PTEN is essential for cell migration but not for fate determination and tumourigenesis in the cerebellum

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Accepted 2 May 2002

SUMMARY

PTEN is a tumour suppressor gene involved in cell cycle control, apoptosis and mediation of adhesion and migration signalling. Germline mutations of PTEN in humans are associated with familial tumour syndromes, among them Cowden disease. Glioblastomas, highly malignant glial tumours of the central nervous system frequently show loss of PTEN. Recent reports have outlined some aspects of PTEN function in central nervous system development. Using a conditional gene disruption approach, we inactivated Pten in mice early during embryogenesis locally in a region specific fashion and later during postnatal development in a cell-specific manner, to study the role of PTEN in differentiation, migration and neoplastic transformation. We show that PTEN is required for the realisation of normal cerebellar architecture, for regulation of cell and organ size, and for proper neuronal and glial migration. However, PTEN is not required for cell differentiation and lack of PTEN is not sufficient to induce neoplastic transformation of neuronal or glial cells

Key words: PTEN, Cerebellum, Cre-LoxP, Lhermitte-Duclos Syndrome, Mouse

INTRODUCTION

The tumour suppressor gene PTEN encodes a lipid phosphatase, which has been shown to play a crucial role in the regulation of adhesion, migration, growth and apoptosis. PTEN germline mutations have been detected in several familial tumour syndromes, the most prominent of which are Cowden disease (CD) and Bannayan-Zonana (BZ) syndrome. These syndromes are characterised by developmental defects, benign tumours/hamartomas and a propensity to develop thyroid and breast cancer (Lloyd and Dennis, 1963). A subset of individuals with CD develops cerebellar gangliocytoma with abnormal and enlarged foliation caused by thickened and broadened layers of dysplastic and disorganised neuronal cells, also known as Lhermitte-Duclos disease (Lhermitte and Duclos, 1920). In addition to the disorganised cerebellar architecture, these individuals can show macrocephalia and mental retardation, and can develop seizures (Padberg et al., 1991).

However, although PTEN germline mutations have been demonstrated to be associated with CD and BZ syndromes, there is a considerable variability in genotype-phenotype correlation in individuals with identified mutations, and large studies have failed to detect PTEN germ line mutations in all individuals with CD and BZ syndrome (Marsh et al., 1998a; Marsh et al., 1998b). The phenotypic variability of specific mutations suggests the involvement of further tumour modifier genes (Marsh et al., 1999).

Several studies have been undertaken to elucidate the role of PTEN during development and tumourigenesis of the central nervous system. First evidence for the importance of PTEN in developmental processes was provided by the analysis of Pten−/− mice, which showed overgrowth of cells and disorganised cell layers in cephalic and caudal regions, resulting in early embryonic lethality (Di Cristofano et al., 1998; Podsypanina et al., 1999). Pten−/− embryonic stem cells fail to form embryoid bodies in vitro and to differentiate when transplanted into recipient mice suggesting a crucial role of PTEN in the differentiation process (Di Cristofano et al., 1998; Podsypanina et al., 1999) and Pten−/− embryos show an extensive proliferation and overgrowth (Stambolic et al., 1998).

Recently, cre loxP mediated inactivation of Pten in neural precursors during midgestation showed severe dysplasia with disturbance of the laminar organisation of the brain.
incompatible with postnatal life. In vitro data however showed that Pten-deficient neural precursor cells have retained the capacity to differentiate into neurones, astrocytes and oligodendrocytes (Groszer et al., 2001) but it remains unclear whether and to what extent Pten-deficient neural stem cells are capable of attaining terminal differentiation in vivo. Instead, inactivation of Pten in cerebellar granule cells leads to progressive increase in cell size and to slight retardation of cell migration (Backman et al., 2001; Kwon et al., 2001).

Loss of heterozygosity of PTEN has been detected in many human cancers, mainly in endometrial and ovarian cancer, and in late stage metastatic tumours and glioblastoma, a malignant brain tumour (Li et al., 1997). Accordingly, mice chimaeric or heterozygous for Pten show an increased spontaneous tumour incidence (Di Cristofano et al., 1998; Podsypanina et al., 1999). Moreover, inactivation of just one Pten allele has been shown to increase proliferation and cell survival and to decrease apoptosis (Di Cristofano et al., 1999; Podsypanina et al., 1999). Therefore, Pten haploinsufficiency has been suggested as an important factor in early selection and expansion of cells during transformation (Di Cristofano and Pandolfi, 2000). We inactivated Pten at two different time points of cerebellar development in a region specific or cell specific fashion, to study the role of Pten in differentiation, migration and neoplastic transformation.

MATERIALS AND METHODS

Generation of Pten conditional mutant mice

Pten conditional mice were generated by introducing loxP recognition sequences into the fourth and the fifth intron of the Pten gene, flanking exon 5. A 9 kb fragment containing a neomycin selection cassette flanked by loxP sites and a gene, flanking exon 5. A 9 kb fragment containing a neomycin sequence 1.3 kb downstream of Exon 5 (Fig. 1) was electroporated into ES cells. Southern blot analysis of targeted clones after homologous recombination was performed on BglII digested genomic DNA and probed with a 32P-labelled exon 4 (‘probe 1’). A 6.6 kb wild-type band (Fig. 1F, lanes 1-3) and a 5 kb band of the recombined allele (Fig. 1F, lane 1) were detected in a subset of clones. Probing with exon 5 (‘probe 2’) of Neurog1/EcoR1-digested genomic DNA resulted in a 6 kb wild-type (Fig. 1G, lanes 1-3) and a 2 kb recombined band in these clones (Fig. 1G, lanes 1, 2). Southern blot analysis of Neurog1/EcoR1-digested genomic DNA of selected clones after transient co-transfection with cre and puromycin expressing plasmids shows a 6 kb wild-type band (Fig. 1H, lanes 1-4) and a 2 kb floxed exon 5 band (Fig. 1H, lane 3) when probed with exon 5. Loss of the neo cassette was confirmed after re-probing the blot with a neo probe (Fig. 1H, lane 3). Mice carrying the Pten lox allele were derived by blastocyst microinjection.

Generation of L7cre transgenic mice

L7cre transgenic mice were generated by microinjection into male pronuclei of fertilised oocytes of FVB mice of a HindIII/EcoRI containing a cre cDNA fragment cloned into exon 4 of the L7 gene [kindly provided by Jim Morgan (Oberdick et al., 1990); Fig. 7A]. Expression patterns were determined by crossing the F1 generation of L7cre mice with ROSA26 reporter mice (Soriano, 1999). Several mouse lines showed an expression restricted to Purkinje cells (see Fig. 7B-D), therefore line L7cre/756 was used for all further experiments. Engrailed 2 transgenic mice (Zinyk et al., 1998) were kindly provided by Alexandra Joyner (Skirball Institute of Biomolecular Medicine, New York University School of Medicine).

Characterisation of mice

Genomic DNA was extracted from tail tips according to standard protocols and amplified with primers Cre1 (5'-ACC AGC CAG TTA TCA ACT C-3') and Cre2 (5'-TAT CGT GCT AGC GAA GAT CTC CAT CTT CCA GCA G-3') yielding a 269 bp product. Thermocycling conditions consisted of 30 cycles of 30 seconds at 94°C, 30 seconds at 56°C and 50 seconds at 72°C. Reactions contained 200 ng template DNA, 0.5 μM primers, 100 μM dNTPs, 9% glycerol, 2.5 U Taq polymerase, 1.8 mM MgCl₂, 1×PCR buffer (Gibco-BRL) in a 20 μl volume.

L7cre 26 mice were screened by PCR using the same conditions described above and primers LZ1 (5'-CGT CAC ACT ACG TCT GAA CG-3') and LZ2 (5'-CGACAGATGATCAGACTTC-3'). Tail DNA from PtenloxP mice was amplified for 30 cycles (30 seconds at 94°C, 30 seconds at 62°C and 50 seconds at 72°C) using primers Pten A (5'-GCG AAA GAA TGG TGC TAC-3') and Pten S (5'-GCC TTA CCT AGT AAA GCA AG-3'). The composition of the PCR mix was identical for all reactions.

Histology, immunohistochemistry and in situ hybridisation

For routine sections, brains were removed, immersion-fixed in 4% buffered paraformaldehyde for at least 4 hours, cut in coronal or sagittal planes and dehydrated through graded alcohols. After paraffin embedding, sections (3 μm) were cut and mounted on coated slides (Super Frost) and routinely stained with haematoxylin and eosin. The following antibodies or antisera were used for immunostaining: glial fibrillary acidic protein (GFAP, polyclonal antiserum 1:300, DAKO), S-100 (polycyclonal rabbit antiserum, 1:2000, DAKO), synaptophysin (polyclonal antiserum 1:300; Zymed), microtubule-associated protein (MAP2, mouse monoclonal antibody 1:500; Roche), phosphorylated neurofilament protein (200 kDa subunit) (mouse monoclonal antibody 1:200, Sigma), neuronal nuclei (NeuN, mouse monoclonal 1:4000; Chemicon), parvalbumin (polycyclonal rabbit antiserum, 1:750, SWANT), calbindin (polyclonal rabbit antiserum, 1:200, Chemicon), p27 (27 kDa polyclonal antiserum 1:500, Santa Cruz), p-Akt (monoclonal antiserum 1:300, Cell Signalling Technology), phosphorylated histone H3 (polycyclonal antiserum 1:1000, Upstate Biotechnology) and BrdU (monoclonal mouse antibody, 1:200, DAKO). A microwave pre-treatment for antigen unmasking was applied for synaptophysin, MAP2, NeuN, parvalbumin, calbindin, p27, p-Akt and BrdU. Detection was accomplished using biotinylated secondary antibodies, streptavidin-peroxidase complex and DAB. The automated NEXES immunohistochemistry machine (Ventana, Tucson, AZ) was used for all antibodies apart from p-Akt and p27.

In situ hybridisation for Math1 (Atoh1 – Mouse Genome Informatics) (probe was a gift from H. Zoghbi, HHMI, Baylor College of Medicine, Houston, TX), proteolipid protein and metabolotropic glutamate receptor 2 (mGLUR2) (probe from S. Nakanishi from Kyoto University, Japan) were carried out as described previously (Marino et al., 2000). The TSA amplification kit was used for Math1 and mGLUR2.

For in situ detection of apoptosis, the Roche TUNEL kit was used.

Immunoblotting

Cerebellar vermis from L7cre; PtenloxP/loxP, L7cre; PtenloxP/lo who generated the wild-type mice was homogenised, and protein extracted in lysis buffer (1% NP 40, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.1% SDS, 0.5% deoxycholic acid, 2 mM EDTA, Complete Protease Inhibitor Cocktail (Roche)) for 30 minutes at 4°C. Protein (30 μg) was resolved on 14% SDS-polyacrylamide gel, transferred to nitrocellulose membrane (Schleicher and Schuell) and probed with the following antibodies: phospho-AKT (polyclonal rabbit antiserum, 1:500, Cell Signalling Technology) and actin (1:500, rabbit polyclonal, Sigma). HRP-conjugated anti-goat secondary antibody was used at 1:5000 (Santa Cruz Biotechnology), and...
blots were visualised using enhanced chemiluminescence (ECL, Amersham). In addition, Chemoluminescent Signals were detected and quantified with a Kodak gel documentation system.

**Whole-mount lacZ staining**

To visualise β-galactosidase activity in organs of reporter mice, tissue slices (2-4 mm) or sagittal cut brains were processed as described elsewhere (Benninger et al., 2000). Paraffin wax-embedded sections of lacZ pre-stained brain slices were cut (3 µm) and counterstained with Nuclear Red or were further processed with standard immunohistochemical techniques using DAB as a chromogen (see above).

**Image acquisition and analysis**

Image acquisition was done with a JVC video camera (1360×1024 pixels) attached to Zeiss Axioskop. Measurements of area or cell number were carried out with AnalySIS software (www.soft-imaging.de). For the calculation of areas, an image was captured at low-power magnification and further processed offline. Cells were marked and counted up by the software on the captured image.

### RESULTS

**Generation and characterisation of En2cre; Pten<sup>LoxP/LoxP</sup> mice**

Pten conditional mice were generated by flanking exon 5 with LoxP sites (Fig. 1A-I). The inserted mutation did not affect the function of the Pten gene and crossing these mice with deleter Cre mice (Schwenk et al., 1995) mimicked the phenotype of the conventional Pten knockout mice (data not shown).

To achieve inactivation of Pten in a region-specific fashion in the developing cerebellum, Pten conditional mutant mice were intercrossed with engrafted 2 (En2 cre) mice (line 22) (Zinyk et al., 1998). This line of En2cre transgenic mice expresses cre in a narrow dorsal domain in the midbrain-hindbrain junction around embryonic day 9.5 (Zinyk et al., 1998). Cells originating from this domain later populate the medial region of the cerebellum, which largely corresponds to the vermis.

Embryonal analysis at day 15.5 (E15.5) of En2cre; Pten<sup>LoxP/LoxP</sup> mice revealed a striking increase in the cell size of all precursor cells resulting in increased organ size of the cerebellar anlage (Fig. 2A,B). In addition to the above-mentioned findings, a complete lack of foliation was observed at postnatal day 1 in En2cre; Pten<sup>LoxP/LoxP</sup> mice (Fig. 2E,F).

In mice older than three weeks, we observed ataxia, impairment of balance and reduction of overall activity. Gross inspection of their brains revealed a lack of foliation and enlargement of the cerebellar vermis (Fig. 3A,B), in keeping with the developmental findings. Histologically and immunohistochemically, all distinctive cell types of the mature cerebellum such as Purkinje cells (calbindin), granule cells (NeuN), stellate (parvalbumin) and basket cells (parvalbumin and neurofilament 200), Golgi cells (mGlur-2 in situ hybridisation) (Ohishi et al., 1993), Bergmann glia (GFAP and S-100) and oligodendrocytes (proteolipid protein in situ hybridisation) were represented and fully differentiated, indicating that PTEN is not required for fate determination and terminal differentiation in vivo. Instead, our experiments show that PTEN provides important cues for cells migration and positioning during development: in mature cerebella, the majority of Purkinje cells were clustered in an area above the fourth ventricle without recognisable orientation, suggesting an impairment of migration and positioning during development (Fig. 3D,K-O). However, several Purkinje cells migrated towards the cerebellar surface, randomly intermingled with granule cells and occasionally reached the surface. Mature granule cells, as identified by NeuN immunostaining, failed to form a proper layer, but were either loosely distributed throughout the deeper cerebellum (Fig. 3H) or failed to migrate inwards, resulting in mature granule cells within the molecular layer (Fig. 3J), in agreement to previous findings (Backman et al., 2001; Kwon et al., 2001). Likewise, Golgi cells (Fig. 4K,L), stellate and basket cells, as well as non

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**Fig. 1.** Generation of mice deficient for PTEN in a tissue specific fashion: (A) organisation of the PTEN wild-type locus; (B) targeting construct used for electroporation into ES cells; (C) targeted locus containing the neomycin resistance gene flanked by LoxP sites; (D) ES cells lacking the neo cassette were generated by transient cre expression, resulting in a Pten<sup>LoxP</sup> allele; (E) tissue-specific Cre-mediated recombination results in loss of exon 5. (F) Southern blot analysis of targeted clones after homologous recombination (BglII digest, probe 1; exon 4) shows a 6.6 kb wild-type band and a 5 kb band of the recombined allele in lane 1. (G) Probing with probe 2 (exon 5) of Neo/I-CfoI-digested genomic DNA resulted in a 6 kb wild-type and a 2.5 kb recombinant band in lanes 1+2. (H) Southern blot analysis (probe 2, NcoI/EcoRI-digested genomic DNA) after transient transfection with a Cre-expressing plasmid shows a 6 kb wild-type band and a 2 kb floxed exon 5 band. (lane 3). (I) Loss of the neo cassette was confirmed after re-probing the blot with a neo probe (lane 3).
neuronal cells (Bergmann glia and oligodendrocytes) (Fig. 4I,J), failed to assume proper position and rather were randomly distributed throughout the cerebellum. Ablation of only one Pten allele in En2cre; Pten<sup>LoxP/LoxP</sup> mice did not result in abnormality of the cerebellum.

### No formation of tumours in <i>En2cre; Pten<sup>LoxP/LoxP</sup></i> mice

Loss of Pten has been described in a variety of malignant human tumours, including glioblastoma (Li et al., 1997; Wang et al., 1997). To assess, whether the lack of one or both alleles of Pten in mature neuronal or glial cells causes dysplasia or true neoplastic transformation, we kept 24 <i>En2cre; Pten<sup>LoxP/LoxP</sup></i> mice under observation for up to 28 weeks. Ataxic gait worsened between 3 and 8 weeks of age and remained steady thereafter. No additional sign of central nervous system damage appeared. Macroscopic examination of the brains confirmed enlargement and malformation of the vermis, as observed in younger animals. Moreover lateral or rostral displacement of the cerebellar hemispheres and of the occipital cortex respectively, was observed (Fig. 3A,B). Morphological analysis showed that dysplastic occasionally bi-nucleated neurons (Fig. 3F, Fig. 4C,D), similar to those found in human cerebellar gangliocytoma, were already present in 7-week-old mice, whereas clearly dysplastic astrocytes (Fig. 4E) were detected after 16 weeks. However, no true neoplastic lesions were detected in these mice. This finding was in line with the lack of proliferating cells in the cerebellum of adult <i>En2cre; Pten<sup>LoxP/LoxP</sup></i> mice, as assessed by immunostaining for the proliferation marker p-histone H3 (data not shown). Our findings clearly demonstrate that lack of Pten is not sufficient to elicit neoplastic transformation neither in neuronal cells nor in glial cells. Surprisingly, Purkinje cells in <i>En2cre; Pten<sup>LoxP/LoxP</sup></i> mice (Fig. 4C) showed cytoplasmic accumulation of neurofilaments similar to tangles and exhibited vacuolation (Fig. 4C), both indicating progressive degeneration. Moreover, the overall number of Purkinje cells continuously decreased over time as shown schematically in Fig. 3K-O, where red dots indicate calbindin-positive Purkinje cells without overt signs of degeneration and green dots represent vacuolated, degenerating Purkinje cells. These findings suggest that the absence of PTEN in Purkinje cells leads to degeneration, rather than to neoplastic transformation.

### Expression pattern of phosphorylated AKT and p27<sup>Kip1</sup> in <i>En2cre; Pten<sup>LoxP/LoxP</sup></i> mice

Loss of PTEN leads to activation of the PI3K pathway and thereby to phosphorylation and activation of Akt (reviewed by Cantley and Neel, 1999). To investigate whether and to what extent activation of Akt is implicated in neuronal and glial abnormalities observed we set out to characterise the expression pattern of phospho Akt (p-Akt) in wild type and compound mutant mice using a p-Akt-specific antibody (Backman et al., 2001; Kwon et al., 2001).

To define the baseline of p-Akt expression in the developing and mature cerebellum, we performed immunohistochemical staining on wild-type brains at various time points. While p-Akt was strongly expressed in Purkinje cells and in granule cell precursors during development (Fig. 2E), a less intense p-Akt expression was noted in Bergmann glia cells and to lesser extent Purkinje cells and Golgi cells in the adult brain. In mutant <i>En2cre; Pten<sup>LoxP/LoxP</sup></i> cerebella at postnatal day 1, we detected a widespread expression of p-Akt, as expected from the architectural abnormalities (Fig. 2). Interestingly, in adult cerebella of <i>En2cre; Pten<sup>LoxP/LoxP</sup></i> mice, strong p-Akt overexpression was observed in a fraction of dysplastic Purkinje cells (data not shown) and in the...
majority of dysplastic and non-dysplastic astrocytes (Fig. 4F). A weaker expression of p-Akt was detected in all other cell types of the cerebellar vermis, which, however, still exceeded the levels in PTEN-expressing cells. In keeping with these findings, immunoblotting of dissected vermis extracts of En2cre; PtenLoxP/LoxP mice showed higher levels of p-Akt than the corresponding wild-type tissue (Fig. 5).

Akt activation affects cell cycle progression through inhibition of p27Kip1 protein levels. Phosphorylation of p27Kip1 by Akt results in its cytoplasmic retention and loss of its growth inhibition. As predicted, p27Kip1 expression levels inversely correlated with those of p-Akt in wild type as well as in mutant cells in the developing cerebellum. However, it should be noted that p27Kip1 levels in adult En2cre; PtenLoxP/LoxP mice did not show a noticeable decrease compared to PTEN-expressing cells in laterally adjacent cerebellar regions. This finding is confirmed by immunoblotting for p27 of the vermis of adult En2cre; PtenLoxP/LoxP mice, which showed no difference from wild-type mice (data not shown).

The thickness of the external granule layer (EGL) during early postnatal development was increased (six or seven cells instead of four or five cells in thickness) in En2cre; PtenLoxP/LoxP mice (Fig. 2G,H). To evaluate whether these cells were undifferentiated precursors that were still proliferating or whether they were already terminally differentiated but non-migrating granule cells, we performed mRNA in situ hybridisation for Math1, a marker of cerebellar granule cell progenitors (Ben-Arie et al., 2000; Helms and Johnson, 1998). We show that a considerable proportion of cells in the thickened EGL of mutant mice are Math1 positive and therefore represent undifferentiated, still actively proliferating, granule cell progenitors.
Cerebellar granule cell proliferation is controlled by p27Kip1 during development and p27Kip1 accumulates in EGL cells shortly before exit from cell cycle and terminal differentiation (Miyazawa et al., 2000). EGL cells in En2cre; PtenLoxP/LoxP mice show a significantly decreased expression of p27Kip1 (Fig. 2I,J). Therefore, activation of Akt and downregulation of p27Kip1 may explain retarded maturation of PTEN deficient EGL cells.

**Cell proliferation and cell death in the cerebellum of En2cre; PtenLoxP/LoxP mice**

Several studies have suggested that PTEN negatively regulates cell growth, and in vitro experiments suggest an increased proliferative potential of Pten−/− neural stem cells (Groszer et al., 2001). We therefore examined cell proliferation at various time points during development using either or BrdU incorporation (Fig. 2C,D) or antibodies against phosphorylated histone H3, which is expressed during M-phase when the chromosomes are fully condensed. Surprisingly, both BrdU and phospho-histone H3 immunostaining showed less proliferation in Pten-deficient cerebella than in wild-type brains. Both the absolute number (i.e. labelled nuclei within the entire sagittal area of the cerebellum) and the relative number (i.e. labelled nuclei as a ratio of all nuclei) were reduced by a factor of two to three at E15.5, P1 and P9 in Pten-deficient cerebella (Fig. 6F). To determine, whether increased size of cerebella in En2cre; PtenLoxP/LoxP mice was caused by augmented cell numbers, increased cell size or both, we calculated cell numbers and cell density from sagittal sections. Surprisingly, overall cell density (i.e. the cell density of an entire sagittal section) was lower in Pten mutant cerebella at E15.5 and P1 (Fig. 6A), probably owing to the lower cellular density of the EGL in mutant animals. However, as the midsagittal area (Fig. 6B) was up to almost twofold larger in mutant cerebella, the absolute cell number was higher in mutant than in wild-type brains (Fig. 6C). TUNEL staining performed on E15.5 and P1 revealed a decreased apoptotic rate in the mutant cerebellum (Fig. 6D). Therefore, the decreased proliferation (Fig. 6F) is ‘compensated’ by a decreased cell death rate, which may explain why similar cell numbers are counted at postnatal day 1 (Fig. 6C).

**Generation and characterisation of L7 cre transgenic mice**

To analyse whether and to what extent migration disturbance, increased cell size and degeneration of Purkinje cells are cell autonomous, rather than a consequence of improper positioning and environment, we set out to inactivate Pten selectively in these cells. To this end, transgenic mice were generated in which Cre was expressed under the control of the...
PTEN function in cerebellar development

L7 promoter, which is mainly expressed in Purkinje cells (Oberdick et al., 1990). Genes resulting from the pronuclear injection of the L7cre construct (Fig. 7A) were screened for integration of the transgene. Transgenic lines were derived from all 11 founder mice (FVB inbred). To investigate the tissue-specific expression and the functionality of the transgene two lines, Tg 753 and Tg 756, were crossed to ROSA26R indicator mice (Soriano, 1999). In situ enzymatic staining for β-galactosidase on brains of adult double transgenic mice revealed staining exclusively in Purkinje cells (Fig. 6B-D). No lacZ expression was detected outside the brain.

While L7cre; PtenLoxP/+ mice showed no cerebellar abnormality, L7cre; PtenLoxP/+ Pten mice exhibited subtle irregularities of Purkinje cell lining but no major architectural disturbances and no clinical phenotype up to 20 weeks of age. All Purkinje cells showed noticeable increase in cell size, including thickening of dendrites and descending axons (Fig. 7E,F), while neighbouring cells (granule cells and Bergmann glia) remained unaffected. At later time points (17 and 20 weeks) Purkinje cells showed signs of degeneration (Fig. 6G). However, we observed that Purkinje cells were retained in large clusters above the fourth ventricle instead of migrating towards the cerebellar surface (Fig. 3K-O).

We show here that PTEN is essential for cell migration and determination of laminar structure of the cerebellum. These results are consistent with the data obtained by inactivation of Pten in neural precursors during midgestation by nestin-mediated cre expression (Groszer et al., 2001). We observed that Purkinje cells were retained in large clusters above the fourth ventricle instead of migrating towards the cerebellar surface (Fig. 3K-O). As PTEN contributes to the regulation of cell adhesion and migration through the MAP kinase/focal adhesion kinases and Shk cascade (Tamura et al., 1999a; Tamura et al., 1998; Tamura et al., 1999b), impaired cell migration and positioning might be a consequence of defective adhesion signalling. It is possible, that impaired migration is due to a deregulated activity of phosphatidylinositol-3-kinase (PI3K). Ming et al. (Ming et al., 1999) have shown that nerve growth cone guidance is mediated by PI3K and there is evidence for an involvement of PI3K in cell migration (Jimenez et al., 2000; Shen and Guan, 2000). However, it has yet to be clarified to what extent the observed migration defects are cell autonomous (i.e. directly caused by Pten deficiency of the migrating cell) or rather indirectly a result of, for example, disturbed radial glia formation, which no longer provide guidance for migrating granule cells.

To date, it is unclear whether Pten plays a role in cerebellar development and terminal differentiation. So far the differentiation capacity of Pten-deficient neural precursor cells into astrocytic, oligodendroglial and neuronal lineages has been shown only in vitro (Groszer et al., 2001). The early postnatal lethality of Nestin-Cre; PtenLoxP/+ mice precluded further analysis. Inactivation of Pten in already committed neuronal populations during postnatal development (Backman et al., 2001; Kwok et al., 2001), although compatible with postnatal life, does not allow us to address this point. Our data demonstrate for the first time that absence of Pten does not impair the capacity of neural progenitors to differentiate into all cell types of the mature cerebellum in vivo.

A surprising finding was the reduced fraction of proliferating cells in cerebella of En2cre; PtenLoxP/+ mice during embryonic and early postnatal development. The proliferation was assessed with an antibody against phosphorylated histone H3, which labels nuclei during the M phase and confirmed by BrdU labelling at E15.5. These findings appear to be in disagreement with previous studies, in which proliferation was increased in Pten-deficient E14 neural sphere cultures and in the ventricular zone of Nestin-cre; PtenLoxP/+ E14.5 embryos (Groszer et al., 2001). When BrdU-positive nuclei were related to the number of all (labelled and unlabelled) nuclei of the entire cerebellar section, a proliferation fraction of 12% was

DISCUSSION

We inactivated Pten during embryonic development in all neuronal and glial cell populations in a precisely defined area of the CNS (the cerebellum). This was achieved by using En2cre transgenic mice, in which cre is active in a narrow dorsal domain in the midbrain-hindbrain junction with a peak expression at embryonic day 9.5 (Zinyk et al., 1998). Cells originating from this domain later populate the medial region of the cerebellum, which corresponds reasonably well to the vermis.

This experimental approach allowed us to study the role of PTEN in cell migration and determination of cytoarchitecture during development. Moreover, as inactivation of Pten was restricted to a specific brain area, we did not elicit a lethal phenotype and therefore the differentiation potential of progenitor cells lacking Pten could be analysed.

Immunoblotting of protein extracts from cerebella of L7cre; PtenLoxP/+; L7cre; PtenLoxP/+; En2cre; PtenLoxP/+ and wild-type mice. Increased levels of phospho-Akt are only detected when Pten is deleted on both alleles in Purkinje cells (L7cre; PtenLoxP/+ mice) or in all cells of the vermis (En2cre; PtenLoxP/+), while wild-type and L7cre; PtenLoxP/+ cerebella show no difference.

![Fig. 5. Immunoblotting of protein extracts from cerebella of L7cre; PtenLoxP/+; L7cre; PtenLoxP/+; En2cre; PtenLoxP/+ and wild-type mice. Increased levels of phospho-Akt are only detected when Pten is deleted on both alleles in Purkinje cells (L7cre; PtenLoxP/+ mice) or in all cells of the vermis (En2cre; PtenLoxP/+), while wild-type and L7cre; PtenLoxP/+ cerebella show no difference.](image-url)
Inhibitor p27. In accordance with this known effect of Akt, we observed a decreased expression of p27 in the broadened EGL in En2cre; PtenLoxP/LoxP mice and only single cells that had migrated inwards expressed p27 at higher levels (Fig. 2LJ). The marked increase in thickness of the EGL shows striking resemblance with the phenotype of p27−/− (Cdkn1b – Mouse Genome Informatics) mice. However, although p27−/− granule cell precursors show increased proliferation when compared with wild-type cells in vitro (Miyazawa et al., 2000), their proliferation rate in vivo has not yet been sufficiently addressed. Downregulation of p27 expression in EGL precursor cells of En2cre; PtenLoxP/LoxP mice might be responsible for many of the effects observed. It has been proposed that p27 acts as a timer in precursor cell division in a dose-dependent fashion (Durand and Raff, 2000). Insufficient levels of p27 in EGL precursors may impair both cell cycle exit and differentiation. In agreement with this interpretation is the prolonged expression of the transcription factor Math1, member of neural basic helix-loop-helix factors controlling neural differentiation. Although expression of p27 in the EGL is generally taken as an indicator for cell cycle exit of inwards migrating precursor cells, the normally organised, though slightly enlarged, cerebellum of p27−/− mice proves that alternate pathways are providing appropriate signals for cell cycle exit, differentiation and migration in p27−/− mice.

A striking feature in En2cre; PtenLoxP/LoxP mice was the chronic progressive loss of Purkinje cells (Fig. 3K-O), which started during early postnatal development and was characterised by vacuolation and accumulation of fibrillary inclusions (Fig. 4C). To analyse whether and to what extent increased cell size and degeneration of Purkinje cells are cell autonomous or whether this is a consequence of improper positioning and environment, we have generated L7cre mice to inactivate Pten selectively in Purkinje cells. L7cre; PtenLoxP/LoxP mice exhibited similar features of Purkinje cell degeneration as En2cre; PtenLoxP/LoxP mice (Fig. 7F,G). We therefore conclude that these effects are likely to be cell autonomous, rather than a consequence of improper migration or micro-environment. Degenerative changes followed by cell loss noted for Purkinje cells point to a possible role of Pten and its downstream targets, in neurodegeneration. However, it is not clear how the observed cell death is brought about, as several studies suggest a role of Akt in promoting cell survival rather than triggering degeneration (Eves et al., 1998; Gary and Mattson, 2001; Tanno et al., 2001; Zhou et al., 2001). However, these findings were obtained in growing rather than in terminally differentiated cells. Therefore it is conceivable that postmitotic neurons, such as Purkinje cells react differently to continuous activation of the Akt pathway: loss of PTEN action on PI3K disinhbits growth factor signalling. Overexpression of Akt may confer hyper-responsiveness to ambient levels of growth factors, for example, by upregulation of insulin-like growth factor 1, which may render specific neuronal populations particularly sensitive to growth factors and Akt-mediated effects.

In two recent studies, GFAP-Cre mice were generated to inactivate Pten selectively in astrocytes. However, GFAP-Cre
was only expressed in a fraction of the astrocytes (Backman et al., 2001; Kwon et al., 2001), whereas Pten inactivation did occur in cerebellar and dentate gyrus granule cells and in some cortical neurons, presumably because of GFAP activity in neural precursor cells during development (Marino et al., 2000). In En2cre; Pten\textsuperscript{LoxP/LoxP} mice, we achieved inactivation of Pten during early embryonic development in precursor cells, giving rise to both glial and neuronal cell types. We show here for the first time that lack of Pten induces dysplasia in mature glial and neuronal cells. Dysplastic changes correlated with strong accumulation of p-Akt, indicating a possible involvement of this pathway in cell dysplasia. Moreover, in contrast to previous findings (Backman et al., 2001; Kwon et al., 2001) we found a considerably p-Akt expression not only in Pten-deficient cells but already in Bergmann glia, Purkinje and Golgi cells of wild-type mice. These findings are supported by baseline p-Akt levels detected in western blots of vermis extracts of wild-type mice. Therefore the preferential involvement of glial and Purkinje cells in dysplasia and degeneration, respectively, might be explained by a further upregulation of an already active pathway.

Loss of heterozygosity for Pten has been detected in many human cancers (Li et al., 1997; Podsypanina et al., 1999; Wang et al., 1997) and in line with these findings, mice chimaeric or heterozygous for Pten show an increased spontaneous tumour incidence (Di Cristofano et al., 1998). Inactivation of just one Pten allele has been shown to increase proliferation and cell survival and to decrease Akt and Parp dependent, Fas-mediated apoptosis in T- and B-lymphocytes (Di Cristofano et al., 1998). Therefore, Pten haploinsufficiency has been suggested to be an important factor in early selection and expansion of cells during transformation (Di Cristofano and Pandolfi, 2000). However, inactivation of one Pten allele during early CNS development in En2cre Pten\textsuperscript{LoxP/+} mice and in Purkinje cells of L7cre; Pten\textsuperscript{LoxP/+} mice failed to result in increases of cell size, deficits in migration and differentiation, dysplasia, or upregulation of p-Akt levels in L7cre; Pten\textsuperscript{LoxP/+} mice (Fig. 5), indicating that development and neoplastic transformation in the CNS are not affected by Pten gene dose. Moreover, we show that inactivation of both Pten alleles in the cerebellum is not sufficient to elicit neoplastic transformation. Hence, this points to a role of PTEN in late tumour progression and invasion, in agreement with previous studies on human tumours.

Both the mouse model presented here and the reports published previously (Backman et al., 2001; Kwon et al., 2001) suggest that malformations in Lhermitte-Duclos disease may originate from diverse cell populations affected by loss of Pten at different stages of development. As cerebellar malformations in Lhermitte-Duclos are a rather heterogeneous entity with high variability of morphological abnormalities, it is most likely that Pten inactivation in precursor cell populations during different stages of development gives rise to variable migration defects associated with increased thickness of foliation, or, in more extreme cases, disorganisation or loss of foliation. Our findings support the view of Kwon and co-workers (Kwon et al., 2001) and Backman and colleagues (Backman et al., 2001) that Lhermitte-Duclos disease is a hamartomatous lesion rather than a neoplastic proliferative disorder. Using this model, we provide further insight into the pathogenesis of CNS malformations associated with germline or somatic mutations of Pten.

We thank Marianne König, Beatrice Pfister and Mauri Peltola for excellent histological assistance, Thomas Rülicke for the microinjection of the L7\textsuperscript{cre} construct, and the staff of the Biologisches...
Zentrallabor for animal caretaking. L7 promoter construct was a gift from Jim Morgan (St Jude Children’s Research Hospital, Memphis, TN); Math1 probe was obtained from H. Zoghbi (HHMI, Baylor College of Medicine, Houston, TX); and the mGluR2 plasmid was received from Shigetada Nakanishi (Kyoto University, Japan). We are grateful to Alexandra Joyner (Skirball Institute of Biomolecular Medicine, New York University School of Medicine New York, NY) for providing us with the Engrailed-2 cre transgenic mice. The work was supported by a grant of the Schweizerischer Nationalfonds (31-64266.00) to S. M. and S. B., and by the Novartis Foundation to S. M.

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