Neonatal estrogenic exposure suppresses *PTEN*-related endometrial carcinogenesis in recombinant mice

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Human endometrial carcinomas, as well as complex atypical hyperplasias (CAH), are estrogen related and frequently have mutations in the *PTEN* gene. However, the mutual contribution of estrogen and *PTEN* mutations to endometrial carcinogenesis *in vivo* is unknown. To address this issue, we investigated whether neonatal estrogenic treatments augment the incidence of CAH and carcinomas in murine *PTEN* (*mPTEN*) heterozygous (+/–) mutant mice, an animal model for endometrial carcinoma. Low doses of diethylstilbestrol (1 ng/g/day), genistein (50 µg/g/day) in phytoestrogens, estriol (E₃) (4 µg/g/day), and vehicle (ethanol and corn oil) were administered subcutaneously daily to neonatal pups from the 1st to 5th day after birth. At 52 weeks of age, the morphological changes in the endometrium, and uterine expression of *Hoxa 10* and *Hoxa 11*, were evaluated. These *Hoxa* genes are abdominal B-type homeobox genes, which normally regulate differentiation of the Müllerian duct. The incidence of CAH and adenocarcinomas of the endometrium was significantly decreased by the neonatal estrogenic treatments in the *mPTEN*+/- mice. Coincidentally, all treatments significantly decreased the stromal cell density, and CAH and adenocarcinomas rarely developed in the epithelium adjacent to the affected endometrial stroma. Moreover, the uterine expression of *Hoxa 10* in mice with neonatal genistein and E₃ treatments, and that of *Hoxa 11* in mice with all treatments, was significantly lower when compared with vehicle alone. Taken together, neonatal estrogenic exposure induced stromal atrophy and/or hyalinization accompanied by repressed expression of *Hoxa 10* and *Hoxa 11*, and exerted an inhibitory effect on *PTEN*-related tumorigenesis. These findings provide new insight into the interaction between endometrial epithelium and stroma in endometrial carcinogenesis *in vivo*.

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Endometrial carcinoma is the most common malignancy of the female genital tract and fifth leading cancer in women worldwide.¹ There are two main pathogenetic types of endometrial carcinoma. Endometrioid adenocarcinoma or type I, the most common type, is frequently related to unopposed estrogen stimulation, that is, polycystic ovarian

syndrome (PCOS), obesity, or exogenous estrogen single use.^{2,3} Serous carcinoma or type II is not related to estrogen stimulation and has a more aggressive clinical course, with a high incidence of metastasis.^{2,3}

Endometrioid adenocarcinoma usually shows microsatellite instability and mutation or methylation in the promoters of DNA mismatch repair genes (*MLH1*, *MSH2*)^{4,5} and mutations in the *K-ras*,^{6,7} *β-catenin*,⁸ and *PTEN* genes.^{9–11} Mutations in *p53* gene are identified as a late event in a small percentage of endometrioid carcinomas, and are often associated with high-grade endometrioid or serous adenocarcinoma.^{12–14} Among the genetic alterations in endometrioid adenocarcinomas, the *PTEN* gene is most

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frequently mutated. This gene is a common tumor suppressor gene that is located on chromosome 10q23, and is frequently associated with loss of heterozygosity in human carcinomas.^{15–17} This mutation has been found in 26–80% of endometrioid carcinomas and in 18–55% of precancerous lesions, that is, complex atypical hyperplasia (CAH).^{18–21} Recombinant mice with a targeted deletion of exons 3–5 of the murine (m) *PTEN* gene on chromosome 19 have been created to understand the biological activity of this tumor suppressor gene.^{22,23} Homozygous mutant mice lacking exons 3–5 of the m*PTEN* gene die in the embryonic stage.²² The mutation of one allele is sufficient to produce neoplasia in some organs, including the prostate, endometrium, lymph node, gastrointestinal tract, thyroid, breast, and thymus, which have phenotypic correlation with tumors arising in patients suffering from Cowden's syndrome.^{24,25} As a particularly high incidence of endometrial hyperplasia as well as adenocarcinomas has been detected, m*PTEN*+/- mice are suitable as an animal model for investigating the role of the *PTEN* gene in endometrial carcinogenesis.²⁶ In an attempt to expand this mouse model, Wang *et al*²⁷ recently generated m*PTEN*+/- and *Mlh1* null (mismatch repair-deficient) double-mutant mice, which accelerated endometrial carcinogenesis.

Estrogens have been implicated as an important etiological factor in endometrial carcinogenesis. There is intense scientific and public interest in the concept that inadvertent and developmental exposure to synthetic estrogenic agents or even to natural substances that possess estrogenic activity may affect carcinogenesis in reproductive organs. Newbold *et al*^{28,29} reported a high incidence of endometrial adenocarcinomas at 78 weeks of age after neonatal exposure to diethylstilbestrol (DES; 1 ng/g of body weight/day) for the 1st to 5th day after birth in CD-1 mice. Besides DES, a low dose of genistein (50 µg/g/day) in phytoestrogens also frequently induced endometrial carcinomas.²⁹ Many human infants are exposed to this compound as a component of soy-based infant formulas. An infant consuming soy-based infant formula would ingest ~9 mg/kg (µg/g) of genistein per day.

This aberrant stimulation by estrogenic agents during the developmental period may disrupt the proper expression of estrogen regulated genes, resulting in altered development of the endometrial stroma, that is, atrophic or hyalinized stroma.^{30–32} Recently, neonatal DES exposure was shown to repress the uterine expression of the abdominal B-type homeobox genes, *Hoxa 10* and *Hoxa 11*, followed by significant loss in stromal development.^{33,34} Furthermore, mice lacking either *Hoxa 10* or *Hoxa 11* exhibited reproductive tract phenotypes resembling those of mice treated with developmental DES exposure.^{35–37} *Hoxa 10* is expressed in endometrial epithelium and stroma, while *Hoxa 11* expression is restricted to the stroma.³⁸ The deve-

lopment of both epithelium and stroma are thought to be under the regulation of *Hoxa 11*.³⁹

The present study was designed to investigate the neonatal estrogenic effect in *PTEN*-related carcinogenesis. Our preliminary study showed that CAH and endometrial carcinomas were observed in 93% (14 of 15 mice) and in 33% (5 of 15 mice), respectively, of m*PTEN*+/- mice at 52 weeks of age (unpublished data). Based on these findings, we examined whether low doses neonatal estrogenic treatments promote the incidence of CAH and endometrial carcinomas in m*PTEN*+/- mice and their wild-type littermates. Surprisingly, the incidence of CAH and adenocarcinomas of the endometrium was dramatically decreased by neonatal estrogenic treatments in m*PTEN*+/- mice. Simultaneously, neonatal estrogenic exposure induced stromal atrophy and/or hyalinization and repressed uterine expression of *Hoxa 10* and *Hoxa 11*. These findings indicate that endometrial stromal cells play an important role in *PTEN*-related tumorigenesis.

Materials and methods

Breeding and Genotypic Analysis Using the Polymerase Chain Reaction (PCR)

The m*PTEN*+/- mice (129 Ola × C57BL/6) with a deletion of exons 3–5 of m*PTEN* were generated.²² Male m*PTEN*+/- mice were mated with 8-week-old wild-type C57BL/6 female mice (Seac Yoshimoto, Fukuoka, Japan) at the Center for Animal Resources and Development, Kumamoto University, Japan. This mating scheme continued until there were eight mice in each group. All animals were maintained in plastic cages in a temperature- and light-controlled room (21–22°C and 12-h light/12-h dark) with adequate food and fresh water. The day the vaginal plug was detected was considered day 1 of pregnancy. Pregnant mice were housed individually in plastic cages. At delivery, female pups were separated for the following estrogenic treatments. All experiments were carried out according to the guidelines in the Laboratory Protocol of Animal Handling, Graduate School of Medical Sciences, Kumamoto University. PCR analysis of tail genomic DNA was used to determine the genotypes of mice according to the published protocol,²² using the following primers: forward—5'-TGCTATGGGATTTCTGCAG-3' and reverse—5'-CAAGCAAAACCAAATTAAGGGCC-3' for the mutant m*PTEN* allele, and forward—5'-ACAGACCTAGGCTACTGCTC-3' and reverse—5'-CTAGAGGCAAGACTTCCGTTC-3', specific for the deleted portion of the m*PTEN* gene.

Neonatal Estrogenic Treatment

DES (Sigma-Aldrich, St Louis, MO, USA), genistein (Sigma-Aldrich) or estriol (E₃) (Sigma-Aldrich) was dissolved in ethanol (Sigma-Aldrich) and corn oil

(Nacalai Tesque, Kyoto, Japan). Female pups were treated daily subcutaneously (s.c.) with the estrogenic agent or vehicle alone from the 1st to 5th day after birth. The doses were 1 ng/g/day of DES, 50 μ g/g/day of genistein, and 4 μ g/g/day of E_3 . In this experiment, *mPTEN*^{+/−} (64 in number) and wild-type mice (64 in number) were divided into four groups of eight animals, which were designated as the DES-treated group, genistein-treated group, E_3 -treated group and vehicle group. First, eight pups of each group of both *mPTEN*^{+/−} and wild-type mice were killed on the 6th day after birth to evaluate the estrogenic activity of DES, genistein, and E_3 . The remaining mice were weaned at 21–22 days of age, housed five per cage, and maintained. Newbolds *et al*^{28,29} reported that the same amount of DES exposure induced endometrial carcinomas at 78 weeks, but not at 52 weeks. Therefore, to evaluate the *PTEN*-related lesions modified by neonatal estrogenic agents, we killed the mice at 52 weeks of age, before the development of carcinomas directly induced by the neonatal estrogenic treatment.

Measurement of Body and Uterine Wet Weights of *mPTEN*^{+/−} and Wild-Type Mice

To evaluate the estrogenic activity of DES, genistein, and E_3 on the uteri, eight female pups per group of the treated animals were killed by inhalation of diethylether (Wako Pure Chemical Industries, Osaka, Japan) on the morning of day 6 after birth. At 52 weeks of age, the female mice were killed by inhalation of diethylether followed by cervical dislocation to evaluate the influence of neonatal estrogenic exposure on their uteri.

In both age groups, body weights were first recorded. On dissection, the uteri were collected quickly, trimmed of extraneous connective tissue, and uterine wet weights then recorded. The percentage ratios of the uterine wet weight to body weight in the estrogenic treatment groups were compared with that of the vehicle group. Data are expressed as the mean $\pm$ standard error (s.e.).

Histological Examination

One horn of the uterus removed from each mouse was stored at -80°C for molecular assay. The other horn was fixed in 10% buffered formalin, embedded in paraffin, and 4- μ m-thick sections cut. Tissue sections were stained with hematoxylin and eosin (H&E) and examined under a light microscope. H&E-stained sections were reviewed by the same gynecological pathologist. The uterine sections were analyzed and diagnosed as endometrial CAH and adenocarcinoma according to the 'World Health Organization Classification of Tumors' for the female genital tract.⁴⁰

Analysis of Stromal Cell Density

The number of endometrial stromal cells was quantified in the H&E-stained sections of all groups using a VM-60 Video Micrometer System (Olympus Co., Tokyo, Japan). The numbers of stromal cells from three random areas (1000 μm^2) of each group were averaged.

Real-Time Quantitative Reverse Transcription-PCR for *Hoxa 10* and *Hoxa 11* Expression

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) from the uterine tissue of one horn from mice of all groups. After RNA extraction, cDNA was generated with 2.5 μ g of total RNA using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions.

Quantification of PCR products was conducted using a fluorescence-based real-time detection method (ABI Prism 7700 Sequence Detection TaqMan System; Applied Biosystems, Foster City, CA, USA). Assay-on-Demand primers and probes were used for *Hoxa 10* and *Hoxa 11*, and an internal control gene, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Applied Biosystems). The PCR was carried out with 2 $\times$ TaqMan Universal PCR master mix (Applied Biosystems), 1.25 μ l of 20 $\times$ Assay-on-Demand (primer and probes), and 2 μ l of cDNA in a 25 μ l final reaction mixture. Conditions consisted of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 15 s of denaturation at 95°C and a combined annealing–extension step at 60°C for 1 min. A negative control, consisting of nuclease-free water in place of template cDNA, was included in each PCR run. The RNA of vas deferens removed from wild-type male mice, which express *Hoxa 10* and *Hoxa 11*,⁴¹ was used as a control calibrator on each plate.

Statistics

Arithmetic means and s.e.'s were calculated for each group. The significance of differences between groups was determined using the protected least significant difference of Fisher's test (ANOVA analysis). $P < 0.05$ was considered significant. Statistical analysis was performed using the statistical software package StatView 5.0 version (SAS Institute Inc., Cary, NC, USA).

Results

Ratio of Uterine Wet Weight to Body Weight at 6 Days after Neonatal Treatment with DES, Genistein, or E_3

In *mPTEN*^{+/−} mice, when compared with vehicle alone, the DES and E_3 treatments resulted in

$166.47 \pm 14.72\%$ and $187.54 \pm 31.21\%$, respectively, gain in the relative ratio of uterine wet weight to body weight (Figure 1, left side; $P < 0.01$). In wild-type mice, both agents also increased the ratio to $163.43 \pm 12.44\%$ and $176.88 \pm 9.40\%$, respectively (Figure 1, right side; $P < 0.01$). Although the ratio was not statistically significant, genistein treatment also increased the uterine weight ratio to $134.40 \pm 9.41\%$ and $142.08 \pm 6.51\%$ in *mPTEN*^{+/−} and wild-type mice, respectively (Figure 1).

Figure 1 Comparison of hormonal activity of DES, genistein, and E_3 in the neonate. Percentage ratio of uterine wet weight of mice at day 6 after birth. The relative ratios were significantly increased in *mPTEN*^{+/−} (left side) and wild-type (right side) mice with DES (D) and E_3 (E) treatments compared to vehicle (V) alone. Genistein (G) treatment nonsignificantly increased the ratio. *Significantly different ($P < 0.01$) from V alone.

Ratio of Uterine Wet Weight to Body Weight at 52 Weeks after Neonatal Treatment of DES, Genistein, or E_3 Treatment

Uterine wet weights and body weights were examined in 31 wild-type mice and 28 *mPTEN*^{+/−} mice. Two of the eight *mPTEN*^{+/−} mice with DES treatment died at 32 and 44 weeks after treatment: one due to lymphoma and the other due to an unknown cause. At 52 weeks of age, myometrial tumors were observed in one wild-type animal with neonatal DES treatment, and in two *mPTEN*^{+/−} mice with neonatal genistein and E_3 treatments. These three mice were excluded from subsequent analysis. Consequently, in *mPTEN*^{+/−} mice, when compared with vehicle alone, the neonatal DES and E_3 treatments significantly reduced the weight relative ratio to $44.53 \pm 8.11\%$ (Figure 2, left side; $P < 0.01$) and $37.71 \pm 8.88\%$ (Figure 2, left side; $P < 0.01$), respectively. In wild-type mice, the neonatal E_3 treatment also decreased the weight ratio to $56.61 \pm 5.05\%$ (Figure 2, right side; $P < 0.05$). Macroscopically, atrophic uteri were observed in both the E_3 treatment and DES treatment mice. The uteri in *mPTEN*^{+/−} mice with neonatal E_3 treatment showed the most atrophy (Figure 2, left side). In contrast, neonatal DES treatment did not induce significant weight loss ($75.19 \pm 10.61\%$) in wild-type mice (Figure 2, right side). In addition, neonatal genistein treatment did not induce signi-

Figure 2 Effects of neonatal treatment with DES, genistein, and E_3 on uterine weight in *mPTEN*^{+/−} and wild-type mice at 52 weeks of age. Percentage ratio of uterine wet weight to body weight of mice. The relative ratios were significantly decreased in *mPTEN*^{+/−} mice with DES (D) and E_3 (E) treatments (left side) and in wild-type mice with E_3 treatment (right side) compared to vehicle (V) alone. Genistein (G) treatment did not induce weight loss. Uteri at killing are shown below the respective graphs. * or **Significantly different ($P < 0.05$, $P < 0.01$) from V alone.

Figure 3 Endometrial lesion profiles in 52-week-old *mPTEN*^{+/-} and wild-type mice without estrogenic treatment. (a) Multifocal CAH in *mPTEN*^{+/-} mice. Magnification $\times 4$, inset, $\times 20$. (b) Normal endometrium in wild-type mice. Magnification $\times 4$, inset, $\times 20$. (c) Well-differentiated adenocarcinoma in two *mPTEN*^{+/-} mice. Magnification $\times 10$, inset, $\times 40$. (d) Poorly differentiated adenocarcinoma in a *mPTEN*^{+/-} mouse. Magnification $\times 10$, inset, $\times 40$.

ficant weight loss in either *mPTEN*^{+/-} mice ($85.50 \pm 16.36\%$) or wild-type mice ($92.63 \pm 5.54\%$) (Figure 2).

Endometrial Lesions in *mPTEN*^{+/-} Mice Without Neonatal Estrogenic Treatments

In *mPTEN*^{+/-} mice without neonatal estrogenic treatments, light microscopic evaluation at 52 weeks of age of H&E-stained sections revealed endometrial lesions characterized by increased architectural complexity and cytological atypia of endometrial glands. The lesions closely resembled in human CAH (Figure 3a). Seven of the eight *mPTEN*^{+/-} mice had multifocal CAH (more than three focal lesions) (Table 1). Notably, age-matched wild-type mice did not develop these lesions (Figure 3b). Two *mPTEN*^{+/-} mice had well-differentiated adenocarcinomas characterized by the proliferation of atypical epithelial cells to varying degrees, and cribriform and confluent glands without intervening stroma (Figure 3c). One *mPTEN*^{+/-} mice had well-differentiated adenocarcinoma coexistent with poorly differentiated adenocarcinoma composed of solid parts in which cells had markedly enlarged, pleomorphic nuclei displaying irregular coarse chromatin and prominent, eosinophilic nucleoli (Figure 3d).

Effect of Neonatal Estrogenic Exposure on Endometrial Epithelium

The incidence of CAH in *mPTEN*^{+/-} mice was reduced by neonatal estrogenic treatment; three of the six mice (50%) with DES treatment, three of the

eight mice (38%) with genistein treatment, two of the eight mice (25%) with E_3 treatment, and seven of the eight mice (88%) with vehicle treatment (Table 1; $P < 0.05$ in genistein and E_3 treatment groups). All of the three DES-treated mice with CAH, one of the three genistein-treated, and one of the two E_3 -treated mice showed focal lesions, while the others had multifocal lesions (Table 1). The total number of individual focal CAH was counted in maximum areas of longitudinal sections of one horn in each mouse. The average number of focal CAH per one

Figure 4 The numbers of focal lesions of CAH in 52-week-old *mPTEN*^{+/-} mice after neonatal estrogenic exposure. Focal CAH were counted in H&E-stained longitudinal sections of one uterine horn per mouse. Each bar shows the mean of focal CAH in each group ($n = 8$), except DES ($n = 6$, 2 mice died at 32 and 44 weeks after treatment). *Significantly different ($P < 0.01$) from V alone. Group V: vehicle alone; group D: DES treated; group G: genistein treated; and group E: E_3 treated.

Table 1 Histopathological findings in the endometria of 52-week-old mice

<i>Estrogenic agents</i>	<i>Vehicle</i>		<i>DES</i>		<i>Genistein</i>		<i>E₃</i>	
<i>Mouse</i>	<i>Wild</i>	<i>mPTEN</i> ^{+/-}	<i>Wild</i>	<i>mPTEN</i> ^{+/-}	<i>Wild</i>	<i>mPTEN</i> ^{+/-}	<i>Wild</i>	<i>mPTEN</i> ^{+/-}
Epithelium								
			* —————					
			NS —————					
Adenocarcinoma	0/8	3/8	0/8	0/6	0/8	1/8	0/8	0/8
			* —————					
			NS —————					
Complex atypical hyperplasia	0/8	7/8	0/8	3/6	0/8	3/8	0/8	2/8
			** —————					
			** —————					
Multiple foci	0/8	7/8	0/8	0/6	0/8	2/8	0/8	1/8
	0/8	0/8	0/8	3/6	0/8	1/8	0/8	1/8
	0/8	0/8	5/8	0/6	0/8	0/8	0/8	0/8
Stroma								
Hyalinization	0/8	0/8	6/8	2/6	4/8	4/8	4/8	0/8
Atrophy	0/8	0/8	1/8	1/6	0/8	0/8	2/8	4/8

NS = nonsignificant, compared with vehicle group.

* $P < 0.05$, compared with vehicle group.

** $P < 0.01$, compared with vehicle group.

uterine horn was significantly lower in *mPTEN*^{+/-} mice with DES, genistein, and *E*₃ treatments than in the mice with vehicle alone (Figure 4; $P < 0.01$). Moreover, while three of the eight (38%) *mPTEN*^{+/-} mice with vehicle treatment had adenocarcinomas (Figure 3c and d), none of the *mPTEN*^{+/-} mice with DES and *E*₃ treatments, and one of the eight (13%) *mPTEN*^{+/-} mice with genistein treatment had adenocarcinomas (Table 1). The incidence of adenocarcinomas in the *mPTEN*^{+/-} mice with DES and *E*₃ treatments was significantly lower than that in the mice with vehicle alone (Table 1; $P < 0.05$). Neonatal estrogenic treatments induced neither CAH nor adenocarcinomas in wild-type mice (Figure 5a–c). As for different epithelial lesions, squamous metaplasia developed in five of the eight wild-type mice with neonatal DES treatment (Figure 5a), but not in the other mice (Table 1).

Effect of Neonatal Estrogenic Exposure on the Endometrial Stroma

Endometrial stroma was characterized by hyalinization or atrophy after neonatal estrogenic treatment. Hyaline was deposited in the endometrial stroma in the *mPTEN*^{+/-} mice with DES treatment (two of the six mice) and genistein treatment (four of the eight

mice) (Figure 5d and e) and in all treated wild-type mice (Figure 5a–c, Table 1). The endometrial stroma was atrophic in DES- and *E*₃-treated wild-type (one of the eight mice with DES treatment and two of the eight mice with *E*₃ treatment) and *mPTEN*^{+/-} mice (one of the six mice with DES treatment and four of the eight mice with *E*₃ treatment) (Figure 5f, Table 1). Neither hyalinization nor atrophy of the stroma was found in any mice with vehicle alone (Table 1). The density of stromal cells was significantly reduced in both *mPTEN*^{+/-} and wild-type mice with DES, genistein, and *E*₃ treatments compared with vehicle alone (Figure 6a and b; $*P < 0.05$ and $**P < 0.01$). In *mPTEN*^{+/-} mice treated neonatally with estrogenic agents, CAH were rare in the hyalinized or atrophic area of the stroma, whereas in the same mice CAH developed in the areas containing sufficient stromal cells (Figure 7).

Expression of *Hoxa 10* and *Hoxa 11* in the Uteri of 52-Week-Old Mice after Neonatal Estrogenic Treatment

Hoxa genes were highly expressed in the uteri of *mPTEN*^{+/-} mice without estrogenic treatment. *Hoxa 10* showed 1.25 ± 0.70 -fold, and *Hoxa 11* 1.75 ± 1.55 -fold, increased expression over that of

Figure 5 Morphological alterations in endometrium in 52-week-old wild-type and *mPTEN*^{+/-} mice neonatally treated with DES, genistein, and *E*₃. (a–c) representative wild-type mice with DES, genistein, and *E*₃ treatments, respectively, showing stromal hyalinization. Squamous metaplasias are only present in mice with DES treatment (a). (d–f) representative *mPTEN*^{+/-} mice with DES, genistein, and *E*₃ treatments, respectively, showing stromal hyalinization and scanty or absence of glands in the stroma. Magnification $\times 4$, inset $\times 20$.

wild-type mice (Figure 8a and b). Neonatal estrogenic exposure significantly decreased the expression level of *Hoxa 10* (0.56 ± 0.42 -fold; Figure 8a; $P < 0.05$) and *Hoxa 11* (0.51 ± 0.44 -fold; Figure 8b; $P < 0.05$) in the uteri of *mPTEN*^{+/-} mice with genistein treatment, *Hoxa 10* (0.50 ± 0.58 -fold; Figure 8a; $P < 0.05$) and *Hoxa 11* (0.45 ± 0.20 -fold; Figure 8b; $P < 0.01$) in the mice with E_3 treatment, and *Hoxa 11* alone in the mice with DES treatment (0.51 ± 0.32 -fold; Figure 8b; $P < 0.05$), when compared with the vehicle mice. No significant differences in the mRNA expression level of these genes were observed among the groups of wild-type mice (Figure 8a and b).

Discussion

In humans, the use of exogenous estrogen as hormone replacement therapy has been suggested by many studies to significantly increase the risk of adenocarcinoma of the endometrium in postmenopausal women.⁴² Tamoxifen, a therapeutic agent used for breast carcinoma, is a potent synthetic estrogen known to increase the risk of developing endometrial carcinoma.⁴³ PCOS with unopposed estrogen action on the endometrium is associated with endometrial carcinoma.⁴⁴ Obesity with unopposed estrogen action is also a well-known risk factor for endometrial carcinomas.⁴⁵ Animal model studies have demonstrated the induction of endometrial carcinomas after neonatal treatment with estrogenic agents, that is, DES,^{28,29,46} genistein,²⁹ 17 β -estradiol,⁴⁷ tamoxifen,⁴⁸ *p*-*t*-octylphenol,⁴⁹ and bisphenol A.⁵⁰ However, the role of genotoxic, epigenetic, and hormonal actions of neonatal estrogenic exposure remains unclear in tumor initiation. Simultaneously, the neonatal estrogenic agents are also well known to induce morphological alterations of the endometrium during development of the reproductive tract in mice.^{30–32}

We first hypothesized that low doses of neonatal estrogenic agents stimulate *PTEN*-related carcinogenesis. Contrary to our expectations, at 52 weeks of

Figure 6 Stromal cell density was calculated by counting the cell number in three fields of vision ($1000 \mu\text{m}^2$) at a magnification of $\times 40$ /section of vehicle-treated (V), DES-treated (D), genistein-treated (G), and E_3 -treated (E) groups of *mPTEN*^{+/-} (a) and wild-type (b) mice. Each bar shows the mean number of stromal cells in each group ($n = 8$, except group D of *mPTEN*^{+/-}; $n = 6$). * or **Significantly different (* $P < 0.05$, ** $P < 0.01$) from V alone.

Figure 7 Photomicrographs of an endometrium of 52-week-old *mPTEN*^{+/-} mice after neonatal exposure to estrogenic agents. H&E-stained tissue sections from a mouse with genistein treatment. CAH of the glands were surrounded by sufficient stromal cells; on the opposite side, there were no CAH in the hyalinized stroma. Magnification $\times 4$, inset $\times 20$.

Figure 8 (a and b) *Hoxa 10* and *Hoxa 11* expression in the uteri of mPTEN^{+/-} and wild-type mice treated neonatally with DES, genistein, and E₂, as detected by real-time RT-PCR. The relative mRNA levels of *Hoxa 10* (a) and *Hoxa 11* (b) are shown in reference to *GAPDH*. Each bar shows the mean of eight mice, except the DES-treated group (D) (mPTEN^{+/-} mice = 5, wild = 7) and E₂-treated group (E) (mPTEN^{+/-} mice = 7). In the DES- and E₂-treated groups, one mouse in each group had myometrial tumors, and RNA was not available for one mouse in the DES-treated group of mPTEN^{+/-} mice. * or **Significantly different (**P* < 0.05, ***P* < 0.01) from V alone. Group G: genistein-treated group.

age, the incidence of CAH and adenocarcinomas of the endometrium had dramatically decreased upon neonatal estrogenic treatment in mPTEN^{+/-} mice. Coincidentally, atrophic and/or hyalinized stroma was often observed in the mice after neonatal estrogenic treatment, and CAH and adenocarcinomas rarely occurred in the epithelium adjacent to the affected stroma. Recent molecular studies have focused on the clustered homeobox genes *Hoxa 10* and *Hoxa 11*. Both are expressed in the developing female reproductive tract, particularly endometrium.^{39,51} The developmental expression pattern of these genes is similar in mice and human.³⁹ Exposure to DES during development has been shown to irreversibly repress the expression of *Hoxa 10* and *Hoxa 11*, and is responsible for the altera-

tions in the developmental program of uterine differentiation.^{33,34,37} The importance of both genes has been explained by studies using null mice. *Hoxa 10* and *Hoxa 11* knockout mice resulted in a significant loss of endometrial stromal cells.^{35,52} Moreover, the neonatal DES-induced repression of *Hoxa 10* and *Hoxa 11* expression in the uterus reduces the number of stromal cells in mice.⁵³ In the present study, exposure to neonatal estrogenic agents, other than DES, reduced *Hoxa 10* and *Hoxa 11* expression in mPTEN^{+/-} mice. The repression of these genes may be responsible for the reduction in stromal cells and the rare development of CAH and adenocarcinomas. Recently, human endometrial and ovarian endometrioid adenocarcinomas have been shown to frequently over express *Hoxa 10*.^{54,55} *Hoxa 10* expression may play another different role in carcinogenesis with endometrioid features.

In the present study, the neonatal estrogenic agents did not enhance endometrial tumorigenesis in mice. Kabbarah *et al*⁵⁶ treated mice carrying knockout alleles for *MLH1* or *MSH2* with DES during the neonatal period, and also failed to develop endometrial carcinomas. Hence, the mechanism of endometrial tumorigenesis induced by neonatal estrogenic exposure most likely differs from that of endometrial carcinogenesis associated with mutations of *PTEN* or DNA mismatch repair genes. Distinct alterations in gene expression following neonatal DES exposure have been reported for *EGF*, *EGF* receptor, *c-fos*, *c-jun*, *c-myc*, *bax*, and *bcl-2*.^{57–59} Although further studies will be required, neonatal estrogenic exposure might affect such genes, and induce carcinomas independently of surrounding stromal cells in adult mice.

Approximately 3 million women in the United States were treated with DES during pregnancy in an attempt to prevent threatened and repeated abortions in late 1940 to early 1970s.⁶⁰ In 1971, Herbst *et al*⁶¹ pointed a strong association between DES use in pregnancy and the occurrence of vaginal clear-cell adenocarcinoma in exposed female offspring. The developmental anomalies of the female reproductive tract including T-shaped hypoplastic uteri and malformation of the cervix were also observed in the DES-exposed offspring.^{61,62} Although 705 cases of clear-cell adenocarcinomas of the vagina and cervix have been reported, there were only two pathologically confirmed case reports of endometrial carcinomas.^{63,64} In a recent cohort study, the incidence of endometrial carcinomas in DES-exposed persons was lower than that of unexposed persons.⁶⁵ These findings in human might be consistent with this study using the mice model.

Taken together, neonatal estrogenic treatments exerted an inhibitory, rather than stimulatory, effect on *PTEN*-related carcinogenesis via stromal alterations. CAH and carcinomas rarely occurred in the atrophic and/or hyalinized stroma, resulting from an irreversible repression of *Hoxa 10* and *Hoxa 11* through these neonatal estrogenic exposures. This

study can be expected to provide new insight into the interaction between the endometrial epithelium and stroma in endometrial carcinogenesis *in vivo*.

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