Development ePress online publication date 8 June 2005

PTEN deletion in Bergmann glia leads to premature differentiation and affects laminar organization

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Accepted 5 May 2005

Development 132, 000-000
Published by The Company of Biologists 2005
doi:10.1242/dev.01891

Summary

Development of the central nervous system is controlled by both intrinsic and extrinsic signals that guide neuronal migration to form laminae. Although defects in neuronal mobility have been well documented as a mechanism for abnormal laminar formation, the role of radial glia, which provide the environmental cues, in modulating neuronal migration is less clear. We provide evidence that loss of PTEN in Bergmann glia leads to premature differentiation of this crucial cell population and subsequently to extensive layering defects. Accordingly, severe granule neuron migration defects and abnormal laminar formation are observed. These results uncover an unexpected role for PTEN in regulating Bergmann glia differentiation, as well as the importance of time-dependent Bergmann glia differentiation during cerebellar development.

Key words: PTEN, Bergmann glia, Cerebellar development, Mouse

Introduction

Precise neuronal functions require highly ordered cell proliferation, differentiation, migration and synaptic specificity. Development of the central nervous system depends on both intrinsic and extrinsic factors that orchestrate neuronal migration to form laminae, the compartments in which neurons are differentiated and specialized. During development of cortical regions in the vertebrate brain, postmitotic neuronal precursors use the radial glial cells as a pathway for migrations required to establish the neuronal laminae (Rakic, 1971; Hatten, 1999). Moreover, recent studies indicate that radial glia are the stem cells for cortical neurons, most notably neurons in the outer layers (Noctor and Kriegstein, 2004). Thus, the molecular control of radial glial differentiation is crucial for the formation of normal cortical architectonics and subsequent synaptic circuitry.

The cerebellar cortex has long provided a model system for studies on glial-guided neuronal migration and on glial differentiation (Ramon y Cajal, 1911; Rakic, 1971; Edmondson and Hatten, 1987; Solecki et al., 2004; Anthony et al., 2004). Bergmann glial cells are first seen in the cerebellar cortex in the late embryonic period, when they express the markers RC2 and BLBP. By birth, the radial glial population has disappeared, replaced by the Bergmann glia, which extend processes from midway through the anlagen to the pial surface. The migration of postmitotic-granule cell precursors begins after birth, with maximal periods of migration between P7 and P12. By P15, the external granule layer (EGL) is no longer evident, as the progenitors have all migrated into the internal granule cell layer (IGL), a zone just deep to the Purkinje neurons.

PTEN (for phosphatases and tensin homolog, deleted on chromosome 10) is a tumor suppressor gene frequently mutated in tumors such as glioblastomas, endometrial carcinomas and advance prostatic cancers (Li et al., 1997; Steck et al., 1997). Although PTEN functions as both protein and lipid phosphatases in vitro (Myers et al., 1998), its major in vivo substrate is PIP3 (Maehama and Dixon, 1998; Sun et al., 1999), a lipid second messenger produced by phosphoinositide 3-kinase (PI3K). PIP3 plays a central role in promoting cell proliferation and survival by activating downstream effectors such as AKT/PKB and mTOR-S6K pathways (Stocker et al., 2002; Stiles et al., 2002; Radimerski et al., 2002). Germline *PTEN* mutations are associated with Cowden disease, Lhermitte-Duclos disease and Bannayan-Zonana syndrome (Marsh et al., 1998; Eng, 2003). In addition to the high risk of cancerous developments, these syndromes share a common feature of disorganized tissue growth with unknown etiology (Maehama et al., 2001).

Our previous study demonstrated that *Pten* deletion in the embryonic brain leads to abnormal histoarchitecture with severe layering defects (Groszer et al., 2001). However, as *Pten* deletion happens in the neural stem/progenitor cells, it is unclear whether the abnormal phenotypes observed are caused by intrinsic migratory defects of *Pten*-null neurons, extrinsic micro-environmental cues provided by *Pten* null radial glia, or perhaps both (Groszer et al., 2001). Recently, Marino et al. (Marino et al., 2002) reported aberrant Purkinje cell

positioning in *Pten* mutant mice, but the mechanism leading to this defect is unknown.

In this study, we have investigated the role of PTEN in postnatal cerebellar laminar formation. We show that the deletion of *Pten* in both neurons and glia leads to severe lamination defects. By specifically deleting *Pten* in Bergmann glial cells, we further demonstrate that PTEN intrinsically controls Bergmann glia differentiation and scaffold organization. Our data suggest that Bergmann glia provide crucial, indispensable and developmental stage-dependent extrinsic cues for cerebellar granule neuron migration and laminar formation. These findings underscore the importance of PTEN as a negative regulator for Bergmann glia differentiation and the essential role of Bergmann glial scaffold for granule neuron migration.

Materials and methods

Generation of brain-specific *Pten* deletion mice

To generate brain-specific *Pten* deletion mice, the *hGFAP-Cre* line (Zhuo et al., 2001) was crossed to $Pten^{loxp/loxp}$ mice (Lesche et al., 2002). Littermates from F2 generations were used for all the experiments described in this study.

Isolation of granule neurons and in vitro migration assay

Cerebellar cells were purified by using a modified procedure described by Hatten (Hatten, 1985). Cerebella were dissected from P6 animals. After removal of meninges, cerebellar tissue was treated with Trypsin-EDTA (Gibco-BRL) and triturated into single cell suspension using fire polished glass pipettes. The cell suspension was applied to a Percoll gradient and separated by centrifugation. Enriched granule neurons were applied to glass bottom microwell dishes (MatTek Corporation) containing shattered Whatman glass fiber filters that are pre-coated with polylysine (Sigma) and laminin (BD Bioscience). Granule neuron migration along the glass fiber was imaged by a Hamamatsu video camera mounted on a Leica inverted microscope and analyzed by computer software (Fishman and Hatten, 1993).

Competitive migration assay was performed as described previously (Hirotsune et al., 1998). Fresh dissociated granule neurons were stained by CellTracker Green dye (Molecular probes) at 20 μM for 45 minutes. Equal numbers of labeled wild-type neurons were mixed with unlabeled mutant neurons (and vice versa) and allowed to form reaggregates overnight before plating on laminin-coated culture chambers. After 24 hours, the reaggregates were fixed with 4% paraformaldehyde and assayed for migration.

For glia-mediated granule neuron migration (Hatten et al., 1986), enriched glia isolated from P6 cerebella were cultured at low density. After 7 days in vitro, cultured glia were free from neuron contamination. Then fresh dissociated granule neurons were applied to glia to yield four different co-culture combinations: (1) wild-type granule neurons/wild-type glia; (2) wild-type granule neurons/mutant glia; (3) mutant granule neurons/wild-type glia; (4) mutant granule neurons/mutant glia. After additional 36 hour incubation, the cells were fixed with 4% paraformaldehyde and subject to immunohistochemical staining with antibodies against Tuj1 (COVANCE) and GFAP (Dako).

Primary granule neuron culture

Granule neurons were obtained from dissociated P6 cerebella as described (Dudek et al., 1997). Cells were grown in DMEM/F12 medium (Gibco) supplemented with 10% calf serum, 25 mM KCl, 30 mM glucose, 5 mM HEPES buffer, 2 mM glutamine and penicillin-streptomycin. Cytosine arabinoside (10 μM) was added 24 hours after plating to prevent proliferation of non-neuronal cells. After culturing 7 days in vitro cells were washed and switched to serum-free medium with or without 25 mM KCl for 24 hours, chromatin condensation was visualized by DAPI staining.

Immunohistochemistry

Immunohistochemical analysis was performed on either 4% paraformaldehyde-fixed paraffin sections (4 μm) or cryosections (10 μm). Immunostaining of BLBP (kindly provided by Dr Heintz at Rockefeller University), BrdU (G3G4, DSHB), Calbindin D-28K (Chemicon, Sigma), DsRed (BD Bioscience), GFAP (Biomeda, DAKO), TAG1 (DSHB), phospho-histone H3 (Upstate), $GABA_A$ receptor alpha6 (Santa Cruz), Tuj1 (COVANCE) and p27 (Santa Cruz) were detected by either Vector MOM Kit or Vector ABC Kit (Vector Laboratories). Whole-mount X-gal staining was performed according to standard protocol.

Western blot analysis

For surveying of PTEN, P-AKT and AKT expression in cerebella, whole-cell extract was prepared by lysing fresh isolated P6 cerebellar granule neurons and glia in RIPA buffer. Cell lysates were subjected to SDS-PAGE electrophoresis and western blot analysis, using antibodies specific for PTEN, P-AKT, AKT (Cell Signaling Technologies), Vinculin or Actin (Sigma) antibodies.

Retrovirus production

Recombinant pantropic replication-incompetent retroviruses were produced according to manufacturer's suggestions (BD biosciences). For generation of retroviral construct, a *Sal*I/*Dra*I fragment containing IRES2-DsRed was isolated from pIRES2-DsRed2 vector and inserted into *Sal*I/*Stu*I sites of the pLNCX2 vector. Next, the *Xho*I/*Not*I fragment containing a nuclear targeting CRE/GFP fusion protein was inserted into the pLNCX2-IRES-DsRed vector to generate the pLNCX2-CRE/GFP-IRES-DsRed retroviral construct.

Preparation and infection of organotypic cerebellar slices

Organotypic cerebellar slices were prepared according to Tomoda et al. (Tomoda et al., 1999). To visualize migrating granule neurons within slices, cerebella from P6 mice were cut coronally into 300 μm slices by a chopper (Stoelting). The slices were transferred onto culture inserts (Millicell; Millipore) and submerged in culture media. Two hours after dissection, retroviruses were applied to medium. Forty-eight to 72 hours after infection, slices were fixed and processed for immunostaining.

Results

Pten deletion results in enlarged brain and severe defects in cerebellar development

During cerebellar development, two waves of neuronal migration are facilitated by radially shaped glia. The first wave occurs between embryonic day 14 and 17 (E14 to E17), when Purkinje neurons migrate outwards through the wall of the cerebellar anlage. The second wave starts postnatally and lasts for approximately 3 weeks when granule neurons migrate inwardly to assume a position beneath Purkinje cells (Hatten, 1999; Wang and Zoghbi, 2001). To study the role of PTEN in cerebellar laminar formation, we crossed *Pten* conditional knockout mice ($Pten^{loxp/loxp}$) (Lesche et al., 2002) with mice bearing an hGFAP-Cre transgene (Zhuo et al., 2001). CRE recombinase expression is known to begin at E13 and can be detected in both cerebellar granule neurons and Bergmann glia but not in Purkinje cells (Zhuo et al., 2001); thus, it is suitable for studying granule neuron migration.

$Pten^{lox/lox}$;*hGfap-cre*+ mice were viable but showed significant macrocephaly. Brain mass increased progressively

until death occurred around postnatal day 21 (P21, M.G., H.W. and X.L., unpublished). Dissection of *Pten* mutant brains revealed a twofold increase in brain mass at P21 (Fig. 1A). To determine the efficiency of *Pten* deletion, we performed western blot analysis on fresh isolated P6 cerebellar granule neurons (95% purity) and glia (70% purity). *Pten* deletion is near complete in granule neurons but to a lesser degree in glial cells of mutant cerebella (Fig. 1B). We also detected prominent CRE immunoreactivity in mutant Bergmann glia throughout postnatal development (data not shown).

AKT is a downstream effector that is negatively regulated by PTEN; increased AKT phosphorylation (AKT-P) is a hallmark of PTEN loss. We examined AKT-P level in both granule neurons and glia. As predicted, AKT-P was significantly increased in both granule neurons and glia from *Pten* mutant cerebella. Interestingly, *Pten* expression level was notably higher in control glia compared with wild-type neurons, whereas AKT-P level was higher in mutant glia than in mutant neurons (Fig. 1B), implying cerebellar glia have stronger PI3K activity than do granule neurons.

Chronological analysis of histological samples revealed a biphasic developmental defect in PTEN mutant cerebella: before P3, PTEN mutant cerebella were significantly larger than controls but had relatively normal EGL and cerebellar lobules (Fig. 1C, parts a-b′). Dramatic impairment occurred after P6 when mutants showed a marked cerebellar enlargement and lack of IGL and folia, the hallmarks of postnatal cerebella (Fig. 1C, parts c-d′). We also observed a time-dependent Purkinje layer defect in mutant mice, demonstrated by immunohistochemical staining with a Purkinje cell specific marker Calbindin (Fig. 1D). Before P3, the positioning of mutant Purkinje cells was largely normal (Fig. 1D, parts a-b′), indicating normal Purkinje cell migration during embryonic development. The first sign of Purkinje layer defect was seen in mutant mice around P4-P6 (Fig. 1D, parts c,c′). By P9, numerous Purkinje neurons, which had previously adopted normal positioning, now were randomly scattered (Fig. 1D, parts d,d′). As *Pten* is not deleted in Purkinje neurons (Zhuo et al., 2002) (data not shown), the aforementioned layering defect could be secondary to the abnormality of granule neuron migration or Bergmann scaffold.

Severe granule neuron layering defects in *Pten$^{lox/lox}$; Gfap-cre+* mice

The lack of the organized IGL, which is composed of postmitotic granule neurons, prompted us to investigate granule neuron migration in *Pten* mutants. Granule neuron precursors are generated at the rhombic lip. Between E13 and E18, the precursors within the EGL proliferate intensively. Shortly after birth, postmitotic granule neurons initiate an

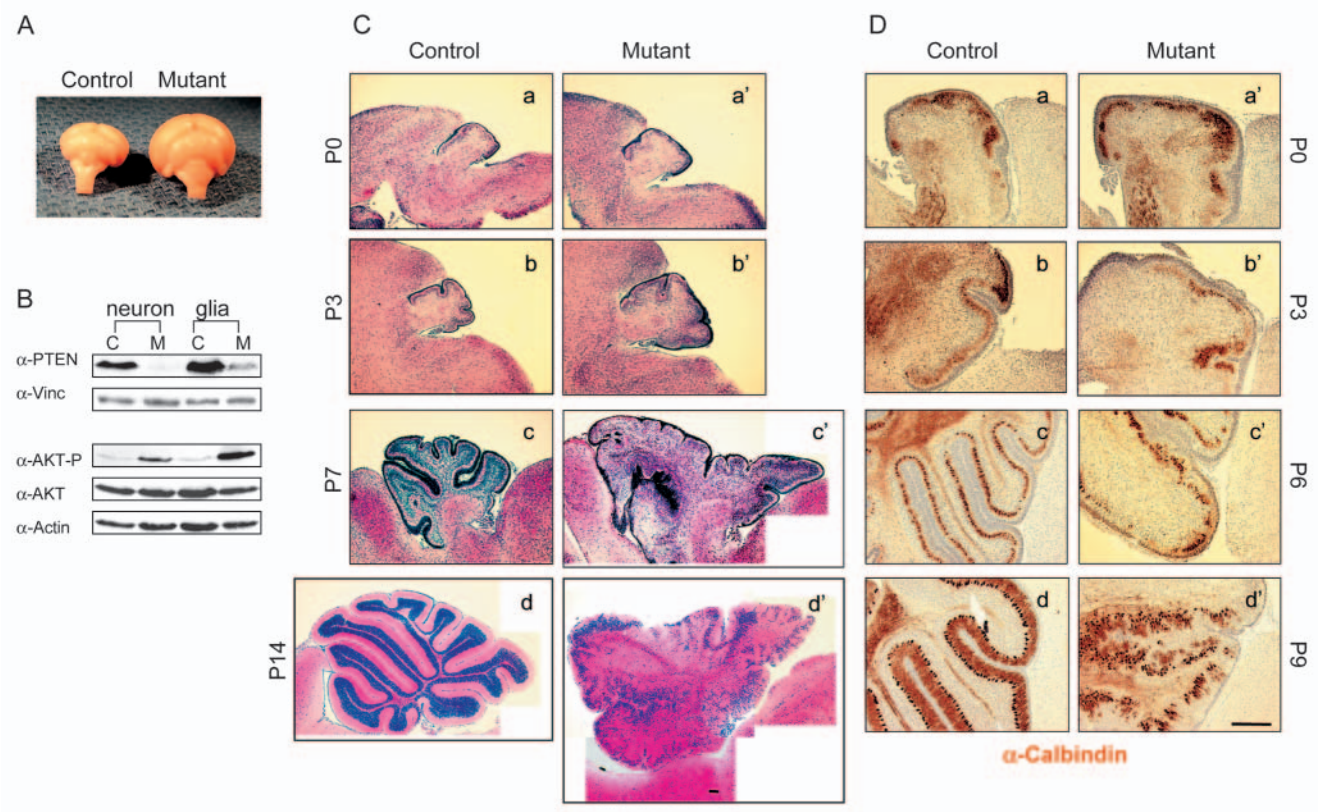

Fig. 1. *hGfap-Cre*-mediated *Pten* deletion leads to macrocephaly and cerebella layering defects. (A) Significant increase of brain mass of *hGfap-cre$^{+/-}$;Pten$^{loxp/loxp}$* mice at P21. (B) Western blot analysis of protein extracts from freshly isolated granule neurons and cerebellar glia at P6. Both granule neurons and glia of *Pten* mutant showed a significant decrease in PTEN expression, accompanied by increased P-AKT levels. (C, a-d′) The sagittal sections revealed a significant increase in size of the cerebella of *Pten* mutants by Hematoxylin and Eosin staining (rostral is upwards and anterior is towards the left). Mutants also showed the loss of foliation and the disruption of the IGL after P6 (C, c′,d′). (D, a′-d′) Calbindin immunostaining showed Purkinje cells misplacement in *Pten* mutant mice over time. At P3, Purkinje cell layer was relatively normal in mutant mice (D, b′). At P9, many ectopic Purkinje cells were seen in *Pten* mutants (D, d′). Scale bar: 100 μm.

inward migration to the IGL; this process persists for ~3 weeks (Hatten, 1999; Wang and Zoghbi, 2001). Histological examination of mutant cerebella revealed large numbers of cells scattered across the molecular layer (ML) (Fig. 2A, part b′) that were immunopositive for p27 (Fig. 2B), a marker for post-mitotic granule neurons. The abnormal granule neuron positioning was not caused by impaired neuronal differentiation because mutants showed a relatively intact TAG1 and $GABA_A$ receptor α6 expression (Fig. 2C,D). Specifically, the timing of $GABA_A$ receptor α6 expression was normal. At P7, no $GABA_A$ receptor α6 expression was seen in either wild-type or mutant mice. At P10, both wild-type and mutants showed detectable $GABA_A$ receptor α6 expression in the IGL. By contrast, the majority of ectopic granule neurons in the ML were negative for $GABA_A$ receptor α6 in the mutants. By P14, IGL and ectopic granule neurons within the ML showed strong $GABA_A$ receptor α6 expression in the mutants. Furthermore, mutants displayed normal Tuj1 expression pattern in the ML, suggesting a normal axon extension (Fig. 2C). We did observe increased TAG1 and Tuj1 expression in the mutants. This may be due to more immature granule neurons, increased soma and axon size, or both, after *Pten* deletion.

We next examined *Pten*-null granule neurons migration. Granule neurons born at P6 were pulse-labeled with BrdU and chased for various time periods. Twenty hours post-labeling, both control and mutant cerebella showed similar numbers of BrdU positive labeled cells in the EGL region (Fig. 3A, parts a,b). By 110 hours, the vast majority of BrdU-positive granule neurons had migrated into the IGL in control littermates (Fig. 3A, part a′). By contrast, mutant mice had a significant number of BrdU-labeled cells retained within the ML (Fig. 3A, part b′ and quantified in right panel). Furthermore, no increase cell division could be detected in the mutant ML by either acute BrdU labeling (Fig. 3A, part b) or Ki67 and phospho-histone H3 immunostaining (Fig. 3B), suggesting that *Pten*-null granule neurons were not proliferating ectopically in the ML region.

Previous studies have indicated that deletion of *Pten* in Purkinje neurons leads to vacuolization, degeneration and progressive loss of Purkinje cells (Marino et al., 2002). To exclude the possibility that there was a loss of granule neurons

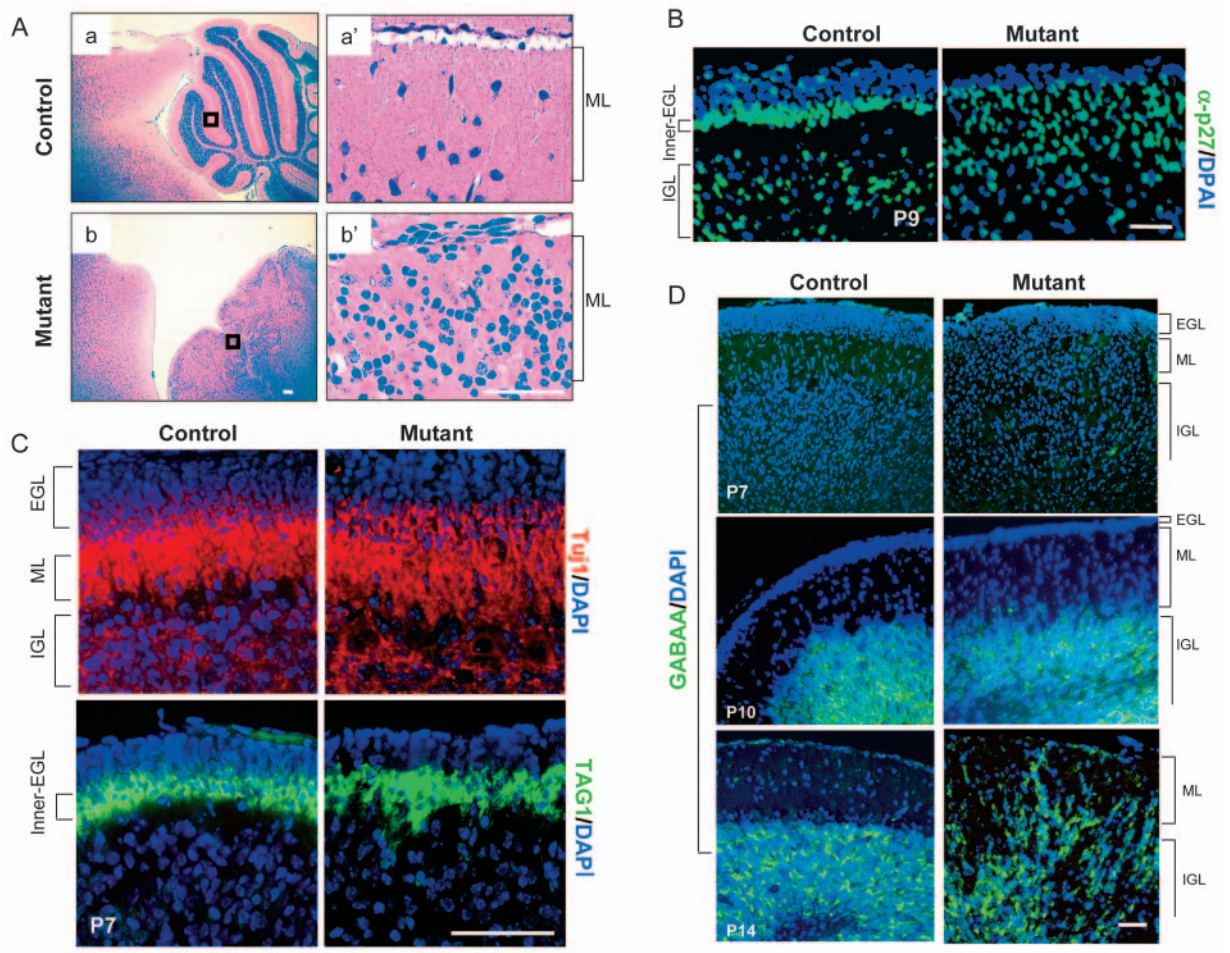

Fig. 2. *hGfap-cre*$^{+/-}$;*Pten*$^{loxp/loxp}$ mice show severe granule neuron ectopia but normal neuronal differentiation. (A) Sagittal section of P14 mouse cerebella (rostral is upwards and anterior is towards the left). High-magnification view of *Pten* mutant cerebella (from framed areas in A, a,b) showed increased number of cells in the ML (A,b′) compared with littermate controls (A,a′). (B) p27 immunostaining confirmed ectopic cells in *Pten* mutants were granule neurons. (C) The expression patterns for axon extension (Tuj1) and early granule neuron differentiation (TAG1). Control versus *Pten* mutant. (D) The expression of $GABA_A$ receptor α6 at different time points. Control versus *Pten* mutant. EGL, external granule layer; ML, molecular layer; IGL, internal granule layer. Scale bar: 50 μm.

either in the IGL or during cell migration, we tested the effect of PTEN on cell survival by culturing isolated granule neurons in the presence or absence of a depolarizing level of potassium. *Pten*-null granule neurons were highly resistant to non-depolarizing level of potassium (5 mM)-induced apoptosis, and the neuron size was significantly enlarged (Fig. 4A, and quantified in Fig. 4B). Similarly, no significant cell death could be detected in mutant brains as measured by TUNEL assay (data not shown).

Pten null granule neurons do not have cell-autonomous migration defects

The granule neuron migration defect seen in *Pten* mutant mice warranted a more detailed examination of their migration properties. Previous studies demonstrated that granule neurons isolated from cerebella could readily migrate on either laminin-coated glass fibers or cerebellar glial fibers, but poorly on fibronectin coated glass fibers (Hatten, 1985; Fishman and Hatten, 1993). We first tested the intrinsic migration properties of *Pten*-null granule neuron migration on laminin coated glass fibers. The migration distance of an individual neuron was recorded with a time-lapse microscope and Fig. 5A showed an example of such a study. Notably the mutant neuron retained a typical migration profile consisting of cycles of extension and contraction on the laminin-coated glass fiber substratum. Comparing 50 migrating neurons from mutants and controls, we found the overall migration velocity was similar (speed of mutant neuron=6.1±4.6 μm/hour; speed of control neuron=6.9±6.3 μm/hour).

To directly test whether *Pten*-null granule neurons could compete with wild-type neurons, we employed a unidirectional neuron migration assay by using cerebellar neuron reaggregates. Previous studies have shown that the maturing granule neurons can use radial neurites generated from reaggregates for their outward migration (Hirotsune et al., 1998). In this experiment, fresh isolated wild-type and *Pten*-null granule neurons were reciprocally labeled with fluorescent dyes and mixed in a 1:1 ratio to form reaggregates before being plated on a laminin-coated dish. The migration distances were measured and quantified from the reaggregate margin to individual labeled granule neuron. No significant differences were observed between wild-type and *Pten*-null neurons (Fig. 5B), indicating that the cell-autonomous migration property of *Pten*-null granule neurons is not altered in the presence of wild-type neurons.

We next studied granule neuron migration in cerebellar organ cultures. P6 $Pten^{lox/lox}$ cerebellar slices were infected by a replication incompetent retrovirus that was engineered to express both a CRE-GFP fusion protein and a DsRed protein bicistronically (see Materials and methods). Because granule neurons are the only cell type that undergoes rapid proliferation in the cerebellar cortex at P6, this retrovirus vector will preferentially infect and delete *Pten* in granule neurons (Tomoda et al., 1999). Forty-eight to 72 hours post-infection, *Pten*-deficient granule neurons (indicated by nuclear GFP expression) exhibited the typical mode of migration, as judged by the morphology of the leading dendrite and trailing axon, indicated by DsRed (Fig. 5C), suggesting that migrating polarity could be maintained in *Pten*-deficient granule neurons in vivo. Thus, the intrinsic migratory properties of *Pten*-null granule neuron does not differ significantly from its wild-type counterparts, based on the individual migration assay, the competition measurement and semi in vivo labeling.

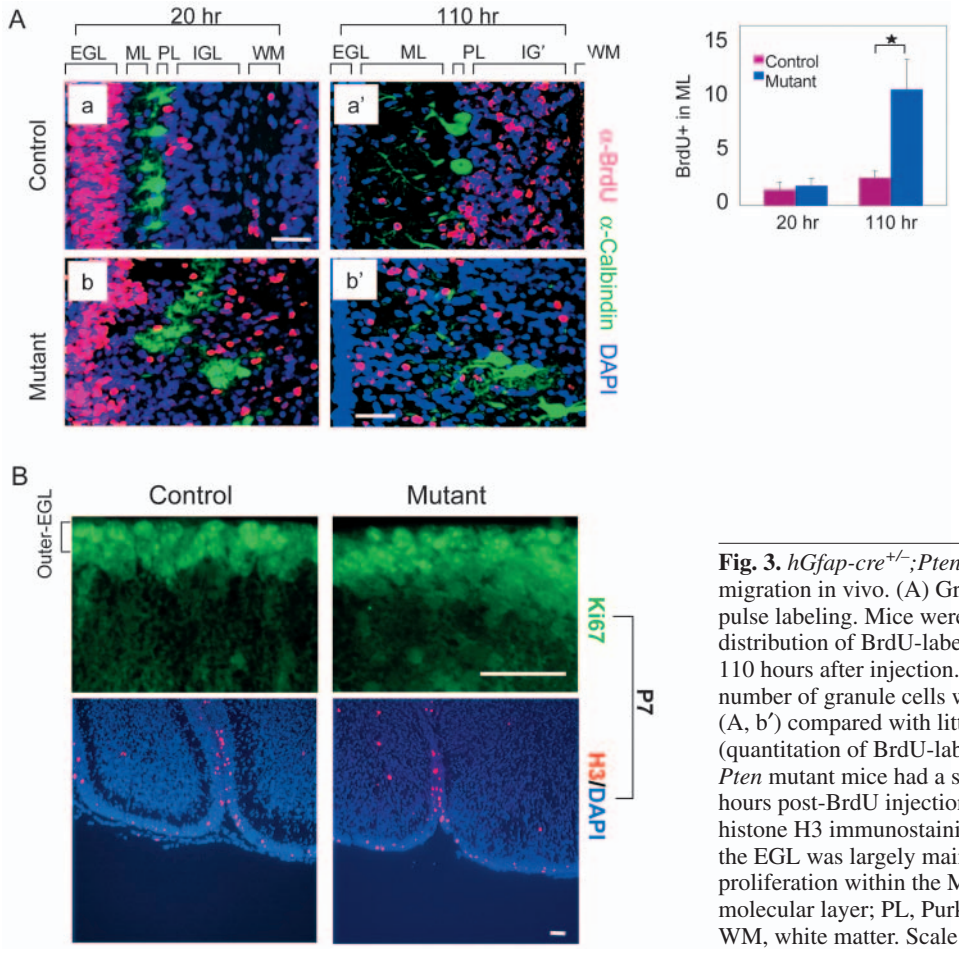

Fig. 3. *hGfap-cre*$^{+/-}$;*Pten*$^{loxp/loxp}$ mice show defects in granule neuron migration in vivo. (A) Granule cell migration was analyzed by BrdU pulse labeling. Mice were injected 100 mg/kg BrdU peritoneally and distribution of BrdU-labeled neurons was determined at 20 hours and 110 hours after injection. At 110 hours post-BrdU labeling, increased number of granule cells was seen in the ML of PTEN mutant mice (A, b′) compared with littermate controls (A, a′). A histogram (quantitation of BrdU-labeled neurons within the ML) indicates that *Pten* mutant mice had a significant increase in cell number at 110 hours post-BrdU injection (★P<0.001). (B) Ki67 and phospho-histone H3 immunostaining indicated that the proliferation zone in the EGL was largely maintained with no apparent ectopic proliferation within the ML. EGL, external granule layer; ML, molecular layer; PL, Purkinje cell layer; IGL, internal granule layer; WM, white matter. Scale bar: 50 μm.

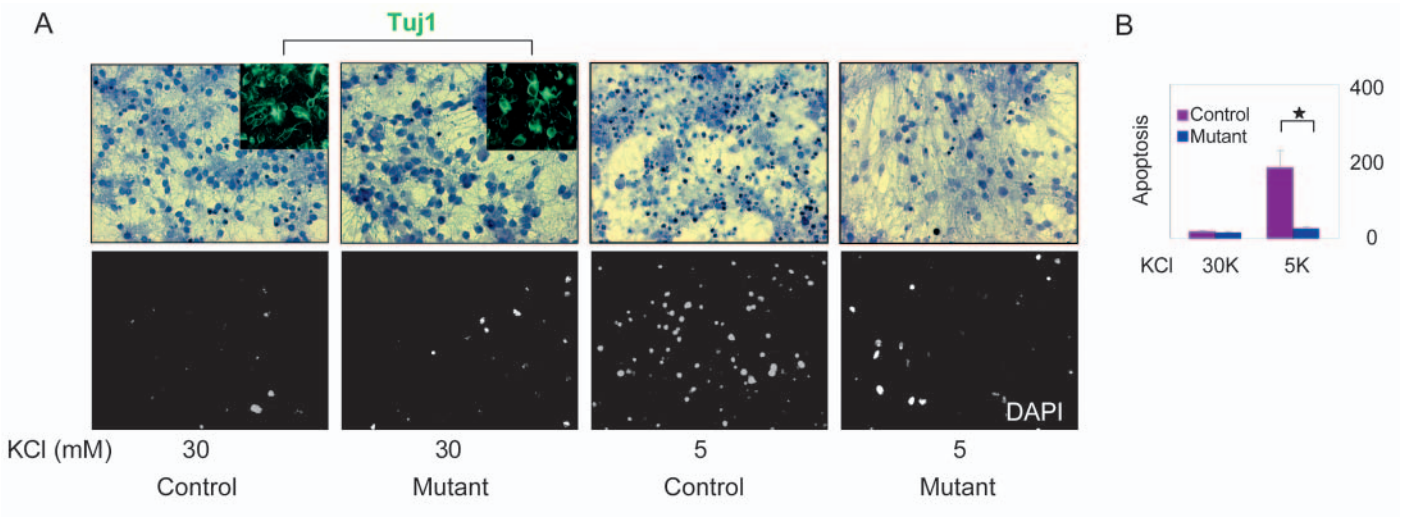

Fig. 4. *Pten*-null neurons are highly resistant to apoptosis. Cerebellar neurons (Tuj1 positive) grown for 7 days were switched to a serum-free medium containing either 30 mM KCl or 5 mM KCl for 24 hours. (A) Neuronal viability was estimated by DAPI staining of the apoptotic nuclei. Fewer apoptotic neurons could be identified in mutants when compared with littermate controls (lower panel). Mutant neurons were also significantly larger than littermate control granule neurons (upper panel). (B) Histogram (quantitation of apoptotic nuclei) indicates *Pten*-null neurons are highly resistant to low K^+-induced cell death under the serum deprivation condition ($^\star P<0.001$).

We next asked whether *Pten* deletion affected neuron-glia interaction. In weaver mice, mutant granule neurons fail to form neuron-glia contacts and display the severe neuron migration defects (Hatten et al., 1986). To test whether the neuron-glia contacts were impaired in *Pten* mutants, we performed the neuron-glia co-culture assays in four different combinations: (1) wild-type granule neurons/wild-type cerebellar glia; (2) wild-type granule neurons/mutant cerebellar glia; (3) mutant granule neurons/wild-type cerebellar glia; (4) mutant granule neurons/mutant cerebellar glia. *Pten*-null granule neurons were found to form tight contacts with both wild-type and mutant glial fibers and to exhibit normal migration profile (Fig. 5D). Furthermore, wild-type granule neurons could associate with mutant astroglia in close apposition. These results suggest *Pten* deletion does not alter neuron-glia contacts during neuronal migration.

Severe Bergmann glia defects in *Pten$^{lox/lox}$; Gfap-cre+* mice

We then turned our attention to cerebellar glial cells in which *Pten* was efficiently deleted. Bergmann glia are the major glial cell type in the cerebellar cortex. During postnatal development, Bergmann glia undergo significant morphological changes to accommodate granule neuron differentiation and migration. At P3, Bergmann glia are BLBP immunoreactive and occupy the outer region of the cerebellar cortex (Fig. 6A). Around P7, Bergmann glia show strong BLBP immunostaining and their cell bodies align roughly in a single layer next to Purkinje cells (Fig. 6B). Subsequently, Bergmann glia extend their fibers that link the EGL and IGL (Fig. 6C), thereby providing a substratum or cue for granule neuron migration (Rakic, 1971). Detailed immunohistochemical analysis revealed a time-dependent Bergmann glial defect in *Pten* mutant cerebella. *Pten*-null Bergmann fibers were significantly enlarged when compared with the controls at P3 (Fig. 6A′). At P7, *Pten*-null Bergmann glial cell bodies were randomly distributed, some had completely lost their contacts to the pial surface and positioned deep within IGL region (Fig. 6B′, white arrowheads). These ectopic Bergmann glia were negative for Ki67 labeling (data not shown), suggesting that they were not generated in situ but, instead, disintegrated from the Bergmann glial layer during the rapid expansion of cerebellar cortex. At P14, *Pten*-deficient Bergmann glia branched out numerous processes and acquired an astrocyte-like cell morphology (Fig. 6C′). Although some Bergmann glia still maintained their endfeet at pial surface, others had withdrawn theirs completely. Accompanying premature Bergmann glia to astrocyte differentiation, numerous granule neurons were evidently retained within the same region (Fig. 6C′, granule neuron nucleus shown in blue). Together, these results suggest that Bergmann glia premature differentiation may affect granule neuron migration and IGL layering formation.

Granule neuron migration defect is secondary to abnormal Bergmann glial layering

Detailed analysis of PTEN mutant cerebella suggested that disruption of Bergmann glial layering might cause the granule neuron migration defect. At P7, concomitant with the mislocalized Bergmann glia in the deep IGL (Fig. 7A, part b), many granule neurons failed to complete their migration and were accumulated within the ML (Fig. 7A, part b′). To directly analyze PTEN function in Bergmann glia development, we constructed a helper-dependent adenovirus (HDA) vector expressing Cre and YFP (HDA.Cre/YFP) (Dorigo et al., 2004). As adenovirus preferentially infects glial cells rather than neurons (Iino et al., 2001), this vector allowed us to delete *Pten* in Bergmann glia. We surgically injected HDA.Cre/YFP into *Pten$^{loxp/loxp}$;Rosa26$^{floxed\text{-}Stop\text{-}lacZ}$* cerebella at P3 and examined at 8 days post-injection. Cre-mediated *Pten* deletion, as indicated by *lacZ*-positive staining (Fig. 7B, part a; area indicated by paired arrows), correlated well with changes in

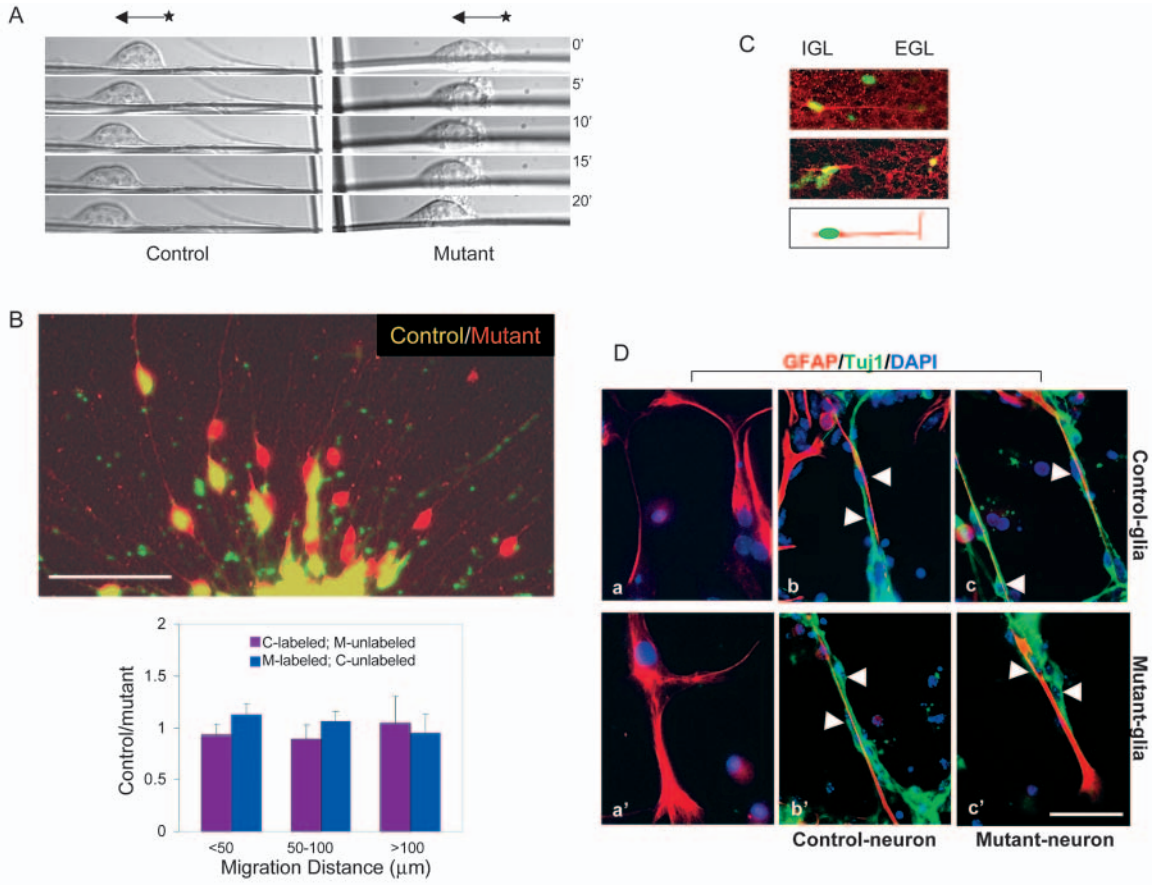

Fig. 5. *Pten*-null granule neurons display normal migratory properties. (A) High-magnification time-lapse video microscopy revealed the migrating neurons attached to laminin-coated glass fiber. (B) Competitive migration assay showed similar migration capacity between *Pten*-null and control granule neurons. Representative neuron migration from reaggregate after a 24 hour incubation was shown on micrograph (top panel). Comparisons of numbers of control versus mutant neurons that migrated to each bin are shown on the histogram (bottom). A value of 1 represents an equal ratio of control and mutant neurons in a specific bin. (C) *Pten*-null granule neurons exhibited the typical migrating profile on organotypic cerebellar slice culture. *Pten*$^{loxp/loxp}$ slices from P6 mice were infected with a recombinant retrovirus expressing CRE-GFP and DsRed. After 48-72 hours incubation, the infected granule neurons with nuclear GFP and cytosol DsRed expression were visualized using a confocal microscope. (D) *Pten*-null and wild-type granule neurons were co-cultured with cerebellar astroglia. (D, a,a′) pure cerebellar glia 7 days in vitro. (b,b′) Wild-type granule neurons were co-cultured with either wild-type or mutant glia for 36 hours, wild-type neurons (Tuj1) formed a close apposition with GFAP-positive mutant glial fibers during migration. (c,c′) Mutant granule neurons were co-cultured with wild-type or mutant glia, and showed normal migrating profile and axon extension along with GFAP-positive astroglial fibers. Arrowheads indicate migrating neurons closely apposite with glial fibers. Scale bars: 50 μm.

Bergmann glial scaffold (Fig. 7B, part b; paired arrows) and retention of granule neurons in the ML region (Fig. 7B, part c; compare red boxed area with blue boxed area, $P<0.05$), although these granule neurons were negative for *lacZ* expression (red boxed area in Fig. 7B, part a). Thus, *Pten* deletion in Bergmann glia can affect granule neuron migration, even when these granule neurons are wild type for *Pten* expression. As a control experiment, HDA.Cre/YFP was also injected into *Rosa26*$^{floxed\text{-}Stop\text{-}lacZ}$ cerebella; no structural changes were observed resulting from viral infection alone (data not shown).

Pten null Bergmann glia premature differentiation is cell-autonomous

We next examined whether Bergmann glial defects were the result of cell intrinsic effect of PTEN loss or extrinsic effect from ectopic positioned granule neurons. We chose to study HDA.Cre/YFP virus-mediated Bergmann fiber changes at a later stage (~P10) to minimize the effect of granule neuron-Bergmann glia interplay on Bergmann fiber morphology (viral injection was performed at P7 + 3~4 days and is required for morphological changes of Bergmann fibers based on our observations). One or 2 weeks after injection, cerebellar specimens were assessed by immunohistochemical staining. As shown in Fig. 8A, we observed a good correlation between YFP expression (in green, upper panel) and *lacZ* expression (in blue, lower panel) in cerebellar tissue, indicating a highly efficient CRE recombinase (YFP^{+})-mediated LoxP excision (*lacZ*$^{+}$, corresponding to *Pten* deletion). In the areas showing strong *lacZ* expression [*lacZ* (+)] (Fig. 8B, lower panels), many Bergmann glial fibers were markedly thickened with increased lateral branching fibers, a characteristic feature of premature Bergmann glia differentiation (Fig. 8B, part a′, 1 week after injection; Fig. 8B, part b′, 2 weeks after injection), when compared with the adjacent *lacZ* (–) area (Fig. 8B, upper panels). Notably, no significant granule cell migration defects could be detected in affected area (Fig. 8B, part c′). Furthermore, Purkinje cell layering was not affected in the *lacZ* (+) area (Fig. 8C, arrows). Taken together, these results indicate that PTEN plays a significant role in Bergmann glia maintenance; the premature differentiation seen in *Pten*-null Bergmann glia is a cell-autonomous event.

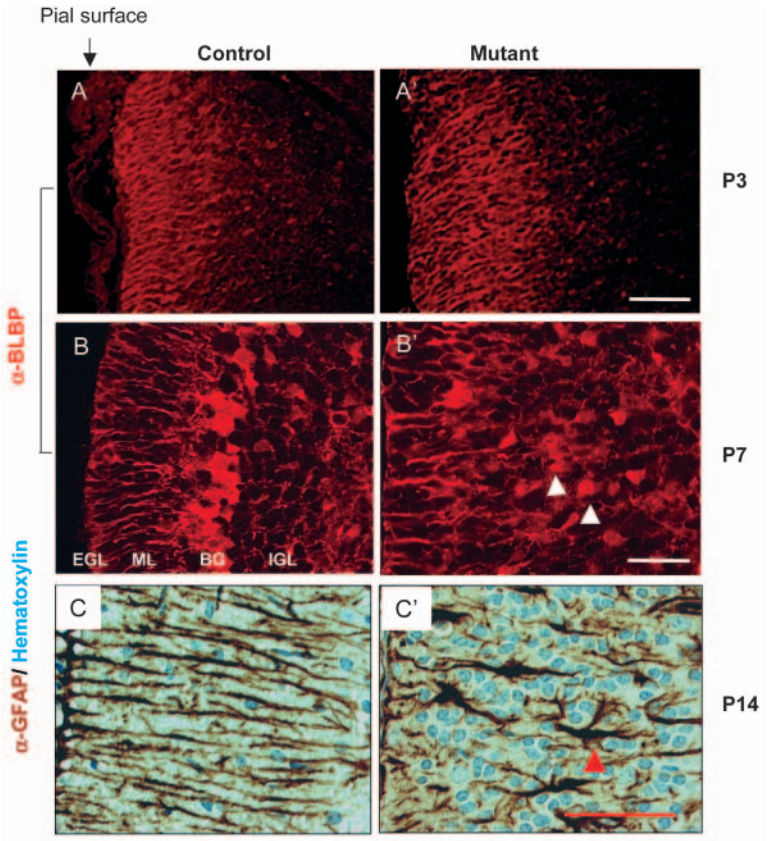

Fig. 6. Developmental stage-dependent Bergmann glia defects in *Pten*-null animals. (A,A′) Sagittal sections immunolabeled with anti-BLBP antibody and revealed significant enlargement of the immature Bergmann glial fibers in *Pten* mutant at P3 (A′). (B,B′) Bergmann glial scaffold defects in *Pten* mutants at P7. In control littermates, BLBP-positive Bergmann glia aligned as a single layer next to Purkinje neurons, whereas many *Pten* mutant Bergmann glia were localized within the deeper IGL region (arrowheads, B′). (C,C′) Bergmann glia prematurely transformed into an astrocytic morphology (arrowhead); shown at P14 in the mutant (C′). Some astrocytic Bergmann fibers still retained end-feet on the pial surface. EGL, external granule layer; ML, molecular layer; IGL, internal granule layer. Scale bars: 50 μm.

Discussion

In this study, we characterized PTEN function during cerebellar development at its proliferating stage by deleting *Pten* in murine cerebellum. We demonstrate that *Pten* deletion in Bergmann glia results in premature differentiation and abnormal anchorage, leading to impaired neuronal migration and laminar organization in the cerebellum.

The cerebellum is the target of numerous gene mutations (Goldowitz and Hamre, 1998). The creation of laminated structure of postnatal cerebellar cortices is achieved by directional migration of committed granule neurons from the EGL to their final destination in the IGL. Many intrinsic and extrinsic factors are known to influence this differentiation process (Hatten, 1999). Therefore, the cerebellum can serve as an excellent model for studying brain morphogenesis. Conditional *Pten* deletion in cerebellum has been reported recently using different Cre lines. Two groups (Backman et al., 2001; Kwon et al., 2001) used a GFAP-CRE line that restricted *Pten* deletion in postmitotic neurons and observed a cell-autonomous increase in neuronal soma size. The third group (Marino et al., 2002) used En2-CRE and L7-CRE lines and observed Purkinje cell positioning defect and degeneration.

In our study, we focus on the role of *Pten* during early postnatal cerebellar development. Our data further extend previous findings and suggest that (1) PTEN intrinsically controls Bergmann glia differentiation, and (2) Bergmann glia provide critical environment cues for granule neuron migration. We speculate that this model may be relevant for explaining some of the mutant phenotypes observed by the previous studies.

Factors controlling granule neuron migration have been extensively studied using gene targeted mouse models. Mutations in *Cdk5*, *Pax6* and *Pafah1b1* genes cause cell-autonomous granule neuron migration aberrations (Ohshima et al., 1999; Engelkamp et al., 1999; Yamasaki et al., 2001; Hirotsune et al., 1998). Mutations in astrotactin and neuregulin (Adams et al., 2002; Rio et al., 1997) affect granule neuron-Bergmann glia interaction and cell migration. The relationship between *Pten* deletion and cell migration defects has been studied recently. PTEN-deficient Dictyostelium cells are poorly chemotactic as result of polarity defects and an inability to restrict gradient sensing (Iijima and Devreotes, 2002; Funamoto et al., 2002). PTEN-deficient mammalian cells, however, are more mobile, although different mechanisms have been proposed (Tamura et al., 1998; Liliental et al., 2000; Raftopoulou et al., 2004). By contrast, the effect of *Pten* deletion on gliophilic migrating granule neurons remains largely unknown. Although dysplastic granule neurons are present in the adult EGL or ML of PTEN mutants (Backman et al., 2001; Marino et al., 2002), their number is relatively small and may not reflect a genuine cell intrinsic migration defect. As demonstrated in this study, *Pten*-null neurons are highly resistant to apoptosis. Thus, the persistent survival of ectopic granule neurons within the ML, which otherwise should be eliminated through apoptosis in wild type, may contribute granule neuron dysplasia seen in these studies (Backman et al., 2001). In support of this assumption, early reports demonstrated an intact ML during early postnatal development (P10), although PTEN expression is significantly downregulated in the inner EGL of *Pten* mutants, suggesting deletion of PTEN in granule neurons prior to migration may not cause a cell-intrinsic migration defect (Backman et al., 2001). To clarify the effect of *Pten* deletion on migrating granule neurons, we performed a systematic investigation on cell intrinsic migration property. We show that *Pten* deletion has minimal effect on intrinsic migratory properties of granule neurons in both in vitro and in vivo settings, suggesting that migration defect of *Pten* mutants is due to changes of extrinsic factors within the local microenvironment.

The intimate structural relationships between migrating granule neurons and Bergmann fibers suggest that elongated radial Bergmann glia serve as a scaffold that is crucial for granule neuron migration (Rakic, 1971). Using genetic model systems, continuing efforts have been made towards the

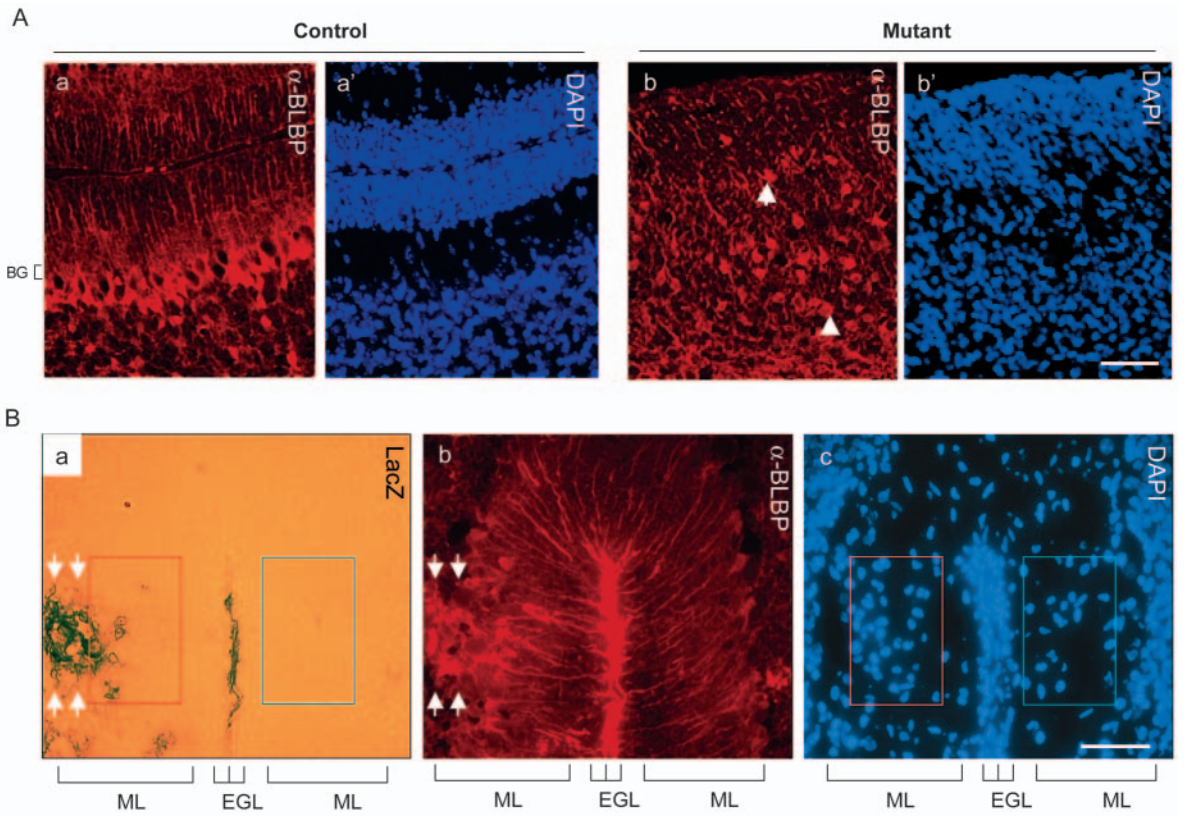

Fig. 7. Bergmann glia scaffold defects lead to granule neuron migration defects. (A) Sagittal sections immunostained with BLBP (a) and counterstained with DAPI (a′). In *Pten* mutants (P7), severe Bergmann glia layering defect (b) coincides with major granule neuron accumulation within the ML (b′). Arrow indicates normal positioned Bergmann glial cell body; arrowhead pointed to ectopic Bergmann glial cell body. (B, a-c) Bergmann glia defects after adenovirus mediated *Pten* deletion were associated with increased granule neurons within the ML. HDA.Cre/YFP virus was injected into $Pten^{loxp/loxp};Rosa26^{floxed\text{-}Stop\text{-}lacZ}$ cerebella at P3 and the tissues were processed 8 days after injection. Bergmann glial scaffold defects were a direct consequence of viral CRE-mediated *Pten* deletion (B, a,b, area indicated by paired arrows) and coincides with granule neuron heterotopia in the ML (B, a-c; red boxed area for injected side; blue boxed area for uninjected control). Granule neurons accumulated within the ML were negative in X-gal staining (red boxed areas in B, a,c). EGL, external granule layer; ML, molecular layer; BG, Bergmann glia layer; IGL, internal granule layer. Scale bars: 50 μm.

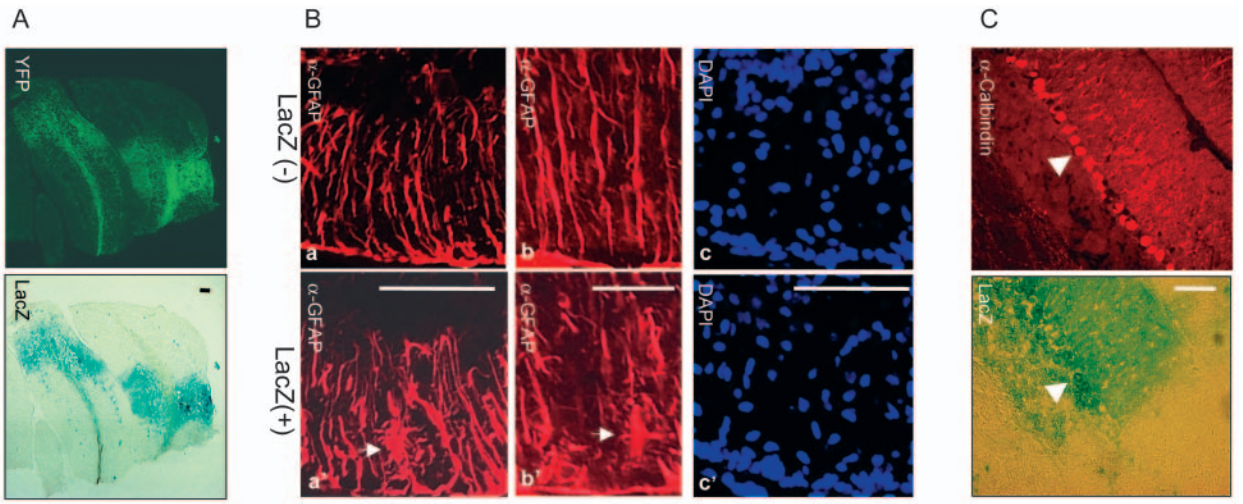

Fig. 8. Bergmann glia premature differentiation is a cell-autonomous event. HDA.Cre/YFP virus was injected into $Pten^{loxp/loxp};Rosa26^{floxed\text{-}Stop\text{-}lacZ}$ at P7 and tissues were analyzed 7 or 14 days after injection. (A) Good correlation between virus infection (YFP, shown in green, upper panel) and CRE recombinase-mediated loxp excision ($lacZ^{+}$, lower panel) in injection site. (B, a-c′) Increased Bergmann fiber thickening and lateral branching (arrow) were evident one week after *Pten* deletion (a′). These Bergmann glia showed astrocytic morphology (arrow) 2 weeks after infection (b′). (c,c′) DAPI staining performed 7 days after injection showed no apparent granule neuron migration defect within the ML; compare *lacZ* (+) (c′) and *lacZ* (–) (c) regions. (C) Purkinje cell positioning was not influenced by Bergmann glia *Pten* deletion after P7. Normally positioned Purkinje cells (arrows) are present in *lacZ* (+) area when compared with the adjacent uninfected *lacZ* (–) area. EGL, external granule layer; ML, molecular layer; BG, Bergmann glia layer; IGL, internal granule layer. Scale bars: 50 μm.

establishment of the indispensable role of Bergmann glial scaffold during granule neuron migration. One of the examples is the studies of weaver mutants; the close association between abnormal Bergmann glial scaffold and impaired granule neuron migration suggested that the morphological change of Bergmann glia was the major cause of granule neuron migration defect (Sidman and Rakic, 1973). However, other studies have demonstrated that weaver Bergmann glia were phenotypically normal in the chimeric studies, and weaver granule neurons failed to form contacts with Bergmann glia (Hatten et al., 1986; Goldowitz, 1989) and express differentiation markers Tag1, suggesting that Bergmann glia scaffold defects in weaver mice might be secondary to mutant granule neurons. The identification of a mutation in the *Girk2* gene as being responsible for the weaver mutant (Patil et al., 1995), which might result in a loss of inwardly rectifying K+ current and cytotoxicity in cerebellar granule cells (Surmeier et al., 1996), further complicated the interpretation of the role of weaver Bergmann glia in granule neuron migration. Thus, it is still not clear whether the migration defect in weaver mutants is due to abnormality of Bergmann glial scaffold or cell-intrinsic ionic changes in granule neurons. Another example is meander tail mice, which have the abnormal Bergmann glial scaffold, but can be rescued in the chimeric study, suggesting they might also be secondary to mutant granule neurons (Hamre and Goldowitz, 1997).

In this study, we provide evidence that the interplay between Bergmann glial scaffold and granule neuron is vital for granule neuron migration and IGL formation. When selectively deleting *Pten* in Bergmann glia by targeted adenovirus injection in vivo at P3, prior to the peak of granule neuron migration (P6-7), we demonstrate that the structural changes in *Pten*-null Bergmann glia are sufficient to affect granule neuron migration. However, we fail to observe a significant granule neuron migration defect after injecting adenovirus at P7, when granule neuron migration is still near its peak. Whereas biochemical changes occur as early as 12-24 hours after *Pten* deletion, the structural changes require at least 3-4 days. These data argue that astrocytic structural changes of *Pten*-null Bergmann glia are likely to be the primary cause of granule neuron migration defects and that the effect of Bergmann glia on granule cell migration is a developmental stage-dependent event. Furthermore, our data suggest that Bergmann glial scaffold may be important for Purkinje cell dendritic arborization. A recent study suggested that Purkinje cell dendritic arborization may rely on Bergmann fibers for proper alignment (Lordkipanidze and Dunaevsky et al., 2005). Consistent with this current study, we also observed a time-dependent Purkinje dendritic defect in PTEN mutants, strictly coupled with the timecourse of Bergmann glia changes. As the Bergmann fibers disappear, the Purkinje dendrites become randomly oriented and lack of fine processes.

Whether PTEN can modulate cell differentiation events remains ambiguous: PTEN loss is not crucial for cell-fate determination in central nervous system (Groszer et al., 2001; Marino et al., 2002), but precocious morphogenesis of mammary gland development (Li et al., 2002) and hair follicle changes (Suzuki et al., 2003) have been observed in *Pten* conditional deleted mammary glands and skin. In the current study, we observed striking morphological changes in Bergmann glia in *hGfap-cre*$^{+/-}$;*Pten*$^{loxp/loxp}$ mice during early postnatal development. The elongated Bergmann glial fibers prematurely develop numerous lateral processes that are indistinguishable from stellate astrocytes. This change highly resembles the cortical radial glia to stellate astrocyte differentiation. By selectively deleting *Pten* in Bergmann glia through targeted adenovirus delivery, we show that the *Pten*-null Bergmann glia premature differentiation is a cell intrinsic event, suggesting PI3K/Akt activity is important for radial glia to astrocyte transformation. In contrast to the dramatic changes in Bergmann glia after *Pten* deletion, we did not observe a significant alternation of cerebellar granule neuron differentiation by examining several differentiation markers. The discrepancy may arise from the relatively short duration of granule neuron differentiation, which could obscure the recognizable changes under current detection means. Alternatively, granule neuron and Bergmann glia may differ in their response to *Pten* deletion in the aspect of cell differentiation.

We thank Drs D. Geschwind, H. Kornblum, H. Vinters and members of our laboratories for helpful comments. This work was supported by grants from the NIH (R01CA107166, U01CA84128-06); from DOD (DAMD PC031130); from the Henry Singleton Brain Research Program and The James S. McDonnell Foundation (to H.W.); and by NIH NS38439 and The Brain Tumor Society (to X.L.).

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