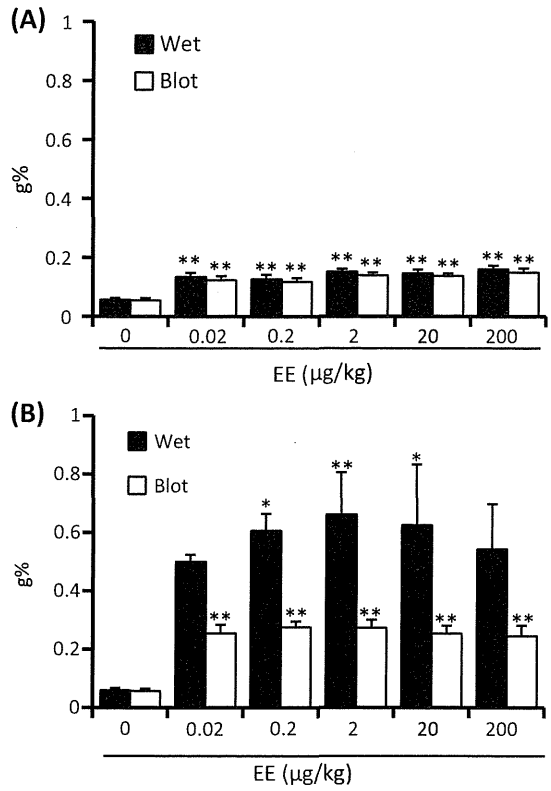

Fig. 1. Relative uterine weights in the uterotrophic assay using immature female rats after single (A) and 3 serial (B) EE injections. *,**: Significantly different from the 0 μg/kg group at $p < 0.05$ and $p < 0.01$, respectively.

were not affected by EE, when compared using the numbers of litter per group. Significant increases in body weight were observed in the 0.02, 0.2, 20, and 200 μg/kg groups during weeks 5–15; however these changes were transient and not dose-dependent. After week 16, there were no intergroup differences in body weight. In the 0.02 μg/kg group, 2 animals were moribund at weeks 36 and 42, and diagnosed with myeloblastic leukemia and carcinoma of the anterior pituitary based on histopathologic examination. These cases were considered to be incidental, since dose-dependency was not found.

The average day of vaginal opening was PND 31 in all groups, when analyzing by individual pups as well as litters; there were no differences among the groups (Table 2). The sequential change in the incidence of normal estrous cyclicity is shown in Fig. 2. In the 0 μg/kg group, all animals had regular 4- or 5-day cycles until 26 weeks of age. Thereafter, the animals with abnormal cycles increased gradually, and the percentage of normal estrous cyclicity at 42 weeks of age was 54%. In contrast, a few animals demonstrated persistent estrus at 7 weeks of age in the 200 μg/kg group. A statistically significant increase in the incidence of abnormal cycles

Table 2
Mean days of vaginal opening in rats exposed to EE during the neonatal period.

EE (μg/kg)	Vaginal opening			
	Per animals	(*n*)	Per litters	(*n*)
0	31.2 ± 1.3[a]	(24)	31.3 ± 0.9	(7)
0.02	31.5 ± 1.6	(24)	31.8 ± 1.3	(8)
0.2	31.6 ± 1.5	(24)	31.5 ± 1.1	(8)
2	31.3 ± 1.0	(24)	31.1 ± 0.6	(8)
20	31.2 ± 0.8	(24)	31.1 ± 0.5	(7)
200	31.5 ± 1.7	(24)	31.4 ± 1.4	(9)

[a] Mean ± SD.

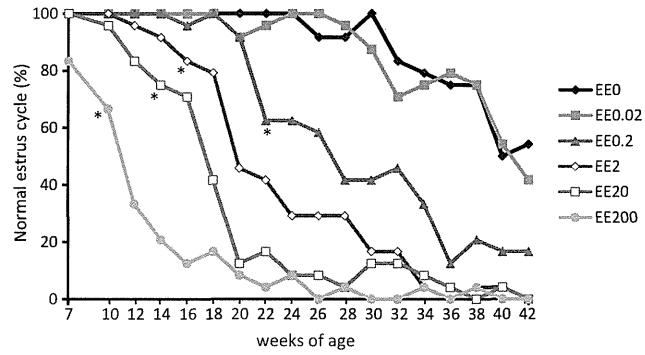

Fig. 2. Sequential change in the incidence of normal estrous cyclicity. $n = 24$ per each group. In the 0.02 μg/kg group, 2 animals were excluded due to tumors at weeks 36 and 42. *: Significantly different from the 0 μg/kg group hereafter at $p < 0.05$ (Fisher's exact test).

was detected in 10-week-old rats at 200 μg/kg, 14-week-old rats at 20 μg/kg, 18-week-old rats at 2 μg/kg and 22-week-old rats at 0.2 μg/kg compared to the 0 μg/kg group. Most of the animals had persistent estrus and the incidence was increased in an age- and dose-dependent fashion. Abnormal cycles other than persistent estrus were continuous proestrus and/or estrus for 3 or 4 days, and persistent diestrus was not found. At 0.02 μg/kg, the incidence of abnormal cycles was similar to the 0 μg/kg group throughout the study.

3.4. Uterine carcinogenicity and histopathology at 10 months of age

The final body and organ weights are summarized in Table 3. The absolute and relative ovarian weights were significantly decreased at ≥2 μg/kg, with a decreasing tendency in the 0.2 μg/kg group when large cysts or masses diagnosed as ovaritis or para-ovarian cysts were excluded. The absolute and relative weights of the uterus were significantly elevated at 0.02 μg/kg.

Based on the histopathologic examination, cystic atretic follicles and loss of the corpus lutea, which suggest anovulation, were observed in most animals at ≥0.2 μg/kg and associated with a decrease in ovarian weight (Table 4). Additionally, interstitial glands were increased in association with these findings and the incidence was significantly elevated in the highest dose group. Similar morphologic changes were also noted in the animals with persistent estrus in the control and 0.02 μg/kg groups. Several primary and antral follicles remained, with no obvious variation among the groups.

The uterine findings are shown in Table 5. Although there were no statistical differences in the incidence and multiplicity of atypical hyperplasia, severe lesions existed in higher dose groups (≥2 μg/kg). Similarly, adenocarcinomas were only observed in the 20 and 200 μg/kg groups. The incidence of cystic endometrial hyperplasia was significantly elevated at 2 and 20 μg/kg. With respect to non-proliferative lesions, squamous metaplasia of the uterine glands was significantly increased from 0.2 μg/kg. At the highest dose, the incidence of adenomyosis was statistically decreased; an animal with disappearance of the uterine lumen was also noted. Endometrial stromal polyps were commonly found in all groups. Fibromas, granular cell tumors, squamous cell hyperplasia of the cervix, and hemangiomas/hemangiosarcomas occurred sporadically in all groups without significant differences.

In the mammary glands, although increased milk secretion was frequently observed, the incidence and severity were similar among the groups (Table 6). The incidence of atypical hyperplasia was only increased statistically in the 20 μg/kg group. Although small in number, neoplastic lesions, such as adenomas

Table 3
Body and organ weights in 10-month-old rats that received neonatal injections of EE.

	EE (μg/kg)					
	0	0.02	0.2	2	20	200
No. of animals examined	24	22[b]	24	24	24	24
Body weight (g)	307.4 ± 28.1[a]	304.3 ± 28.7	318.7 ± 31.8	298.7 ± 31.7	312.6 ± 41.6	316.8 ± 41.7
Ovaries[c]						
(mg)	76.0 ± 24.4	74.7 ± 21.4	60.0 ± 18.2	44.9 ± 7.4**,d	55.6 ± 12.6*,d	53.3 ± 19.1**,d
(mg%)[e]	25.0 ± 9.2	24.7 ± 7.3	19.1 ± 6.8	15.1 ± 3.3**,d	17.8 ± 4.2[d]	17.0 ± 5.8**,d
Uterus[c]						
(g)	1.16 ± 0.54	2.13 ± 1.32*	1.06 ± 0.59	1.10 ± 0.40	1.19 ± 0.27	1.12 ± 0.57
(g%)	0.38 ± 0.21	0.73 ± 0.51*	0.34 ± 0.21	0.38 ± 0.17	0.39 ± 0.12	0.36 ± 0.19

* Significantly different from the 0 μg/kg group at 0.05.
** Significantly different from the 0 μg/kg group at 0.01.
[a] Mean ± SD.
[b] Number of effective animals was reduced to 22 due to 2 animals bearing tumors of the pelvic cavity and pituitary.
[c] 2 animals per group were excluded from measurement of organ weight due to perfusion.
[d] 1 animal in the 2 μg/kg group, 2 animals in the 20 μg/kg group and 1 animal in the 200 μg/kg group that were histologically diagnosed with ovaritis or para-ovarian cysts were excluded.
[e] Ovarian weight (mg)/body weight (g) × 100.

Table 4
Histopathologic findings of the ovaries observed in rats that received neonatal injections of EE.

	EE (μg/kg)					
	0	0.02	0.2	2	20	200
No. of animals examined	24[a]	24[b]	24[a]	24[a]	24[a]	24[a]
Cystic atretic follicles	9 (38%)	7 (32%)	19 (79%)**	24 (100%)**	23 (96%)**	23 (96%)**
Loss of corpus lutea	6 (25%)	4 (17%)	17 (71%)**	21 (88%)**	19 (79%)**	23 (96%)**
Increase of interstitial glands	7 (29%)	3 (14%)	12 (50%)	8 (33%)	12 (50%)	15 (63%)*

* Significantly different from the 0 μg/kg group at 0.05 (Fisher's exact test).
** Significantly different from the 0 μg/kg group at 0.01 (Fisher's exact test).
[a] All animals were autopsied at 44-week-old.
[b] 2 animals were examined at 36- and 42-week -old, and the others were autopsied at 44-week-old.

Table 5
Histopathologic findings of the uterus observed in rats that received neonatal injections of EE.

	EE (μg/kg)					
	0	0.02	0.2	2	20	200
No. of animals examined	24[b]	24[c]	24[b]	24[b]	24[b]	24[b]
Proliferative lesions						
Atypical hyperplasia	13 (54%)	20 (83%)	16 (67%)	19 (79%)	17 (17%)	20 (83%)
Slight	7	15	6	10	6	7
Moderate	6	5	10	5	7	9
Severe	0	0	0	4	4	4
Multiplicity of atypical hyperplasia[a]	1.08 ± 0.28	1.05 ± 0.22	1.25 ± 0.45	1.21 ± 0.42	1.24 ± 0.44	1.35 ± 0.59
Cystic endometrial hyperplasia	8 (33%)	11 (46%)	14 (58%)	22 (92%)**	19 (79%)**	16 (67%)
Adenocarcinoma	0	0	0	0	3 (13%)	2 (8%)
Other lesions						
Squamous metaplasia	1 (4%)	0	9 (38%)*	11 (46%)**	12 (50%)**	7 (29%)*
Adenomyosis	5 (21%)	11 (46%)	4 (17%)	3 (13%)	1 (4%)	0*
Disappearance of lumina	0	0	0	0	0	1 (4%)

* Significantly different from the 0 μg/kg group at 0.05 (Fisher's exact test).
** Significantly different from the 0 μg/kg group at 0.01 (Fisher's exact test).
[a] The average number per rat with hyperplasia (mean ± SD).
[b] All animals were autopsied at 44-week-old.
[c] 2 animals were examined at 36- and 42-week-old, and the others were autopsied at 44-week-old.

and fibroadenomas, were found at 20 and 200 μg/kg. At ≧0.2 μg/kg, some acini exhibiting oxyphilic and hypertrophic changes, like normal mammary glands of male rats (Fig. 3), and the incidence of oxyphilic cells was increased in a dose-dependent fashion. There were no intergroup differences in hyperplasia, adenomas and carcinomas of the anterior pituitary (data not shown). No significant findings were noted in the vagina, adrenal glands, liver, thymus, brain, and thyroid.

3.5. Sex related hormone level at 10 months of age

The serum P4 level was significantly lowered at ≧2 μg/kg (Fig. 4). When compared by the cycle pattern, the level of P4 in animals showing persistent estrus was generally lower than that in animals showing normal cycle, although there were large fluctuations between individual rats. There were no intergroup differences in the serum levels of any other hormones.

Table 6
Histopathologic findings of the mammary glands observed in rats that received neonatal injections of EE.

	EE (μg/kg)					
	0	0.02	0.2	2	20	200
No. of animals examined	24[a]	24[b]	24[a]	24[a]	24[a]	24[a]
Increased milk secretion	15 (63%)	18 (75%)	18 (75%)	13 (54%)	17 (71%)	18 (75%)
Slight	11	16	10	7	7	8
Moderate	2	1	8	6	6	9
Severe	2	1	0	0	4	1
Atypical hyperplasia	3 (13%)	2 (8%)	3 (13%)	2 (8%)	11 (46%)*	7 (29%)
Lobular hyperplasia	0	0	2 (8%)	2 (8%)	3 (13%)	2 (8%)
Ductal hyperplasia	0	0	0	0	1 (4%)	0
Adenoma	0	0	0	0	1 (4%)	1 (4%)
Fibroadenoma	0	0	0	0	1 (4%)	0
Oxyphilic cells/virilization	0	0	6 (25%)*	5 (21%)*	8 (33%)**	13 (54%)**

* Significantly different from the 0 μg/kg group at 0.05 (Fisher's exact test).
** Significantly different from the 0 μg/kg group at 0.01 (Fisher's exact test).
[a] All animals were autopsied at 44-week-old.
[b] 2 animals were examined at 36- and 42-week -old, and the others were autopsied at 44-week-old.

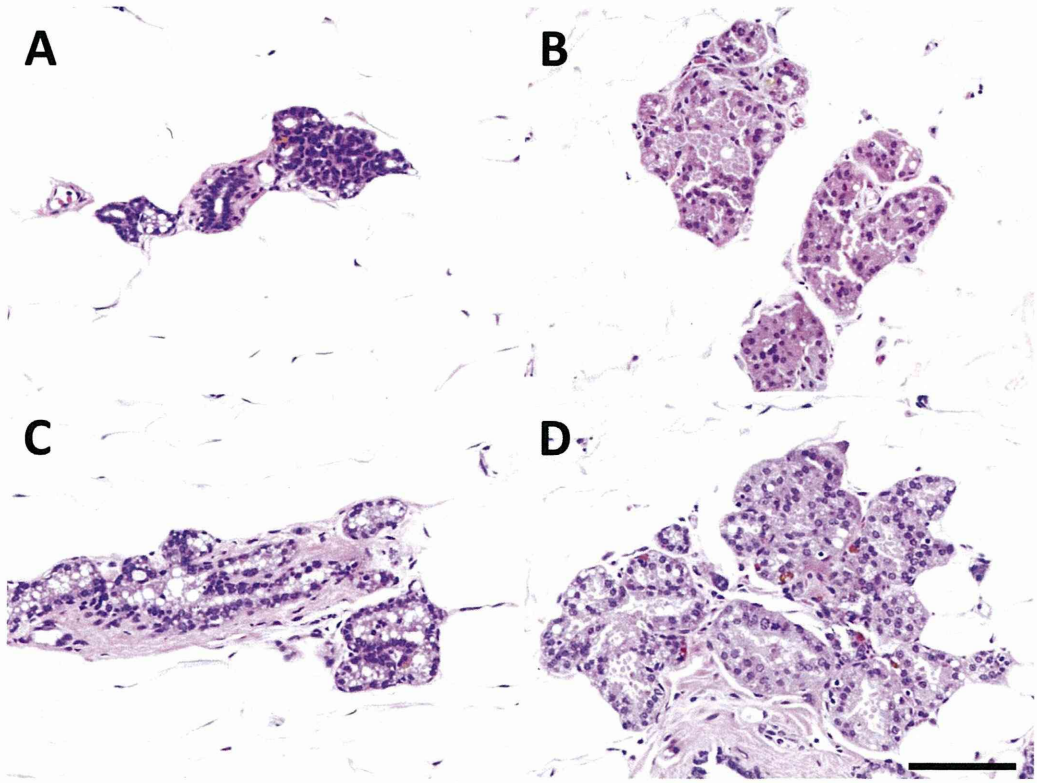

Fig. 3. Oxyphilic change of the mammary glands observed in 10-month-old female rats exposed to EE during the neonatal period. Normal mammary gland of a female rat in the 0 μg/kg group, which was lined by 1–2 layers of low cuboidal epithelium (A). In intact adult males, the acini are composed of large, pale-staining, foamy, and vacuolated cells (B). At ≧0.2 μg/kg, some acini exhibited oxyphilic and hypertrophic changes, resembling normal mammary glands of male rats (C, 0.2 μg/kg; D, 200 μg/kg). Bar = 100 μm.

4. Discussion

In the present study, a single injection of EE (0–200 μg/kg) during the neonatal period in rats did not affect body weight growth and puberty. The average day of vaginal opening was PND 31 in all groups; however, after sexual maturation, animals demonstrating abnormal estrous cycles were significantly increased from 0.2 μg/kg, and it was shown that delayed adverse effects were inducible by several hours of exposure to EE during the neonatal period. Most animals had persistent estrus, indicating anovulation. Although abnormal cycles occur spontaneously in aging animals, it was notable that the onset and incidence of abnormal cycles were accelerated in a dose-dependent fashion in the EE-treated groups. Based on the results of the uterotrophic assay, the dose of EE associated with delayed effects was within the dose range with estrogenic activity. In addition, the existence of a threshold in delayed effects was suggested because the early onset of abnormal cycles did not occur in the 0.02 μg/kg group. In agreement with our previous study using Donryu rats exposed to DES [2], estrous cyclicity was regarded as a very useful indicator of delayed toxic effects on the female reproductive tract, which clearly demonstrated age- and dose-dependent effects. Since the exposure time to EE is very limited compared to DES, it is considered that the present model is more sensitive than DES model. Abnormal cycles began at 10–22 weeks of age, and therefore it was considered that detection of such effects would be difficult using required reproductive toxicity studies, including extended one-generation reproductive toxicity study, by regulatory bodies/governmental authorities because

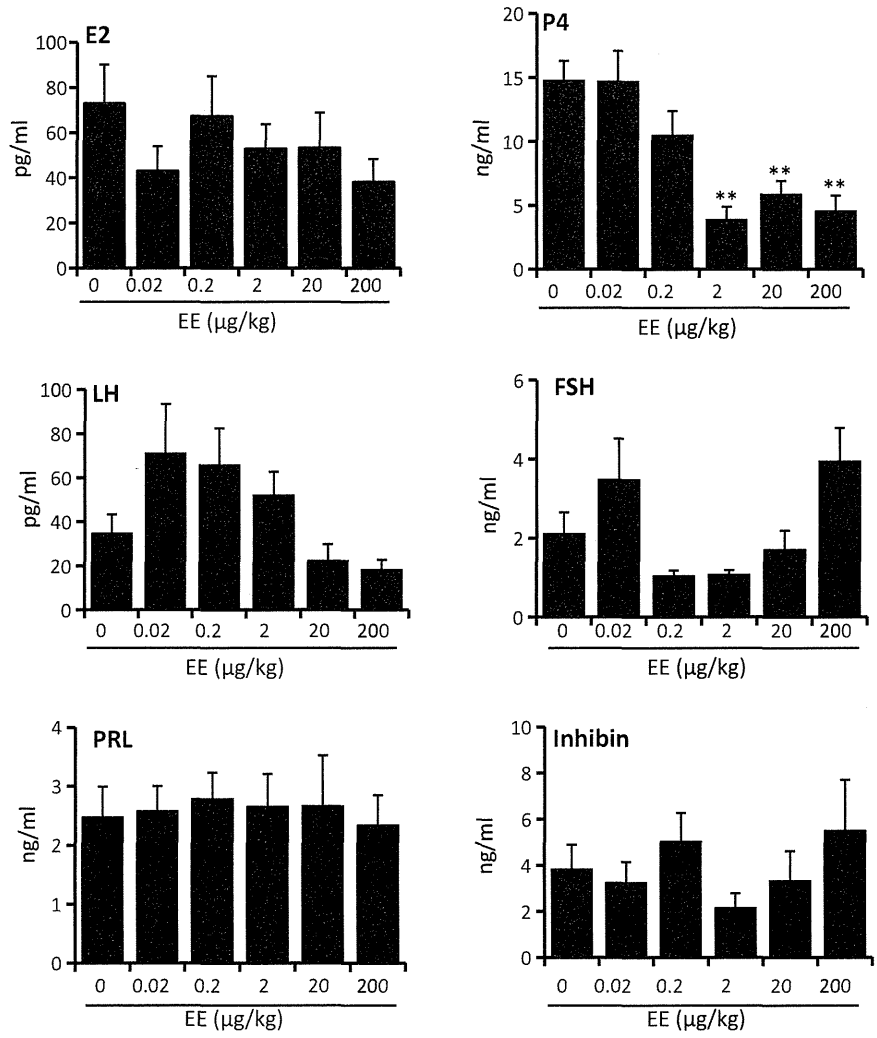

Fig. 4. Serum level of sex-related hormones at 10 months of age. Data are the mean ± SEM. $n = 22$ per each group. In the 0.02 μg/kg group, 2 animals were excluded due to tumors at weeks 36 and 42. **: Significantly different from the 0 μg/kg group at $p < 0.01$.

there are no studies covering the neonatal period to middle age.

To examine the long-term effects of neonatal exposure to EE, we performed histopathologic examinations of female reproductive organs at 10 months of age. A decrease or decreasing tendency of ovarian weight existed from the 0.2 μg/kg group, and histopathologic examinations revealed cystic atretic follicles and loss of corpus lutea in the same groups, which suggests anovulation. Such findings were also noted in the animals showing persistent estrous in the control and 0.02 μg/kg groups. Therefore, the morphologic changes induced by delayed adverse effects in the ovary were regarded as changes related to aging, rather than abnormalities specific to EE-treatment. In contrast, there was no obvious variation in the remaining primary and antral follicles and serum hormone levels secreted by follicles, such as E2 and inhibin, suggesting that anovulation is not caused by dysfunction of the ovary and depletion of reserve follicles.

Although no statistical differences were noted in the incidence and multiplicity of uterine atypical hyperplasia and adenocarcinoma, there was a tendency toward increased severity of lesions at ≧2 μg/kg. Moreover, the incidence of cystic endometrial hyperplasia was significantly elevated at 2 and 20 μg/kg. In these groups, the serum P4 level was significantly lowered due to a loss of corpus lutea by persistent anovulation. Because the serum E2 level was unchanged, the estrogen:progesterone (E:P) ratio was elevated in these groups after the onset of abnormal estrous cyclicity. A prolonged increase in the E:P ratio is regarded as an important factor for the development of endometrial adenocarcinoma in rodents, as well as humans [12,18–20]. Therefore, an increasing tendency toward severity of uterine proliferative lesions in the higher dose groups might be caused by an elevated E:P ratio. Similarly, previous studies reported that the early onset of persistent estrus and an increase in uterine adenocarcinoma were observed in Donryu rats that received a single injection of DES or high-dose *p*-*t*-octylphenol, an estrogenic chemical, during the neonatal period [2,21]. In addition, a significant increase in squamous metaplasia was apparent from 0.2 μg/kg, the dose associated with delayed adverse effects in estrous cyclicity. Prolonged estrogen exposure has been associated with squamous metaplasia in rats for many years; however, the precise underlying mechanism is not known [22]. Therefore, it is thought that the findings observed in the current study reflect hormonal imbalance (a reduction in P4 relative to E2) due to the early onset of anovulation.

At the highest dose of EE, disappearance of the lumen occurred in one case. Similar morphologic abnormalities were reported in rats that had neonatal exposure to high doses of DES [2]. Adenomyosis is commonly observed in aging GALAS rats, and the incidence is 12–40% in the 1- or 2-year toxicity studies in our laboratory (data not shown). In the present study, the incidences of adenomyosis in the control and low- or middle-dose groups were similar to

our background data, but the incidence in high-dose groups was decreased. It is known that early postnatal exposure to estrogenic compounds can suppress uterine gland genesis and expression of estrogen receptors, and can alter the uterine response to estrogen [23–25]. Therefore, development of uterine glands might be affected by neonatal exposure to EE, especially at high doses, and lead to a decrease in adenomyosis. Although the absolute and relative weights of the uterus were significantly elevated at 0.02 μg/kg, the toxicologic significance is not known because there were no histologic findings related to the increase in uterine weight and uterine weights in the higher dose groups were not changed.

Neonatal exposure to EE did not affect the proliferative lesions in the anterior pituitary and serum level of pituitary hormones, including PRL. In addition, there were no intergroup differences in the incidence and severity of milk secretion in the mammary glands. In the 20 μg/kg group, the incidence of atypical hyperplasia was increased, and neoplastic lesions, such as adenomas and fibroadenomas, were only found from 20 μg/kg. Neonatal exposure to DES in rats has been reported to affect mammary carcinogenesis, although the types of tumors induced varied by dose and timing [26]; the cause remained undetermined, but the increase in atypical hyperplasia at 20 μg/kg, the inducible level of delayed effects, might be related to EE-treatment. In contrast, an increase in acini exhibiting oxyphilic and hypertrophic changes (virilization) was observed from 0.2 μg/kg in a dose-dependent manner. It has been reported that virilization of mammary acini appeared in female rats that received neonatal administration of DES [26,27]. In these studies, oxyphilic changes were noted in rats administered DES from PND 0-14 and PND 0-5, whereas rats administered DES from PND 6-14 did not exhibit such a change. Therefore, the critical period of endocrine disruptors affecting mammary morphology was thought to be from 0 to 5 days after birth. In a recent report similar to our study, changes in the mammary glands such as milk accumulations and hyperplasia were found in adult rats received single neonatal exposure to EE [28]. Accordingly, although precise mechanisms remain unknown, it was thought that neonatal exposure to EE is likely to have some effects on development of mammary glands directly and resulted in oxyphilic changes in the current study.

The critical mechanism underlying the early onset of abnormal estrous cycles is unknown; however, dysfunction of the ovulation center in the hypothalamus is presumed to be a possible mechanism regulating anovulation, because there were no intergroup differences in the remaining follicles in the ovary and the serum levels of pituitary hormones, such as FSH, LH, and PRL, at 10 months of age. Kisspeptin, which is expressed in specific neurons in the anteroventral periventricular nucleus and arcuate nucleus of the hypothalamus, is widely recognized to play a critical role in female reproductive function, including regulation of ovulation and estrous cyclicity [29,30]. It has been reported that neonatal injection of estradiol benzoate to male and female rats results in a dose-dependent decrease in hypothalamic kiss-1 mRNA levels in the prepubertal stage, which is linked to lowering of serum LH concentrations [31]. Therefore, neurons expressing kisspeptin might be a target of neonatal exposure to EE. Research to elucidate the relationship between the expression of the kiss-1 gene and delayed adverse effects is now in progress in our laboratory.

5. Conclusion

In summary, our results clearly demonstrated that neonatal exposure to EE at doses of 0.2–200 μg/kg, which exert estrogenic activity *in vivo*, induces early onset of anovulation in a dose-dependent fashion after sexual maturation. Estrous cyclicity is regarded as a very useful indicator of delayed adverse effects on the female reproductive tract. Dysfunction of the ovulation center in the hypothalamus is presumed to be a possible mechanism underlying the early onset of anovulation based on the lack of abnormalities in the remaining follicles and pituitary hormones, although the precise mechanism has not been delineated. As a long-term effect derived from the early onset of anovulation, it is suggested that prolonged estrogen exposure might increase the risk for uterine carcinogenesis. In contrast, there was a possibility that neonatal exposure to EE could directly affect development of mammary glands.

Conflict of interest statement

The authors have no conflict of interest.

Acknowledgments

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Thickened area of external granular layer and Ki-67 positive focus are early events of medulloblastoma in *Ptch1*$^{+/-}$ mice

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ABSTRACT

Patched1 (Ptch1) encodes a receptor for Sonic hedgehog (Shh) and is major gene related to human medulloblastoma (MB) in the Shh subgroup. MB is thought to arise from residual granule cell precursors (GCPs) located in the external granular layer (EGL) of the developing cerebellum. As the detailed preneoplastic changes of MB remain obscure, we immunohistochemically clarified the derived cell, early events of MBs, and the cerebellar developmental processes of *Ptch1*$^{+/-}$ (Ptch1) mice, an animal model of human MB of the Shh subgroup. In Ptch1 mice, the earliest proliferative lesions were detected at PND10 as focal thickened areas of outer layer of the EGL. This area was composed of GCP-like cells with atypia and nuclei disarrangement. In the latter cerebellar developmental period, GCP-like cell foci were detected at high incidence in the outermost area of the cerebellum. Their localization and morphological similarities indicated that the foci were derived from GCPs in the EGL. There were two types of the foci. A Ki-67-positive focus was found in Ptch1 mice only. This type resembled the GCPs in the outer layer of EGL characterized by having proliferating activity and a lack of neuronal differentiation. Another type of focus, Ki-67-negative, was observed in both genotypes and exhibited many of the same features of mature internal granule cells, suggesting that the focus had no preneoplastic potential. Due to morphological, immunohistochemical characteristics, our results indicate that the focal thickened area of EGL and Ki-67-positive foci are preneoplastic lesions of MB.

1. Introduction

Medulloblastoma (MB) is the most common malignant tumor in children which shows tremendous biological and clinical heterogeneity (Dhall, 2009; Hatten and Roussel, 2011; Jones et al., 2012). MB in humans is classified into four subtypes with distinct clinical, biological, and genetic profiles (Aref et al., 2012; Ellison et al., 2011; Jones et al., 2012; Kool et al., 2012; Mohan et al., 2012; Northcott et al., 2011). Molecular analysis of Sonic hedgehog (Shh) tumors in humans revealed activation of the Shh signaling pathway due to the loss of *Patched1 (Ptch1)* and mutations in other components of the Shh pathway. Approximately as high as 30% of MBs have mutations in Shh pathway components (Bhatia et al., 2012; Crawford et al., 2007; Klesse and Bowers, 2010; Oliver et al., 2005; Roussel and Hatten, 2011; Wang et al., 2012). *Ptch1* encodes a receptor for Shh, Patched1 (Ptch1), and is one of the major genes related to MB formation in humans (Dhall, 2009; Raffel, 2004). A subset of MBs has been identified with allelic loss of chromosome 9q22, a region that contains *Ptch1* (Dhall, 2009; Raffel, 2004). Pathway activation is triggered by binding of Shh to Ptch1, which in the absence of Shh suppresses the activity of Smoothened (Smo). Shh binding to Ptch1 or mutational inactivation of Ptch1 relieves the inhibition of Smo culminating in the activation of one or more of the Gli1 transcription factors that regulate the expression of downstream targets (Huse and Holland, 2010; Roussel and Hatten, 2011). Inappropriate activation of the Shh pathway is accepted as a cause of familial cancer due to inherited mutation of the *Ptch1* gene, which has been identified as responsible for nevoid basal cell carcinoma syndrome (Dhall, 2009; Klesse and Bowers, 2010).

Heterozygous *Ptch1* knockout mice (Ptch1 mice) display many of the typical symptoms of nevoid basal cell carcinoma syndrome, also known as Gorlin syndrome, including skeletal abnormalities, neural tube closure defects, a generalized over-growth, and predisposition to tumor formation (Corcoran and Scott, 2001; Hahn et al., 1999; Raffel, 2004). In addition, the Ptch1 mouse strain displays a high yield (14% up to 30%) of MB that resembles human

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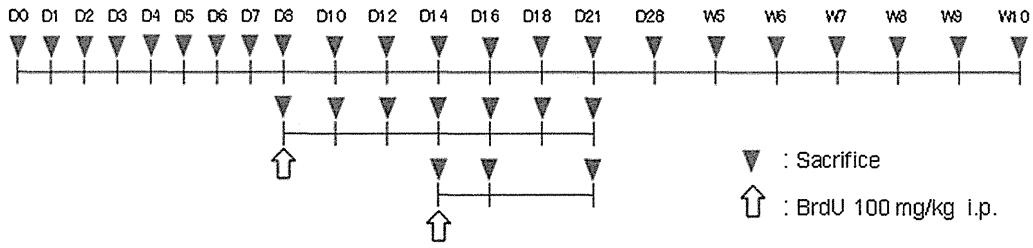

Fig. 1. Experimental design. Schedules for necropsy and the administration of BrdU are illustrated. Each time point represents at least 2 wild-type mice and 6 Ptch1 mice from over 2 dams. D, postnatal day; W, postnatal week.

MB of the Shh subgroup (Goodrich et al., 1997; Lau et al., 2012; Wetmore et al., 2000). In the mice, homozygous loss of *Ptch1* results in embryonic lethality at 9.5–10.5 days after fertilization (Goodrich et al., 1997). Thus, heterozygous *Ptch1* knockout mice have been used as a model for nevoid basal cell carcinoma/Gorlin syndrome including human MB, rhabdomyosarcoma, and basal cell carcinoma (Corcoran and Scott, 2001; Dyer, 2004; Hahn et al., 1999; Pazzaglia, 2006; Wu et al., 2011). Although Ptch1 mice are a valuable model for evaluation of drug efficacy and modulating effects of additional gene mutations, chemicals, or irradiation on brain tumor formation in childhood, the long latent period of 9 to over 12 months for assessment results in clinical signs of increased intracranial pressure (ataxia, decreased movement, paresis of hind limbs, enlarged occipital prominence, hunched back, and/or poor grooming) and death (Ayrault et al., 2009; Briggs et al., 2008; Ecke et al., 2008; Farioli-Vecchioli et al., 2007; Kimura et al., 2005; Pazzaglia et al., 2006, 2009; Pogoriler et al., 2006; Takahashi et al., 2012; Uziel et al., 2005; Wetmore et al., 2001). Therefore, detection of early indicators of MBs such as preneoplastic lesions in Ptch1 mice and evaluation with changes as an indicator of MB in short-term studies is needed.

To find early indicators of tumors in childhood, detailed investigation of normal developmental processes of target organs can be useful. Human MBs are thought to be derived from residual granule cell precursors (GCPs) located in the external granule cell or external granular (germinal) layer (EGL) of the cerebellum, although GCPs migrate inward to form the internal granule cell or internal granular layer (IGL) during normal cerebellar development (Behesti and Marino, 2009; Haldipur et al., 2012; Roussel and Hatten, 2011). The processes of cerebellar and MB development in Ptch1 mice are not well-defined.

This study was conducted to clarify the derived cell and early events of MBs, and cerebellar developmental processes in Ptch1 mice. We examined cerebella of Ptch1 mice and wild-type littermates sequentially during postnatal day (PND) 0 to 10 weeks of age.

2. Materials and methods

2.1. Animals

Ptch1 heterozygous knockout mice, generated by replacing exon 1 and 2 of the ptch1 gene with a LacZ/neomycin cassette (Goodrich et al., 1997), were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained in our laboratory. They were housed in polycarbonate cages with wood chip bedding and maintained in an air-conditioned animal room (temperature 24 ± 1 °C, relative humidity 55 ± 5%, 12-h light–dark cycle) with basal diet (CRF-1, Oriental Yeast Co., Tokyo, Japan) and tap water available *ad libitum*. The experimental protocol using animals was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

2.2. Necropsy

To examine following morphologic analysis necropsy was performed according to protocol (Fig. 1). Ptch1 and wild-type littermate mice were euthanized under deep anesthesia with isoflurane. 2–11 wild-type mice and 6–19 Ptch1 mice were analyzed at each time point from at least two litters.

2.3. Genotyping

Animals were genotyped by PCR amplification of genomic DNA extracted from the tail. The wild type allele was distinguished with primers 5′-CTG CGG CAA GTT TTT GGT TG-3′ and 5′-AGG GCT TCT CGT TGG CTA CAA G-3′, which yield a 200-bp PCR product. The mutant allele was detected using primers 5′-GCC CTG AAT GAA CTG CAG GAC G-3′ and 5′-CAC GGG TAG CCA ACG CTA TGT C-3′, which yield a 479-bp PCR product.

2.4. BrdU labeling

To examine migration of GCPs, a single intraperitoneal injection of 100 mg/kg body weight of 5-Bromo-2′-deoxyuridine (BrdU, CAS No. 59-14-3 Sigma–Aldrich, MO, USA) in saline (Otsuka Pharmaceutical Factory, Inc., Japan) was given to mice at PND8 and 14. Animals treated with BrdU at PND8 were euthanized as above 1.5 h after the injection and at PND10, 12, 14, 16, 18, and 21. Animals treated with BrdU at PND14 were euthanized 1.5 h after injection and at PND16 and 21 (Fig. 1).

Table 1
Primary antibodies used for immunohistochemistry.

Antigen	Clone	Concentration/dilution	Antigen retrieval	Visualization system	Source
BrdU	BU1/75(ICR1)	1 μg/mL	Autoclave	LSAB	AbD serotec
Ki-67	TEC-3	30 μg/mL	Autoclave	LSAB	Dako
NeuN	A60	3 μg/mL	Autoclave	Polymer	Millipore
$p27^{kip1}$	EP233(2)Y	1:2000	Autoclave	Polymer	Abcam
Nestin	Rat-401	3 μg/mL	Autoclave	Polymer	Millipore
CyclinD1	EPR2241(IHC)-32	1:300	Autoclave	Polymer	Millipore
GFAP	Polyclonal	1 μg/mL	Microwave	Polymer	Dako
Calbindin-D-28K	CB-955	3 μg/mL	Microwave	Polymer	Sigma–Aldrich

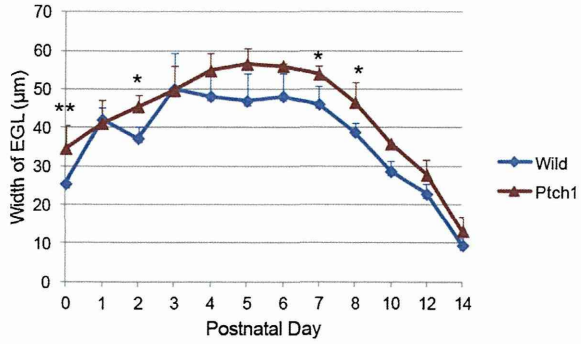

Fig. 2. Changes in the width of EGL in developing cerebellum of Ptch1 and wild-type mice from PND0 to PND14. *, **: Significantly different from wild-type mice at $P<0.05$ and $P<0.01$, respectively.

2.5. Tissue processing

After detailed necropsy, brains were removed and fixed in 10% neutral buffered formalin. Midsagittal sections of cerebella were routinely processed for paraffin embedding, sectioned and stained with hematoxylin and eosin. The prepared histopathological specimens were examined under light microscopy.

2.6. Morphometric assessment

Photomicrographs of midsagittal sections of the cerebellum were taken with a digital camera attached to microscope (DP71, Olympus Corp., Tokyo, Japan), and then measurement was performed using image analysis software (WinROOF, Version 5.7.1, Mitani Corp., Tokyo, Japan). The numbers of wild-type and Ptch1 mice measured at each time point were 3–6 obtained from 2 to 5 litters (mainly 3–5 litters) except for Ptch1 mice at PND10. At PND10, five mice were obtained from the same dam. The width of the EGL of each mouse was determined by five measurements selected at random from the entire cerebellum (PND0 to 2) or 4th/5th cerebellar lobules (PND3 to 14).

2.7. Immunohistochemistry

Antibodies used for immunohistochemistry included monoclonal rat anti-BrdU (AbD serotec, Oxford, UK), monoclonal rat anti-mouse Ki-67 (Dako Cytomation, Glostrup, Denmark) as proliferation marker, monoclonal mouse anti-NeuN (Millipore, MA, USA) as a mature granule cell marker, monoclonal rabbit anti-$p27^{kip1}$ (Abcam, Tokyo, Japan) as a postmitotic granule cell marker, monoclonal mouse anti-Nestin (Millipore) as a neuronal stem cell marker, monoclonal rabbit anti-CyclinD1 (Millipore) as a proliferating GCPs marker, polyclonal rabbit anti-GFAP (Dako Cytomation) as a Bergmann glia marker, and monoclonal mouse

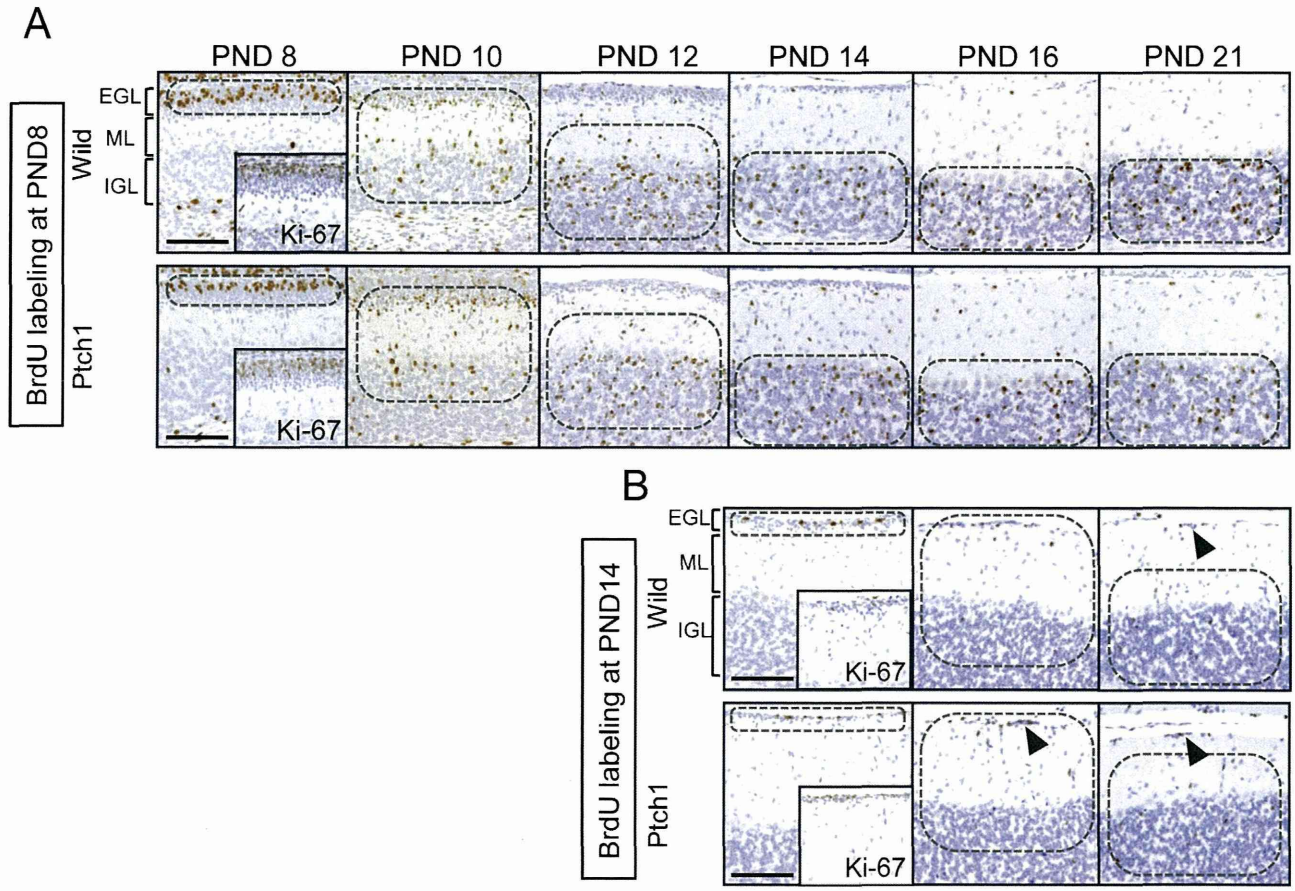

Fig. 3. Migration of GCPs of wild-type and Ptch1 mice from PND8 to PND21. (A) Sequential migration of GCPs labeled with BrdU at PND8 (1.5 h after injection) of wild-type (top row) and Ptch1 mice (bottom row). BrdU-positive cells were observed in Ki-67-positive, proliferating outer layers of the EGL 1.5 h later (PND8). At PND10 (2 days after injection), most of the BrdU-positive cells were localized in the inner layer of the EGL, molecular layer (ML), and IGL. The EGL almost disappeared at PND16 and most of the BrdU-positive cells finished migrating into the IGL by PND21. (B) Sequential migration of GCPs labeled with BrdU at PND14 of wild-type (top row) and Ptch1 mice (bottom row). BrdU-positive cells were observed in 1–3 layers of Ki-67-positive, proliferating cells of the EGL at PND14 (1.5 h after injection). From PND16 onwards when most of GCPs finished migrating from the EGL, small foci with GCP-like cells labeled with BrdU were detected in the outermost regions of cerebellar cortex (arrowhead). Circle indicates major location of BrdU-labeled GCPs. Scale bar: 100 μm.

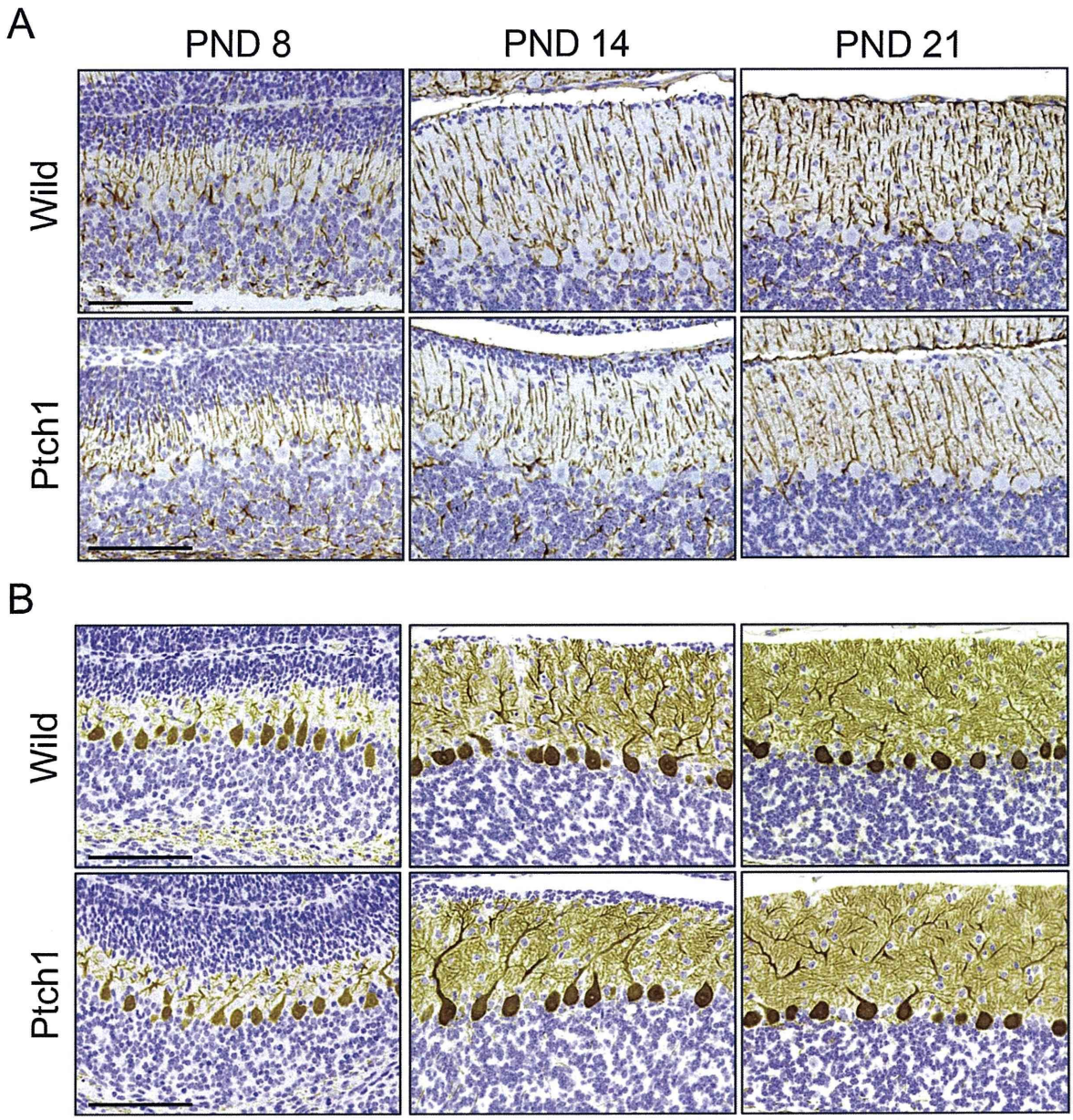

Fig. 4. Bergmann glia and Purkinje cells in the developing cerebellum of wild-type and Ptch1 mice. (A) Bergmann glia of wild-type (top row) and Ptch1 mice (bottom row) stained with anti-GFAP antibody at PND8, 14, and 21. No morphological abnormalities were observed in Ptch1 mice compared to wild-type mice. (B) Purkinje cells of wild-type (top row) and Ptch1 mice (bottom row) stained with anti-Calbindin-D-28K antibody at PND8, 14, and 21. No morphological abnormalities were observed in Ptch1 mice compared to wild-type mice. Scale bars: 100 μm.

anti-Calbindin-D-28K (Sigma–Aldrich, MO, USA) as a Purkinje cell marker. A labeled streptavidin-biotin method was applied for anti-BrdU and Ki-67 antibodies using polyclonal rabbit anti-rat biotinylated IgG (Dako Cytomation) and streptavidin-conjugated horseradish peroxidase (Dako Cytomation). A polymer method was applied for the rest of the primary antibodies using Histofine Simple Stain kit (Nichirei Biosciences Inc., Tokyo, Japan). The immunoreactions were visualized by peroxidase-diaminobenzidine reaction. The sections were then counterstained lightly with hematoxylin. Table 1 provides details of protocols for the immunohistochemistry and information of the antibodies.

2.8. Statistical analysis

The width of the EGL was analyzed by Student's *t*-test following a test for equal variance. The value of one litter per dam or average value of some litters from the same dam was statistically analyzed at PND0, 1, 2, 3, 7, 8, 12 and 14.

3. Results

3.1. General remarks

There were no significant differences in body weight from PND0 to PND21 among the genotypes of intact animals (data not shown). Mortality and body weight of mice were not affected by injection of BrdU. No clinical signs were detected in wild-type and Ptch1 mice. At necropsy, swelling of cerebellum and obscurity of lobular structure with a lack of cerebellar foliation which were diagnosed as MB microscopically were observed in Ptch1 mice at PND28 and W5. Hydrocephalus showing slight dilatation of ventricles of cerebrum and masses of skeletal muscles near the ribs or sternums