, S.J., 1993. Benign breast disease. Resolved and unresolved issues. Cancer 71, 1187–1189.

Couse, J.F., Dixon, D., Yates, M., Moore, A.B., Ma, L., Maas, R., Korach, K.S., 2001. Estrogen receptor-α knockout mice exhibit resistance to the developmental effects of neonatal diethylstilbestrol exposure on the female reproductive tract. Dev. Biol. 238, 224–238.

Daikoku, T., Song, H., Guo, Y., Riesewijik, A., Mosselman, S., Das, S.K., Dey, S.K., 2004. Uterine Msx-1 and Wnt4 signaling becomes aberrant in mice with the loss of leukemia inhibitory factor or Hoxa-10: evidence for a novel cytokine-homeobox-Wnt signaling in implantation. Mol. Endocrinol. 18, 1238–1250.

Dale, T.C., 1998. Signal transduction by the Wnt family of ligands. Biochem. J. 329, 209–223.

Dunn, T.B., Green, A.W., 1963. Cysts of the epididymis, cancer of the cervix, granular cell myoblastoma, and other lesions after estrogen injection in newborn mice. J. Natl. Cancer Inst. 31, 425–455.

Forsberg, J.-G., 1969. The development of atypical epithelium in the mouse uterine cervix and vaginal formix after neonatal oestradiol treatment. Br. J. Exp. Pathol. 50, 187–195.

Herbst, A.L., Ulfelder, H., Poskanzer, D.C., 1971. Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. N. Engl. J. Med. 284, 878–881.

Huelsken, J., Birchmeier, W., 2001. New aspects Wnt signaling pathways in higher vertebrates. Curr. Opin. Genet. Dev. 11, 547–553.

Iguchi, T., 1992. Cellular effects of early exposure to sex hormones and antihormones. Int. Rev. Cytol. 139, 1–57.

Iguchi, T., Takasugi, N., Bern, H.A., Mills, K.T., 1986. Frequent occurrence of polyovular follicles in ovaries of mice exposed neonatally to diethylstilbestrol. Teratology 34, 29–35.

Iguchi, T., Watanabe, H., Katsu, Y., Mizutani, T., Miyagawa, S., Suzuki, A., Kohno, S., Sone, K., Kato, H., 2002. Developmental toxicity of estrogenic chemicals on rodents and other species. Congenit. Anom. (Kyoto) 42, 94–105.

Jeays-Ward, K., Dandonneau, M., Swain, A., 2004. Wnt4 is required for proper male as well as female sexual development. Dev. Biol. 279, 431–440.

Karantza, V., 2011. Keratins in health and cancer: more than mere epithelial cell markers. Oncogene 30, 127–138.

Kispert, A., Vainio, S., McMahon, A.P., 1998. Wnt-4 is a mesenchymal signal for epithelial transformation of metanephric mesenchyme in the developing kidney. Development 125, 4225–4234.

Kitajewski, J., Sassoon, D., 2000. The emergence of molecular gynecology: homeobox and Wnt genes in the female reproductive tract. Bioessays 22, 902–910.

Liu, X., Liu, T., Slusarski, D.C., Yang-Snyder, J., Malbon, C.C., Moon, R.T., Wang, H., 1999. Activation of a frizzled-2/β-adrenergic receptor chimera promotes Wnt signaling and differentiation of mouse F9 teratocarcinoma cells via Gαo and Gαt. Proc. Natl. Acad. Sci. U.S.A. 96, 14383–14388.

Lustig, B., Behrens, J., 2003. The Wnt signaling pathway and its role in tumor development. J. Cancer Res. Clin. Oncol. 129, 199–221.

Lyons, J.P., Mueller, U.W., Everett, C., Fang, X., Hsieh, J.C., Barth, A.M., McCrea, P.D., 2004. Wnt-4 activates the canonical β-catenin-mediated Wnt pathway and binds Frizzled-6 CRD: functional implications of Wnt/β-catenin activity in kidney epithelial cells. Exp. Cell Res. 298, 369–387.

Ma, L., Benson, G.V., Lim, H., Dey, S.K., Maas, R.L., 1998. *Abdominal B (AbdB) hoxa* genes: regulation in adult uterus by estrogen and progesterone and repression in Müllerian duct by the synthetic estrogen diethylstilbestrol (DES). Dev. Biol. 197, 141–154.

Majumdar, A., Vainio, S., Kispert, A., McMahon, J., McMahon, A.P., 2003. Wnt11 and Ret/Gsnf pathway cooperate in regulating ureteric branching during metanphiric kidney development. Development 130, 3175–3185.

Maye, P., Zheng, J., Li, L., Wu, D., 2004. Multiple mechanisms for Wnt11 mediated repression of the canonical Wnt signaling pathway. J. Biol. Chem. 279, 24659–24665.

McLachlan, J.A., Newbold, R.R., Bullock, B.C., 1980. Long-term effects on the female mouse genital tract associated with prenatal exposure to diethylstilbestrol. Cancer Res. 40, 3988–3999.

Miller, C., Pavlova, A., Sassoon, D.A., 1998. Differential expression patterns of Wnt genes in the murine female reproductive tract during development and the estrous cycle. Mech. Dev. 76, 91–99.

Miyagawa, S., Katsu, Y., Watanabe, H., Iguchi, T., 2004a. Estrogen-independent activation of ErbBs signaling and estrogen receptor α in the mouse vagina exposed neonatally to diethylstilbestrol. Oncogene 23, 340–349.

Miyagawa, S., Suzuki, A., Katsu, Y., Kobayashi, M., Goto, M., Handa, H., Watanabe, H., Iguchi, T., 2004b. Persistent gene expression in mouse vagina exposed neonatally to diethylstilbestrol. J. Mol. Endocrinol. 32, 663–677.

Moll, R., Divo, M., Langbein, L., 2008. The human keratin: biology and pathology. Histochem. Cell Biol. 129, 705–733.

Nakamura, T., Katsu, Y., Watanabe, H., Iguchi, T., 2008. Estrogen receptor subtypes selectively mediate female mouse reproductive abnormalities induced by neonatal exposure to estrogenic chemicals. Toxicology 253, 117–124.

Newbold, R.R., McLachlan, J.A., 1982. Vaginal adenosis and adenocarcinoma in mice exposed prenatally or neonatally to diethylstilbestrol. Cancer Res. 42, 2003–2011.

Newbold, R.R., Bullock, B.C., McLachlan, J.A., 1985. Progressive proliferative changes in the oviduct of mice following developmental exposure to diethylstilbestrol. Terat. Carcinog. Mutag. 5, 473–480.

Pandur, P., Lasche, M., Eisenberg, L.M., Kuhl, M., 2002. Wnt-11 activation of a non-canonical Wnt signaling pathway is required for cardiogenesis. Nature 418, 636–641.

Parr, B.A., McMahon, A.P., 1998. Sexually dimorphic development of the mammalian reproductive tract requires Wnt-7a. Nature 395, 707–710.

Pavlova, A., Boutin, E., Cunha, G., Sassoon, D., 1994. Msx1 (Hox-7.1) in the adult mouse uterus: cellular interactions underlying regulation of expression. Development 120, 335–346.

Saitoh, A., Ansen, L.A., Vogel, J.C., Udey, M.C., 1998. Characterization of Wnt gene expression in murine skin: possible involvement of epidermis-derived Wnt4 protein in cutaneous epithelial–mesenchymal interactions. Exp. Cell Res. 243, 150–160.

Sassoon, D., 1999. Wnt genes and endocrine disruption of the female reproductive tract: a genetic approach. Mol. Cell. Endocrinol. 158, 1–5.

Smalley, M.J., Dale, T.C., 1999. Wnt signaling in mammalian development and cancer. Cancer Metast. Rev. 18, 215–230.

Smalley, M.J., Dale, T.C., 2001. Wnt signaling and mammary tumorigenesis. J. Mammary Gland Biol. Neoplasia 6, 37–52.

Stark, K., Vaninio, S., Vassileva, G., McMahon, A.P., 1994. Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. Nature 372, 679–683.

Suzuki, A., Sugihara, A., Uchida, K., Sato, T., Ohta, Y., Katsu, Y., Watanabe, H., Iguchi, T., 2002. Developmental effects of perinatal exposure to bisphenol-A and diethylstilbestrol on reproductive organs in female mice. Reprod. Toxicol. 16, 107–116.

Takasugi, N., Bern, H.A., 1964. Tissue changes in mice with persistent vaginal cornification induced by early postnatal treatment with estrogen. J. Natl. Cancer Inst. 33, 855–865.

Takasugi, N., Bern, H.A., DeOme, K.B., 1962. Persistent vaginal cornification in mice. Science 138, 438–439.

Tsai, M.J., O'Malley, B.W., 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Ann. Rev. Biochem. 63, 451–486.

Tsukamoto, A.S., Grosschedl, R., Guzman, R.C., Parslow, T., Varmus, H.E., 1988. Expression of the int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in male and female mice. Cell 55, 619–625.

van Noort, M., Clevers, H., 2002. TCF transcription factors, mediators of Wnt-signaling in development and cancer. Dev. Biol. 244, 1–8.

Vainio, S., Heikkia, M., Kispert, A., Chin, N., McMahon, A.P., 1999. Female development in mammals is regulated by Wnt-4 signaling. Nature 397, 405–409.

Wang, Y., Macke, J.P., Abella, B.S., Andreasson, K., Worley, P., Gilbert, D.J., Copeland, N.G., Jenkins, N.A., Nathans, J., 1996. A large family of the product of the Drosophila tissue polarity gene frizzled. J. Biol. Chem. 271, 4468–4476.

Watanabe, H., Suzuki, A., Kobayashi, M., Lubahn, D.B., Handa, H., Iguchi, T., 2003. Similarities and differences in uterine gene expression patterns caused by treatment with physiological and non-physiological estrogens. J. Mol. Endocrinol. 31, 487–497.

Watanabe, H., Suzuki, A., Goto, M., Lubahn, D.B., Handa, H., Iguchi, T., 2004. Tissue-specific estrogenic and non-estrogenic effects of a xenoestrogen, nonylphenol. J. Mol. Endocrinol. 33, 243–252.

Watanabe, H., Suzuki, A., Mizutani, T., Khono, S., Lubahn, D.B., Handa, H., Iguchi, T., 2002. Genome-wide analysis of changes in early gene expression induced by oestrogen. Gene Cells 7, 497–507.

Weber-Hall, S.J., Phippard, D.J., Niemeyer, C.C., Dale, T.C., 1994. Developmental and hormonal regulation of Wnt gene expression in the mouse mammary gland. Differentiation 57, 205–214.

Willert, K., Nusse, R., 1998. β-Catenin: a key mediator of Wnt signaling. Curr. Opin. Genet. Dev. 8, 95–102.

Wodarz, A., Nusse, R., 1998. Mechanisms of Wnt signaling in development. Ann. Rev. Biol. 14, 59–88.

Yu, H., Pask, A.J., Shaw, G., Renfree, M.B., 2006. Differential expression of WNT4 in testicular and ovarian development in a marsupial. BMC Dev. Biol. 6, 44.

Zhu, H., Mazor, M., Kawano, Y., Walker, M.M., Leuug, H.Y., Armstrong, K., Waxman, J., Kypta, R.M., 2004. Analysis of Wnt gene expression in prostate cancer: mutual inhibition by WNT11 and androgen receptor. Cancer Res. 64, 7918–7926.

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REGULAR ARTICLE

Hedgehog signaling plays roles in epithelial cell proliferation in neonatal mouse uterus and vagina

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Abstract Both the uterus and vagina develop from the Müllerian duct but are quite distinct in morphology and function. To investigate factors controlling epithelial differentiation and cell proliferation in neonatal uterus and vagina, we focused on Hedgehog (HH) signaling. In neonatal mice, Sonic hh (*Shh*) was localized in the vaginal epithelium and Indian hh (*Ihh*) was slightly expressed in the uterus and vagina, whereas all Glioma-associated oncogene homolog (*Gli*) genes were mainly expressed in the stroma. The expression of target genes of HH signaling was high in the neonatal vagina and in the uterus, it increased with growth. Thus, in neonatal mice, *Shh* in the vaginal epithelium and *Ihh* in the uterus and vagina activated HH signaling in the stroma. Tissue recombinants showed that vaginal *Shh* expression was inhibited by the vaginal stroma and uterine *Ihh* expression was stimulated by the uterine stroma. Addition of a HH signaling inhibitor decreased epithelial cell proliferation in organ-cultured uterus and vagina and increased stromal cell proliferation in organ-cultured uterus. However, it did not affect epithelial differentiation or the expression of growth factors in organ-cultured uterus and vagina. Thus, activated HH signaling stimulates epithelial cell proliferation in neonatal uterus and vagina but inhibits stromal cell proliferation in neonatal uterus.

Keywords Hedgehog · Uterus · Vagina · Cell proliferation · Organ culture · Mouse (female C57BL/6J)

Introduction

Female reproductive tracts originate from the Müllerian duct. The middle to upper portion of the Müllerian duct develops into the oviduct and uterus and the lower portion, which is connected to the urogenital sinus, develops into the vagina in embryonic and neonatal mice. Kurita (2010) has shown that the Müllerian vagina extends to the caudal end of the body by day 8, suggesting that, in adults, both the uterus and vagina have developed from the Müllerian duct. However, the uterus and vagina are quite distinct in their morphology and function. The uterine epithelium (UE) is composed of a simple columnar luminal epithelium and a glandular epithelium. The Müllerian vaginal epithelium (VE) consisting in pseudostratified columnar cells develops into a stratified cuboidal epithelium with markers of stratification, namely transformation related protein 63 (TRP63) and keratin 14 (KRT14) expression (Forsberg 1965; Nakajima et al. 2011). *Trp63* is necessary for differentiation into stratified squamous epithelium since, in *Trp63* knockout mice, the

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Müllerian VE forms a uterus-like single layer of columnar epithelium (Kurita et al. 2004).

Tissue recombination of the epithelium and stroma has been performed in order to investigate the mechanism of epithelial differentiation from the Müllerian duct. The UE combined with the vaginal stroma (VS) of neonatal mice differentiates from a simple columnar epithelium into a TRP63- and KRT14-positive stratified squamous epithelium (Cunha 1976; Kurita et al. 2004; Nakajima et al. 2011). Similarly, the VE combined with the uterine stroma (US) shows a TRP63- and KRT14-negative single layer of columnar cells typical of the UE. These data indicate that epithelial-stromal signaling decides epithelial fate, differentiation and growth in the female reproductive tracts of developing mice. This epithelial cell differentiation in the uterus and vagina can be induced by the mesenchyme up to day 7 (Cunha 1976), suggesting that epithelial cell differentiation in the uterus and vagina is completed around day 7.

The Hedgehog (HH) family has been well studied as one of the paracrine regulators in epithelial-stromal interactions in developing intestine, limb, lung and prostate (Pepicelli et al. 1998; van Tuyl and Post 2000; Ramalho–Santos et al. 2000; McMahon et al. 2003; Madison et al. 2005; White et al. 2006; Jiang and Hui 2008). The HH family includes Sonic HH (SHH), Indian HH (IHH) and Desert HH (DHH). Binding of a HH ligand to a transmembrane receptor, namely patched 1 (PTCH1) or PTCH2, relieves the transmembrane protein called smoothened (SMO) and HH signaling is then activated through the transcription factors, Glioma-associated oncogene homolog 1 (GLI1) and GLI2 (Cohen 2003; Shaw and Bushman 2007). GLI3 is known as a transcriptional repressor balancing and refining GLI1 and GLI2 activation. The mRNA expression of *Gli1* and *Ptch1* increases in response to HH signaling activation and therefore, those genes are useful as markers of HH signaling activation.

In the developing prostate, *Shh* is localized in the epithelium, whereas *Gli1*, *Ptch1* and *Ptch2* are localized in the stroma (Lamm et al. 2002). In the organ-cultured developing prostate, the addition of SHH decreases the number of ductal tips, epithelial cell proliferation and TRP63-positive basal cells, whereas these parameters are increased by the HH signaling inhibitor, cyclopamine (Freestone et al. 2003; Wang et al. 2003; Berman et al. 2004). Loss-of-function in *Gli2* increases the number of TRP63-positive basal cells (Doles et al. 2006). These data indicate that epithelial SHH activates HH signaling in the stroma and then inhibits epithelial differentiation and cell proliferation in the developing prostate. However, Lamm et al. (2002) have shown that HH signaling in the developing prostate stimulates epithelial cell proliferation. Thus, the role of HH signaling in epithelial cell proliferation and ductal morphogenesis is complicated and seems to depend on the developmental stage (Vezina and Bushman 2007). In addition, epithelial SHH stimulates the stromal expression of transforming growth factor β1 (*Tgfβ1*), bone morphogenetic protein 4 (*Bmp4*) and *activin βA-subunit* and inhibits the stromal expression of fibroblast growth factor 10 (*Fgf10*) in the developing prostate, suggesting that activated HH signaling in the stroma can affect the epithelium through the regulation of the expression of these genes (Wang et al. 2003; Pu et al. 2004).

In the uterus, constitutive activation of SMO causes the epithelium to contain vagina-like stratified cells in adults and wingless-related MMTV integration site 5a (*Wnt5a*) expression is stimulated at day 24 (Franco et al. 2010a; Migone et al. 2011). Thus, HH signaling regulates epithelial differentiation in the immature and adult uterus. However, the role of HH signaling in the neonatal mouse uterus and vagina has not yet been investigated. In order to investigate epithelial differentiation and cell proliferation in the female reproductive tracts of neonatal mice, we have examined the ontogenic mRNA expression and localization of genes related to HH signaling in the uterus and vagina and the effects of cyclopamine on epithelial differentiation and cell proliferation in the uterus and vagina grown *in vitro*. Furthermore, in order to investigate the epithelial-stromal interactions, the mRNA expression of the *Shh* and *Ihh* has also been examined in the tissue recombinant.

Materials and methods

Animals

Female C57BL/6J mice (CLEA, Tokyo, Japan) were given a commercial diet (MF, Oriental Yeast, Tokyo, Japan) and tap water *ad libitum* and were kept at 22±1.0°C under a 12 h light/12 h darkness regime in artificial illumination (lights on: 0800–2000). Animals were maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals approved by our Institutional Animal Care Committee. Uteri and vaginae of 0- and 2-day-old mice containing undifferentiated epithelium and those of 15-day-old mice containing differentiated epithelium were examined.

Mice were ovariectomized at day 90. At 7 days after surgery, ovariectomized mice were given a single subcutaneous injection containing 0.1 μg/25 g body weight of 17β-estradiol (E2, Sigma, St. Louis, MO, USA) dissolved in sesame oil or sesame oil alone. At 16 h after a single injection of E2, vaginae were collected for real-time reverse transcription with the polymerase chain reaction (RT-PCR).

Epithelial-stromal separation, tissue recombination, and grafting

Epithelial-stromal separation, tissue recombination and grafting were performed as described previously (Bigsby et al. 1986; Nakajima et al. 2011). Uteri and vaginae of 2- and 15-day-old

mice were cut, placed into 1% trypsin (Becton, Dickinson, Franklin Lakes, NJ, USA) in Hanks' balanced salt solution (HBSS; Sigma) and digested at 4°C for 90 min. Trypsin action was stopped by addition of fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA). UE, US, VE and VS were separated mechanically by using fine surgical forceps, immediately frozen in liquid nitrogen and stored at -80°C for RT-PCR.

For tissue recombination, UE recombined with US (UE+US) or VS (UE+VS) or VE recombined with VS (VE+VS) or US (VE+US) from 2-day-old mice were placed on agar plates and allowed to adhere at 37°C in a 5% CO_2 atmosphere overnight. Tissue recombinants were grafted under the renal capsules of 60- to 90-day-old C57BL/6J mice. At the time of grafting, all host mice were ovariectomized. They were then killed to harvest tissue recombinants at days 5 and 7 post-grafting, because transition from the UE to the VE and *vice versa* in tissue recombinants was completed at day 7 post-grafting (Nakajima et al. 2011). These tissue recombinants were placed immediately in liquid nitrogen and stored at -80°C for real-time RT-PCR.

Organ culture system

An organ culture system for neonatal uterus and vagina was modified according to previous reports (Ootani et al. 2009; Nakajima et al. 2011). Eight volumes of Cellmatrix type I-A (Nitta Gelatin, Osaka, Japan) were mixed with 1 volume 10× Dulbecco's Modified Eagle's Medium/Nutrient Mixture Ham's F-12 (DMEM/F12; Sigma) and then 1 volume 200 mM HEPES buffer containing 262 mM $NaHCO_3$ and 0.05 N NaOH was added to the mixture. This cold gelation mixture (200 μl) was poured into inner millicell cell culture inserts (Millipore, Bedford, Mass., USA) placed into the well of a 24-well plate and allowed to gel at 37°C. Uteri and vaginae of 0-day-old mice were cut and placed into HBSS. Tissues were washed three times in HBSS and mixed with fresh 200 μl cold gelation mixture. Tissues and gelation mixture were overlaid onto a base of gelled collagen in each cell culture insert and allowed to gel at 37°C. Subsequently, 200 μl 20% FBS in DMEM/F12 (Invitrogen) was added to each well and tissues were cultured at 37°C in a humidified, 5% CO_2/air atmosphere for 2 or 5 days. Medium was changed at day 3 of culture. Cyclopamine (10 μM; Enzo Life Sciences, Farmingdale, NY, USA) was added to the medium from day 0 of culture (Freestone et al. 2003; Wang et al. 2003; Berman et al. 2004). For immunohistochemistry of 5-bromo-2'-deoxyuridine (BrdU), uteri and vaginae were cultured with 18% FBS in DMEM/F12 containing 0.1 mg/ml BrdU (Sigma) for 2 h before fixation.

Immunohistochemistry of TRP63, KRT14 and BrdU

Immunohistochemistry of TRP63, KRT14 and BrdU was performed on organ-cultured uteri and vaginae. For TRP63 and KRT14, samples (n=7) were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4) at 4°C, whereas BrdU samples (n=7–10) were fixed in 10% formalin neutral buffered solution (Wako Pure Chemical Industries, Osaka, Japan), both as described previously (Kim et al. 2009; Nakajima et al. 2011). Hematoxylin was used for counterstaining. Cells immunoreactive for BrdU were counted under a light microscope with a 40× objective lens. BrdU-positive cells were counted randomly in 600 cells in each of the UE, US, VE and VS.

RNA isolation and RT-PCR

UE, US, VE and VS of 2- and 15-day-old mice were homogenized in TRIzol (Invitrogen). Total RNA was purified by using an RNeasy total RNA kit (Qiagen, Hilden, Germany) and reverse-transcribed into cDNA. To determine the expression of *Shh*, *Ihh*, *Dhh*, *Ptch1*, *Ptch2*, *Gli1*, *Gli2*, *Gli3*, *Krt8*, or *vimentin*, an aliquot of cDNA was amplified with specific primers (see Supplemental Table) derived from mouse mRNA sequences. Reverse transcription and PCR were carried out by using the Takara RNA PCR Kit (AMV; Takara Bio, Otsu, Japan). Peptidylprolyl isomerase A (*Ppia*) was chosen as an internal standard. Ten UE, US, VE and VS of 2-day-old mice and two UE, US, VE and VS of 15-day-old mice were pooled for RNA isolation at each point. Two independent experiments were carried out for each study.

Real-time RT-PCR

Real-time RT-PCR was preformed as described previously (Nakajima et al. 2011). Total RNA was isolated from uteri and vaginae of 2- or 15-day-old mice, vaginae of oil- or E2-treated 90-day-old mice and UE+US, UE+VS, VE+VS and VE+US tissue recombinants at days 5 or 7 post-grafting and from organ-cultured uteri and vaginae for 2 or 5 days. To determine the expression of *Shh*, *Ihh*, *Dhh*, *Gli1*, *Gli2*, *Gli3*, *Ptch1*, *Ptch2*, *Tgf1*, *Bmp4*, *activin βA-* or *βB-subunit*, *Fgf7*, *Fgf10*, or *Wnt5a*, an aliquot of cDNA was amplified with specific primers (see Supplemental Table) derived from mouse mRNA sequences. *Ppia* was chosen as an internal standard. Twelve uteri and vaginae of 2-day-old mice, four uteri and vaginae of 15-day-old mice, a vagina of a 90-day-old mouse, a tissue recombinant at days 5 or 7 of post-grafting and five organ-cultured tissues were pooled for RNA isolation at each point. Three to seven independent experiments were carried out.

Statistical analyses

Data are expressed as means±SE. Differences were estimated by using an analysis of variance followed by appropriate *post hoc* tests. A two-tailed Student's t-test or Welch's t-test was used for the comparison of two means. Differences were considered significant at $P<0.05$.

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

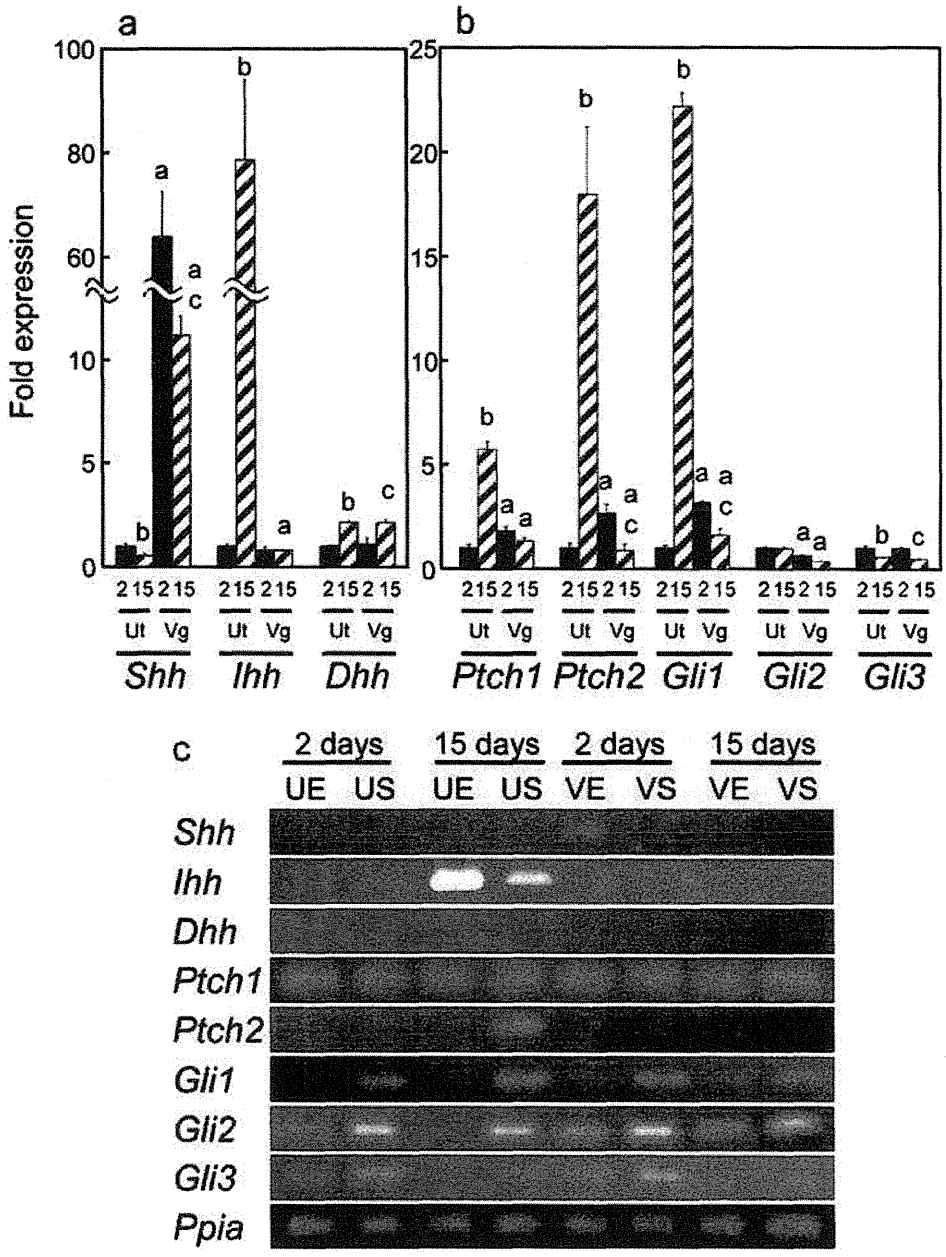

Fig. 1 mRNA expression levels of genes for various Hedgehog types, transmembrane receptors and transcription factors (*Shh*, *Ihh*, *Dhh*, *Ptch1*, *Ptch2*, *Gli1*, *Gli2* and *Gli3*) in the uterus and vagina at days 2 and 15 (**a**, **b**) were analyzed by real-time reverse transcription with the polymerase 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.

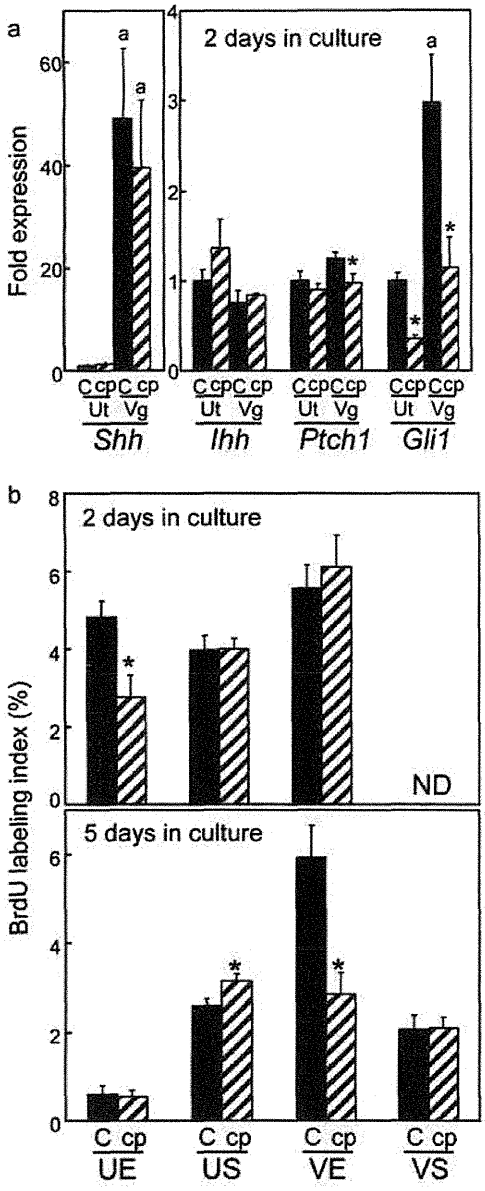

Fig. 2 *Shh*, *Ihh*, *Ptch1* and *Gli1* mRNA expression levels in organ-cultured uterus and vagina cultured for 2 days in control medium (C) or medium containing 10 μM cyclopamine (cp) were analyzed by real-time RT-PCR (**a**). Data are expressed relative to mRNA expression of the organ-cultured uterus 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.

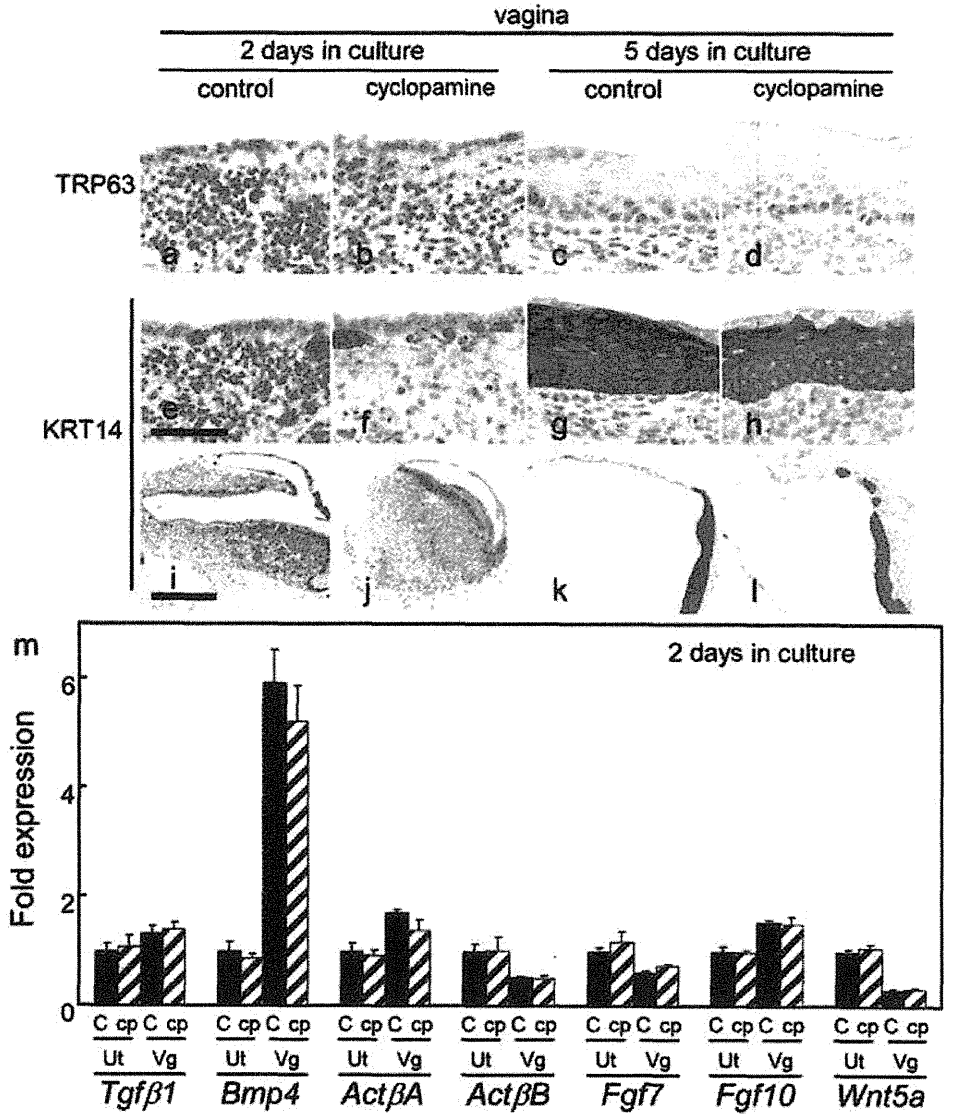

Fig. 3 Expression (*brown*) of TRP63 (**a-d**) and KRT14 (**e-l**) in the epithelium of organ-cultured vagina cultured for 2 days (**a**, **b**, **e**, **f**, **i**, **j**) or 5 days (**c**, **d**, **g**, **h**, **k**, **l**) in control medium (**a**, **c**, **e**, **g**, **i**, **k**) or medium containing 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)

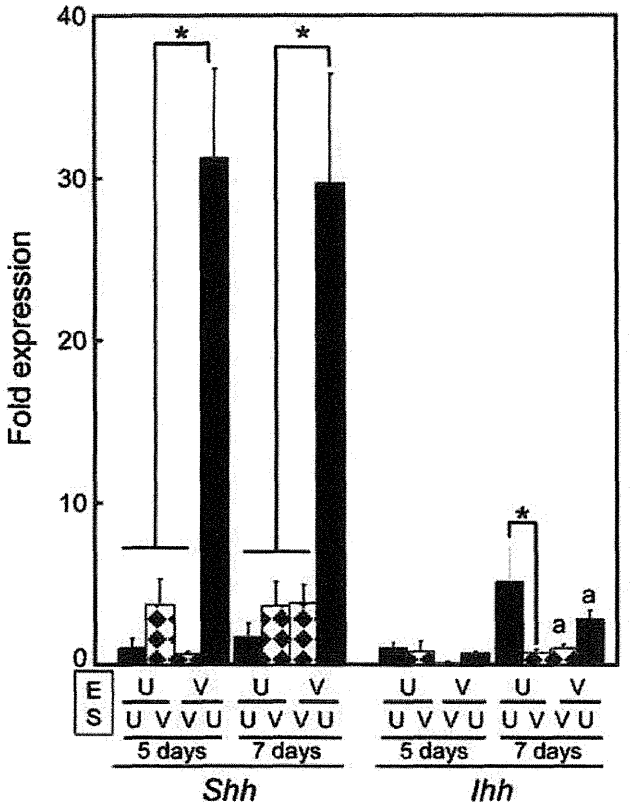

Fig. 4 *Shh* and *Ihh* mRNA expression levels in tissue recombinants at days 5 or 7 post-grafting as analyzed by real-time RT-PCR (E: epithelium, S: stroma, U: uterus, V: vagina). Data are expressed relative to mRNA expression of UE+US at day 5 of post-grafting; relative expression = 1.0 (*$P<0.05$,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

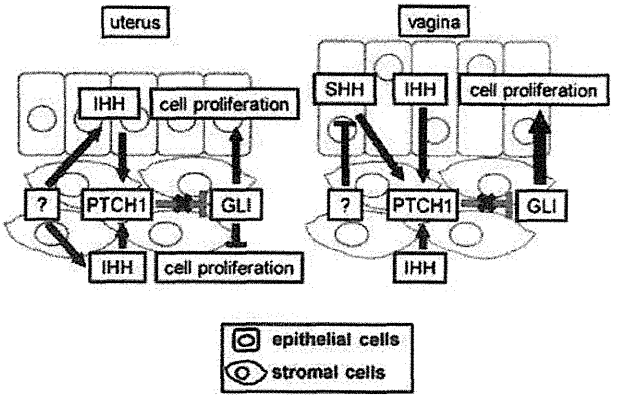

Fig. 5 Representation of the epithelial-stromal regulation of neonatal uterine or vaginal cell proliferation by HH signaling