

Fig. 2. Changes in the width of EGL in developing cerebellum of Ptch1 and wild-type mice from PND0 to PND10. *, **: 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

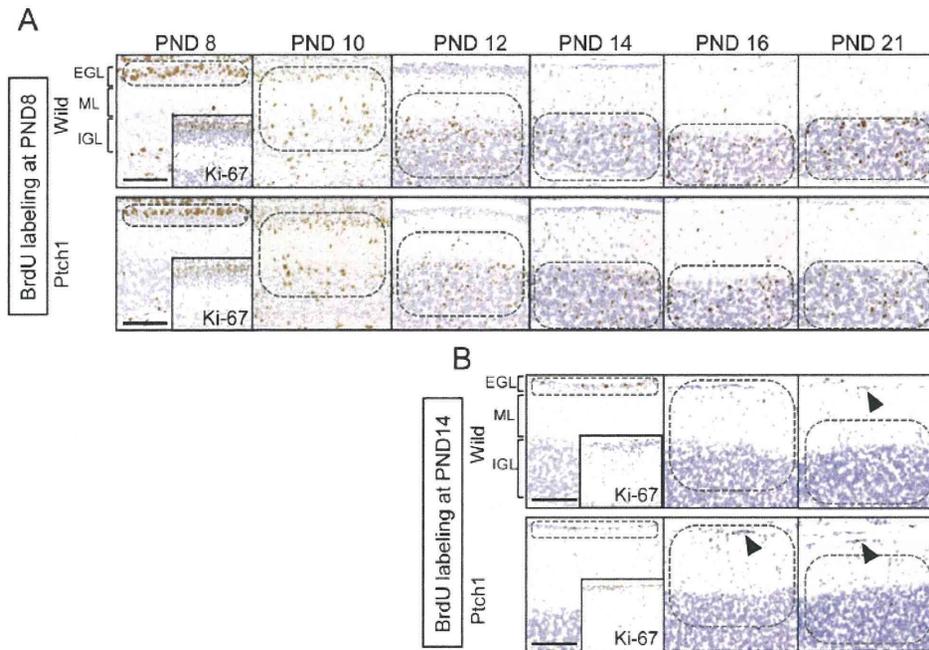


Fig. 3. Migration of GCPs of wild-type and Ptch1 mice from PND8 to PND21. (A) Sequential migration of GCPs labeled with BrdU at PND8 (1.5 h after injection) of wild-type (top row) and Ptch1 mice (bottom row). BrdU-positive cells were observed in Ki-67-positive, proliferating outer layers of the EGL 1.5 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 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.

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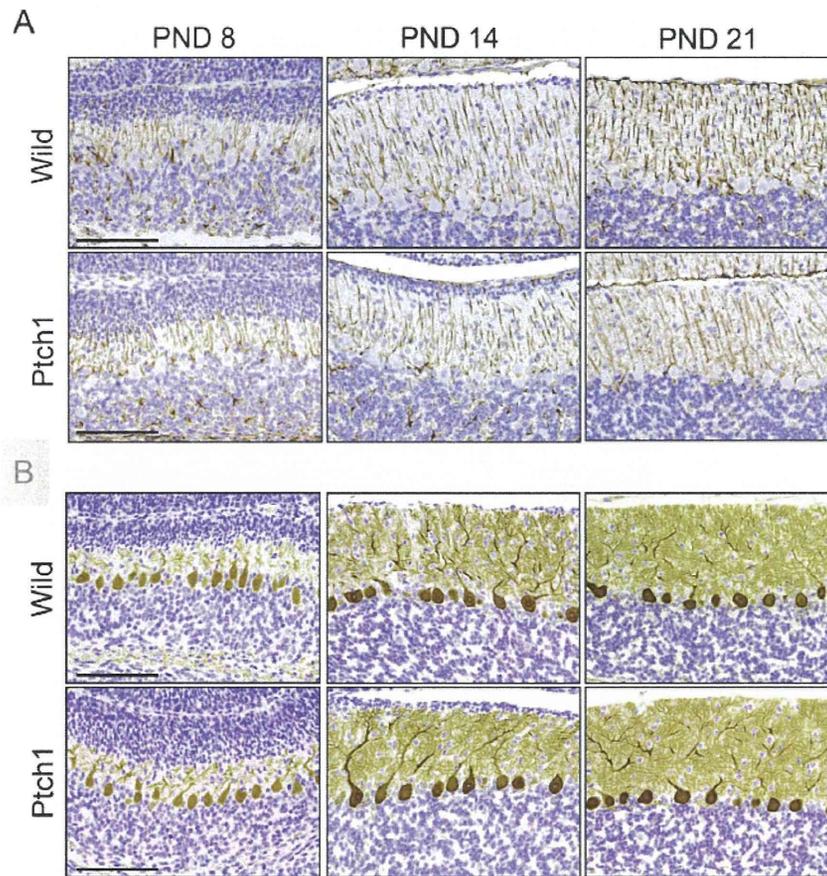


Fig. 4. Bergmann glia and Purkinje cells in the developing cerebellum of wild-type and *Ptch1* mice. (A) Bergmann glia of wild-type (top row) and *Ptch1* mice (bottom row) stained with anti-GFAP antibody at PND8, 14, and 21. No morphological abnormalities were observed in *Ptch1* mice compared to wild-type 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 μ m.

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

171 2.8. Statistical analysis

172 The width of the EGL was analyzed by Student's *t*-test following
173 a test for equal variance.

174 3. Results

175 3.1. General remarks

176 There were no significant differences in body weight from PND0
177 to PND21 among the genotypes of intact animals (data not shown).
178 Mortality and body weight of mice were not affected by injection
179 of BrdU. No clinical signs were detected in wild-type and *Ptch1*
180 mice. At necropsy, swelling of cerebellum and obscurity of lobular
181 structure with a lack of cerebellar foliation which were diagnosed
182 as MB microscopically were observed in *Ptch1* mice at PND28 and
183 W5. Hydrocephalus showing slight dilatation of ventricles of cere-
184 brum and masses of skeletal muscles near the ribs or sternums
185 and in the abdominal muscles were noted after PND3 in *Ptch1*
186 mice only. These intramuscular masses were diagnosed micro-
187 scopically as rhabdomyosarcomas. Macroscopically, there was no

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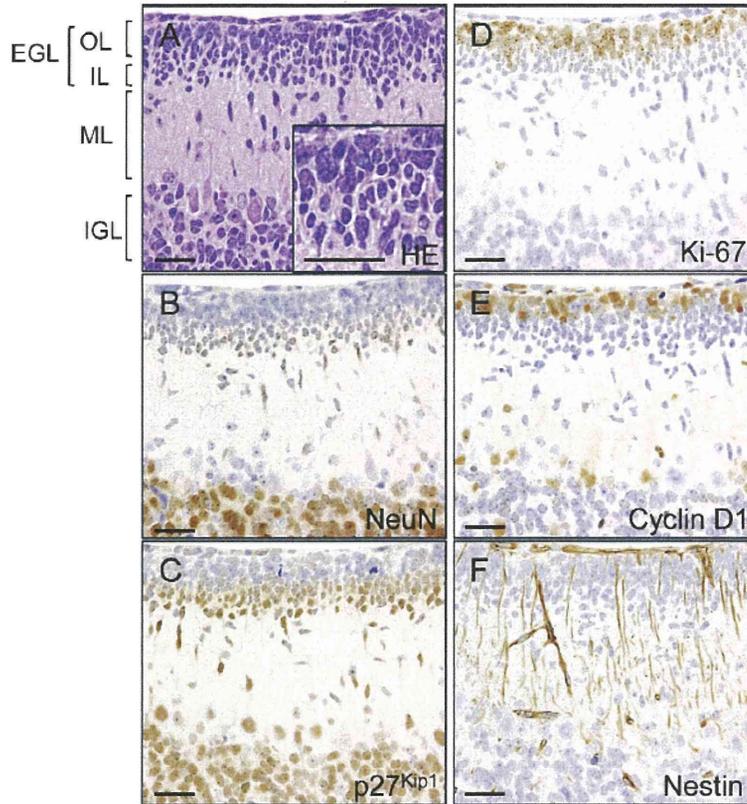


Fig. 5. Morphology and immunohistochemistry of the developing cerebellum. Representative morphology and immunohistochemistry of the developing cerebellar cortex from *Ptch1* mice (PND10). Their characteristics were common to both genotypes. (A) HE staining of the cerebellar cortex. EGL with asterisk was magnified (inset). (B) NeuN was weakly positive in the inner layer of the EGL (IL) and migrating granular cells in the molecular layer (ML) and strongly positive in the IGL. (C) p27^{kip1} was strongly positive in the IL and migrating granular cells in the ML and positive in the IGL. (D) Granular cells in the outer layer of the EGL (OL) were positive for Ki-67. (E) Granular cells in the OL were positive for Cyclin D1. (F) Positive staining for Nestin was observed radially from the surface of the cerebellum to the IGL in a straight line. Scale bars: 25 μm.

188 metastatic lesion of MB and rhabdomyosarcomas in tumor-bearing
189 mice.

190 3.2. Sequential changes in the width and migration process of the
191 EGL

192 Postnatal changes in the width of the EGL in wild-type and *Ptch1*
193 mice are shown in Fig. 2. The width of the EGL peaked at PND5 to
194 7 and the width in *Ptch1* mice was greater than in wild-type mice
195 during PND5 to 10.

196 Fig. 3 shows localization of BrdU-positive cells injected in
197 mice at PND8 (Fig. 3A) and PND14 (Fig. 3B), respectively. GCPs in
198 the outer layer of the EGL were the only cell type to be positive
199 for BrdU in the EGL. In animals injected with BrdU at PND8,
200 BrdU-positive cells were observed in the outer layer of the EGL
201 which was positive for Ki-67 1.5 h after the injection (Fig. 3A and
202 A insets). BrdU-positive EGL cells gradually migrated into the IGL
203 accompanied by thinning of the EGL in both genotypes. At PND16,
204 only one layer of the EGL was present and then disappeared at
205 PND21 in both genotypes.

206 3.3. Sequential changes of Purkinje cells and Bergmann glia in the
developing cerebellum

207 Purkinje cells and Bergmann glia are major components of the
208 cerebellum and have important roles in cerebellar development.
209 Long processes of the radial glial cells, Bergmann glia, were visual-
210 ized by immunohistochemistry with anti-GFAP antibody (Fig. 4A).
211 For both genotypes, there was no difference in the density or
212 extending direction of the processes at PND0 to 21. In addition,
213 Purkinje neurons (including dendritic arbors) were stained with
214 anti-Calbindin-D-28K antibody. From qualitative microscopic
215 inspection a difference in the number, alignment, or arborization
216 of Purkinje cells was not detected between the genotypes during
217 postnatal cerebellar development.

218 3.4. Morphology and immunohistochemical characteristics of
granule cells in the developing cerebellum

219 Morphology and immunohistochemical reactions to antibodies
220 related to cell proliferation and neuronal differentiation revealed

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Table 2
Morphological characteristic of granular precursor cells located in EGL, molecular layer, and IGL in developing cerebellum at PND0 to 14.

	EGL		Molecular layer	IGL
	Outer layer	Inner layer		
Histopathology	Medium-large size Round-polygonal	Small size Oval-elongate (vertical)	Small size Oval-spindle (vertical)	Medium size Round
Immunohistochemistry				
Ki-67	+	-	-	-
CyclinD1	+	-	-	-
NeuN	-	±	±	+ / ++
p27 ^{kip1}	-	++	+ / ++	+
Nestin	+	+	+	± / - ^b

-, negative; ±, weakly positive; +, positive; ++, strongly positive. These characteristics shown in the table are common to all time points measured (PND0 to 14); except for Nestin staining in the IGL.

^a Until PND10.

^b Day after PND10.

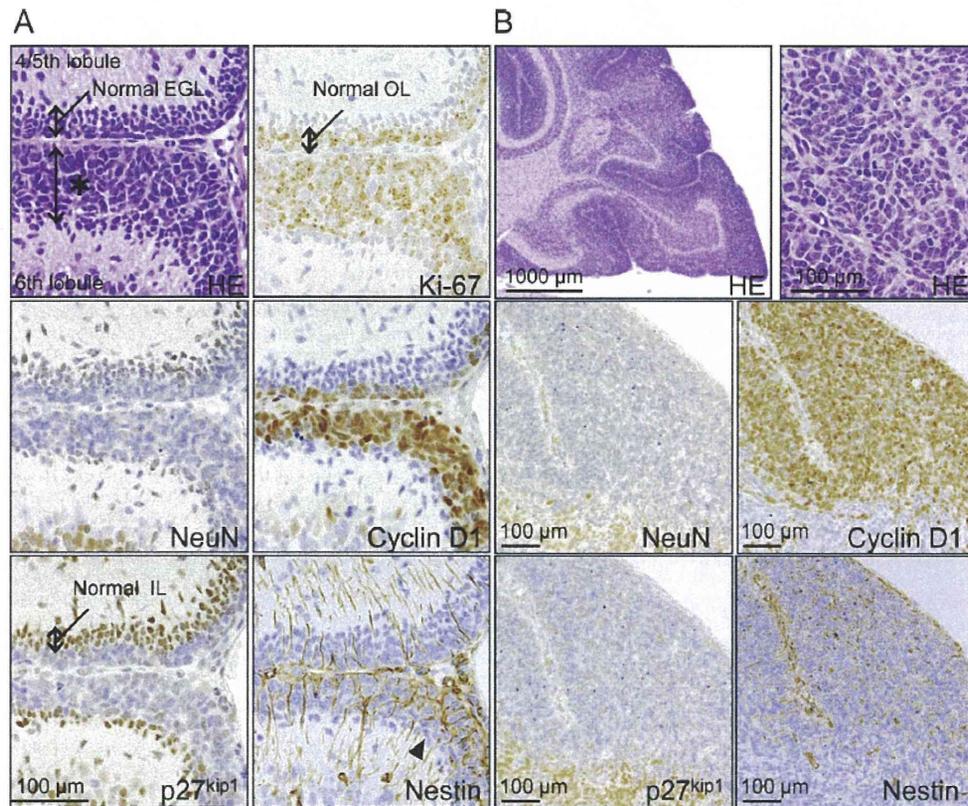


Fig. 6. Proliferative lesions in the developing cerebellum in *Ptch1* mice at PND10 and 12. (A) A thickened proliferative lesion (asterisk) in the outer layer of the EGL (6th lobule) in *Ptch1* mice at PND10. The 4th/5th lobule showed normal EGL structure. In this lesion, the outer layer of the EGL stained with Ki-67 and cyclinD1 was expanded. The inner layer of the EGL (IL) stained with NeuN and p27^{kip1} was thinned compared to the normal IL in the 4th/5th lobule. Positive staining for Nestin was observed intracellularly in all directions in this lesion (arrowhead). Scale bar: 100 μ m. (B) MB in *Ptch1* mice at PND12. Higher magnification of HE staining showed that proliferating cells resembled thickened lesions of the outer layer of the EGL.

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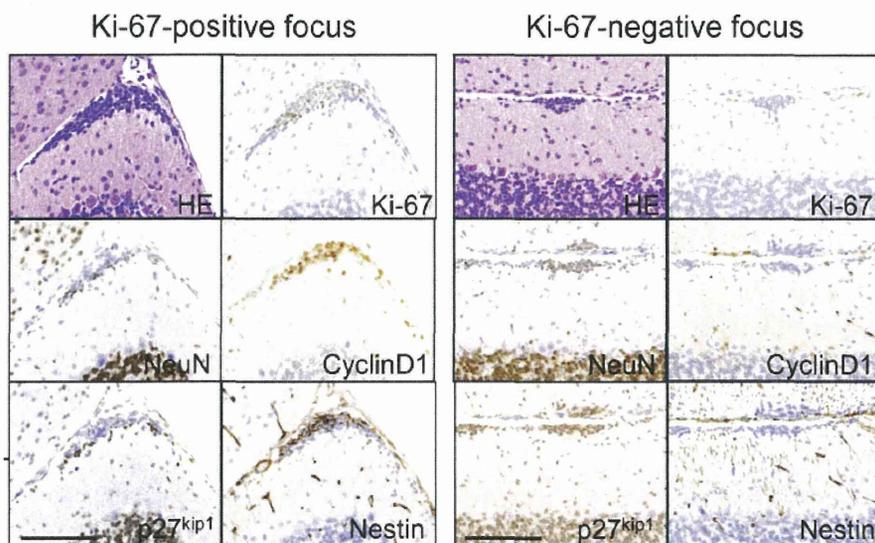


Fig. 7. Immunohistochemical characterization of foci composed of GCP-like cells. Ki-67-positive (left) and Ki-67-negative (right) foci of Ptch1 mice. Ki-67-positive foci were composed of medium to large-sized, round to polygonal nuclei that were negative or weakly positive for NeuN and p27^{kip1} and positive for cyclin D1 and nestin, indicating unclear differentiation into neuronal cells. Ki-67-negative foci were composed of round and small nuclei that were positive for NeuN and p27^{kip1} and negative for Cyclin D1 and nestin, indicating differentiation into neural cells similar to IGL cells. Scale bar: 50 μ m.

223 that the structure of the developing cerebellum was common to
224 both genotypes (Table 2 and Fig. 5). Nuclei of GCPs in the outer layer
225 of the EGL were medium to large-sized and round to polygonal.
226 These cells were positive for Ki-67 and Cyclin D1 and negative for
227 NeuN and p27^{kip1}. Inner layer EGL nuclei were small-sized, oval to
228 elongate, and arranged vertically. In this layer, granular cells were
229 negative for Ki-67 and Cyclin D1, weakly positive for NeuN and
230 strongly positive for p27^{kip1}. Migrating granular cells in the molecu-
231 lar layer were oval to spindle shaped small cells arranged vertically
232 and had the same staining profiles as inner layer EGL nuclei. Gran-
233 ular cells with medium-sized round nuclei in the IGL surrounded
234 eosinophilic mossy fibers. Those were negative for Ki-67 and Cyclin
235 D1 and positive to strongly positive for NeuN and p27^{kip1}. Positive
236 staining for nestin was observed radically throughout the cerebellar
237 cortex, and weak staining in the IGL remained until PND10.

238 3.5. Changes in the developing cerebellum and derived cells

239 Proliferative lesions were observed in the developing cerebel-
240 lum in Ptch1 mice. Proliferation of GCP-like cells was observed
241 in a thickened area of the EGL, which was continuous from the
242 normal EGL on and after PND10 in Ptch1 mice (Fig. 6A, aster-
243 isk). The proliferative lesions substituted for the inner layer of the
244 EGL (Fig. 6A). Moreover, early occurrence of MB was detected at
245 PND12 (Fig. 6B). In both proliferative lesions, large-sized, round
246 to polygonal cells with atypia were main components. Immuno-
247 histochemically, the cells were positive for Cyclin D1 and nestin
248 and weakly positive or negative for NeuN and p27^{kip1}. Inter-cellu-
249 lar irregular nestin staining was distinctive of these lesions (Fig. 6A
250 arrowhead and B), as nestin-positive fibers were arranged regularly
251 and radically in a straight line in the normal EGL (Figs. 5F and 6A).
252 Histopathology and immunohistochemistry of these proliferative
253 lesions resembled GCPs in the outer layer of the EGL of the normal
254 cerebellum.

249 After PND16, the time when migration of EGL cells to the IGL
250 has almost completed, foci with GCP-like cells were detected in the
251 outermost region of the cerebellar cortex in the cerebella of mice
252 from both genotypes (Figs. 3B and 7). In the mice injected with
253 BrdU at PND14, BrdU-positive cells were detected in the EGL 1.5 h
254 after treatment (Fig. 3B) and BrdU-positive cells had migrated into
255 the deep molecular layer and the IGL by PND21 (Fig. 3). As some of
256 the GCP-like cells of the foci in mice injected with BrdU at PND14
257 were positive for BrdU after PND16, the cells were considered to be
258 derived from the residual GCPs in the EGL (Fig. 3B, arrowhead). The
259 residual foci were clearly classified into two types: Ki-67-positive
260 and Ki-67-negative (Fig. 7). Ki-67-positive foci were composed of
261 atypical cells whose nuclei were medium to large-sized and round
262 to polygonal in shape (Fig. 7, left). The cells were immunohisto-
263 chemically negative or weakly positive for NeuN and p27^{kip1} and
264 positive for cyclin D1 and nestin (Fig. 7, left). Conversely, Ki-67-
265 negative foci were composed of cells with round and small shaped
266 nuclei. The cells were positive for NeuN and p27^{kip1} and negative
267 for nestin and cyclin D1, indicating differentiation into neural cells
268 (Fig. 7, right). Some of Ki-67-negative foci contained a strongly
269 eosinophilic area resembling mossy fibers among the nuclei (Fig. 8,
270 asterisk) and they were comparable to the structure of the IGL.

271 3.6. Relationship between residual foci of GCPs and MBs

272 The incidences of Ki-67-positive or negative foci and MBs were
273 investigated from PND16 up to 10 weeks of age in both genotypes
274 (Fig. 9). MBs were divided into two types: a focal MB occupying one
275 to two lobules of the cerebellum was defined as a small MB and an
276 advanced MB spreading over three or more lobules was defined as a
277 large MB. In Ptch1 mice, Ki-67-positive foci, small MB, and large MB
278 were observed and most of the mice had Ki-67-negative foci. The
279 peak of Ki-67-positive foci was up to PND21, the completion period
280 of cerebellar development. The incidences of MBs were comparable

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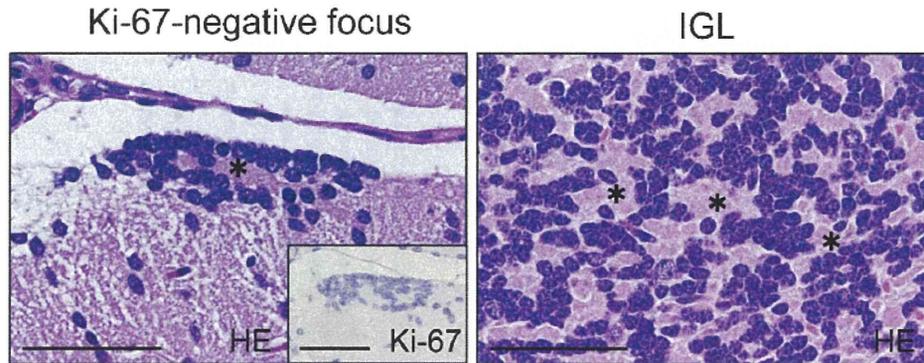


Fig. 8. Ki-67-negative foci showing differentiation into the IGL. HE staining of Ki-67-negative focus (left) and the IGL (right) of Ptch1 mice at PND21. An eosinophilic mossy fiber-like substance (arrowhead) was surrounded by nuclei of Ki-67-negative focus. Mossy fibers (asterisk) were observed among the nuclei of granular cells of the IGL. Scale bar: 50 μ m.

287 between ages. In wild-type mice, no proliferative lesions, neither
288 Ki-67-positive foci nor MBs other than Ki-67-negative foci, were
289 detected at any age (Fig. 9). The incidence of Ki-67-negative foci
290 decreased with increased age in wild-type mice. The number of Ki-
291 67-negative foci per animal was larger in Ptch1 mice as compared to
292 wild-type mice (data not shown).

293 Examination for localization of each change revealed that more
294 than 70% of Ki-67-negative foci were localized in the β -10th lobules
295 of the cerebellum in both genotypes (Fig. 10). In addition, more than
296 50% of Ki-67-positive foci and small MBs were also observed in the
297 β -10th lobules in Ptch1 mice (Fig. 10).

298 4. Discussion

299 The present study was performed to clarify derived cell and early
300 changes of MBs in Ptch1 mice using immunohistochemistry. We
301 also compared cerebellar developmental processes in Ptch1 mice to
302 wild-type mice. Currently, human MBs are classified as four distinct
303 subtypes by molecular studies (Ellison et al., 2011; Northcott et al.,
304 2011; Jones et al., 2012; Kool et al., 2012). Tumors of Ptch1 mice are
305 thought to be equivalent for those of the Shh subgroup in human
306 (Lau et al., 2012). Therefore, MBs in Ptch1 mice will be good tool for
307 testing new drugs targeting the Shh pathway. Additionally, detailed
308 investigation of the cerebellar development and early changes of

309 MB in Ptch1 mice will be beneficial to understanding pathogenesis
310 of human MBs.

311 During the experimental period, Ptch1 mice showed no clinical
312 signs except development of hydrocephalus and rhabdomyosarcoma
313 in some cases, an outcome that has been previously described
314 (Corcoran and Scott, 2001; Wetmore et al., 2000; Svård et al., 2009).
315 Although the pathogenesis of hydrocephalus in Ptch1 mice has not
316 been fully understood, impaired cilia function of the ependymal
317 cells might be related (Gavino and Richard, 2011).

318 The present study demonstrated that a single injection of BrdU
319 was useful in pursuing the sequential migrating process of GCPs
320 from the EGL to the IGL. Similar to results of another study (Thomas
321 et al., 2009), we observed that the migration process in Ptch1 mice
322 was the same as that in wild-type mice and that the proliferation of
323 GCPs increased in Ptch1 mice resulting in a slightly wider EGL layer.
324 No clear evidence was obtained, but the increased proliferation
325 in the EGL in Ptch1 mice might be related to the high potential for
326 MB described below. Shh signaling regulates the expansion of the
327 pool of GCPs (Roussel and Hatten, 2011; Wang et al., 2012).
328 Reduced levels of Ptch1 expression in the cerebellum of Ptch1
329 mice might be caused by loss of one allele resulting in increased
330 proliferation of GCPs (Corcoran and Scott, 2001; Goodrich et al.,
331 1997; Gupta et al., 2010; Toftgard, 2000; Yang et al., 2008). In
332 addition, an extended duration of GCP localization in the EGL might

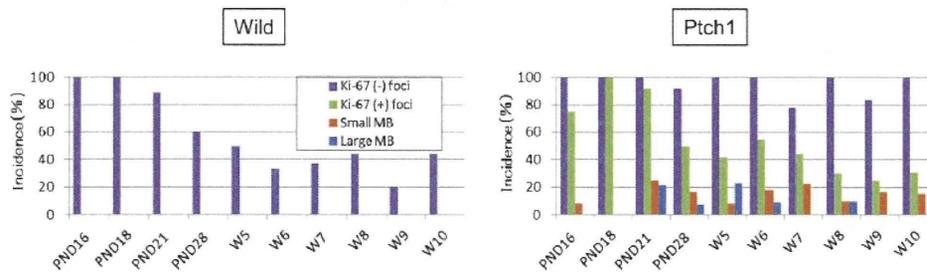


Fig. 9. Incidences of Ki-67-negative (-) and positive (+) foci and MBs in wild-type and Ptch1 mice. Incidences (%) were calculated as follows; number of animals which have specific lesion (e.g. Ki-67-negative foci) in the cerebellum relative to the total number of animals examined. Ki-67-negative foci were observed in both genotypes, but in higher incidence in Ptch1 mice throughout the examination period. Ki-67-positive foci had a tendency to decrease with aging. Small MB and large MB had no tendency to increase with aging.

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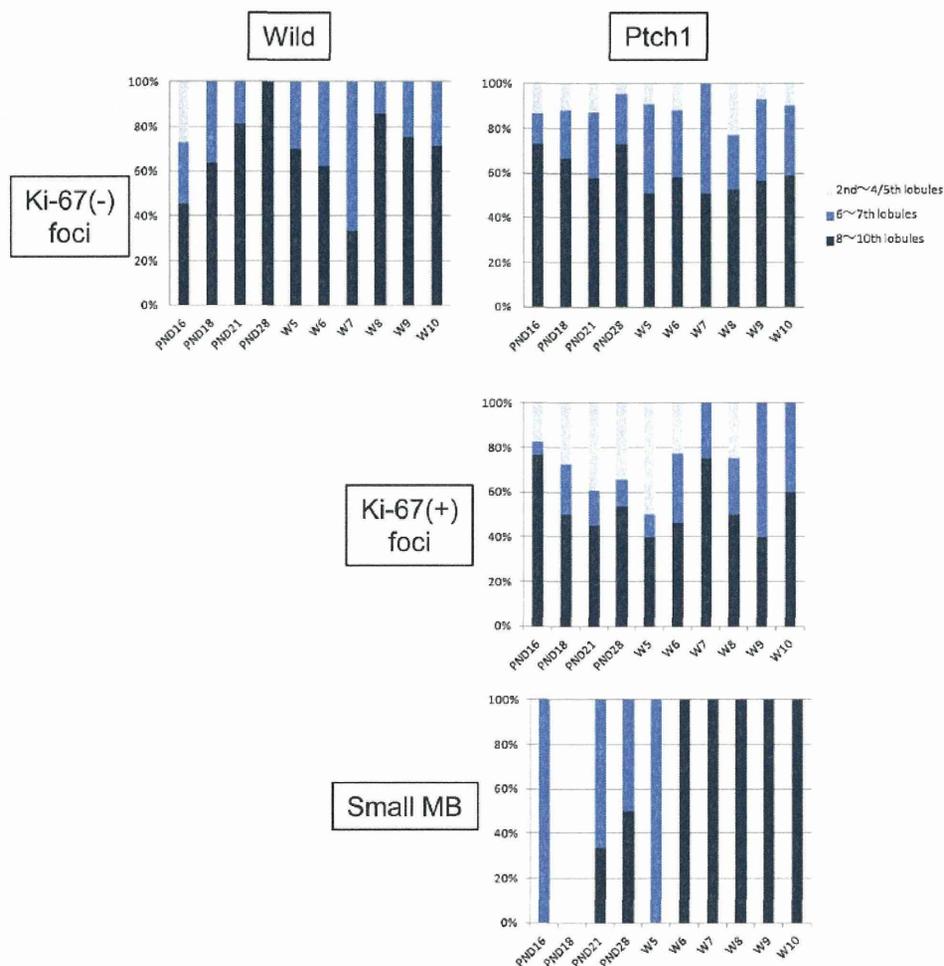


Fig. 10. Location of Ki-67-negative (-) and positive (+) foci and small MBs in the cerebellum of wild-type and Ptch1 mice. Incidences (%) were calculated as follows: total number of specific lesion per cerebellar lobule of all animal examined relative to total number of the lesion in the whole cerebellum of all animals examined.

339 increase opportunities for additional mutations which may lead to
334 tumorigenesis (Corcoran and Scott, 2001; Wetmore et al., 2000).

336 Proliferative lesions such as preneoplastic lesions of MBs during
337 3 weeks after birth in Ptch1 mice were described as rare occurrences
338 in previous reports (Kim et al., 2003); however, our detailed
339 and sequential examination revealed that proliferative lesions and
340 MBs had already been detected in Ptch1 mice within 3 weeks
341 after birth. The morphology and immunohistochemistry of MBs at
342 PND12 indicated that MBs arose from GCPs of the outer layer of the
343 EGL during cerebellar development. The cells of the earliest
344 proliferative lesion, focal thickened area of the EGL, showed the same
345 immunohistochemical profiles as GCPs of the outer layer of the EGL.
346 As the thickened area had cellular atypia and disarrangement compared
347 to normal GCPs, it was considered to be preneoplastic of MB. The appearance of focal thickened proliferative lesions was limited

339 during PND10 to PND14 when the EGL was detectable in this study.
334 It may be that those lesions were recognized as Ki-67-positive foci
335 or small MBs after PND16 because normal GCPs neighboring the
336 lesions disappeared due to migration into the IGL after PND16.

337 We also found another preneoplastic lesion of MB derived from
338 residual GCPs as Ki-67-positive focus only in Ptch1 mice. In previous
339 reports, MBs were thought to arise from residual GCPs located at the
340 surface of the cerebellum (Corcoran and Scott, 2001; Goodrich et al.,
341 1997). In our study, the foci of GCP-like cells were found on the surface
342 of the cerebellar cortex and only detectable on and after PND16.
343 They were clearly divided into two types by the morphological and
344 immunohistochemical profiles and genotypes. Observation of the
345 EGL in BrdU-treated animal revealed that both types of the foci with
346 BrdU-positive cells were composed of residual GCPs. The cells of Ki-
347 67 positive foci had the same profiles as granular cells of the outer

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layer of the EGL, indicating that they were undifferentiated and had high proliferating potential. The other focus, Ki-67-negative type, was composed of cells showing neuronal differentiation with a lack of proliferating activity. They resembled granular cells of the IGL or the inner layer of the EGL indicating that the cells were already differentiated into mature granular cells. The Ki-67-negative foci were detected in both genotypes. In addition, more than half of the Ki-67-positive foci were located in the β -10th lobules of the cerebellum as well as in small MBs in *Ptch1* mice. Although MBs are thought to be derived from residual GCPs located in the EGL of the cerebellum (Behesti and Marino, 2009; Roussel and Hatten, 2011), these results strongly suggest that residual GCPs in the Ki-67-positive foci were preneoplastic lesions. A high yield of Ki-67-positive foci may be useful as an easily detectable and confidential indicator of MB. These preneoplastic lesions might be useful as early markers to detect modulation effects of chemicals to shorten experimental periods.

The observation of a higher incidence of Ki-67-negative residual foci in *Ptch1* mice as compared to wild-type mice and a reduced incidence of Ki-67-negative residual foci with increased age in wild-type mice was unexpected. These findings may be attributable to smaller Ki-67-negative foci in wild-type mice and a much lower number per animal as compared to *Ptch1* mice, although their incidences were 100%. Chances to find the foci in a cross section of the cerebellum may have been reduced with increased age in wild-type mice due to cerebellar growth.

Purkinje cells secrete the mitogen *Shh* for GCP proliferation in the EGL during early phases of cerebellar development (Roussel and Hatten, 2011). After the GCPs lost cell proliferating activity, they migrate along radial fibers of Bergmann glia (Roussel and Hatten, 2011). It has been reported that abnormal development of Purkinje cells and Bergmann glia can disrupt proliferation, migration, and differentiation of GCPs (Adams et al., 2002; D'Arca et al., 2010; Rakic and Sidman, 1973; Schwartz et al., 1997). Our immunohistochemical results demonstrate no abnormal morphology in Bergmann glia or Purkinje cells, indicating that the MB formation process during cerebellar development might be intrinsic to the GCPs rather than abnormalities in Bergmann glia and Purkinje cells.

In conclusion, a thickened area of the EGL and Ki-67-positive foci were considered to be the early preneoplastic lesions of MBs derived from GCPs in the EGL in *Ptch1* mice. These were distinguishable by immunohistochemistry with proliferation and neuronal differentiation markers. Preneoplastic lesions of MBs may serve as useful indicators and as substitutions for advanced MBs in the evaluation of drug efficacy and modulating effects of additional gene mutations, chemicals, and irradiation. Importantly, the Ki-67-positive focus is an easily detectable and confidential indicator due to higher occurrences in the third week after birth.

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