AD_____

Award Number: DAMD17-00-1-0010

TITLE: The Function of PTEN Tumor Suppressor Gene in Prostate Cancer Development

PRINCIPAL INVESTIGATOR: Hong Wu, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of California, Los Angeles
Los Angeles, California 90095-1735

REPORT DATE: March 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	March 2002	Annual (15 Feb 01 - 14 Feb 02)

4. TITLE AND SUBTITLE
The Function of PTEN Tumor Suppressor Gene in Prostate Cancer Development

5. FUNDING NUMBERS
DAMD17-00-1-0010

6. AUTHOR(S)
Hong Wu, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, Los Angeles
Los Angeles, California 90095-1735

E-Mail: Hwu@mednet.ucla.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

Prostate cancer is the most common malignancy in men. Studying the biology of prostate cancer and development of new therapies are hampered by a lack of insight into the molecular basis of the disease and appropriate animals models. The recently identified tumor suppressor gene PTEN is a promising candidate for being involved in prostate cancer since it is frequently deleted in prostate cancer, especially in advanced or metastatic forms.

To study the function of PTEN in prostate cancer development, we have deleted Pten gene and generated an animal model system. Mice lacking one allele of Pten gene developed prostate abnormalities, ranging from hyperplasia to malignant carcinomas, starting from the 8^{th} month. To accelerate this process, we have generated $Pten^{loxp/loxp}$ mice, which will allow us to delete Pten specifically in the prostate glands. We are currently breeding the $Pten^{loxp/loxp}$ mice with prostate specific Cre transgenic mice. We have also generated a TAT-Cre fusion protein which will allow us to focally delete Pten by surgical injection into the prostate. This study will not only allow us to better understand the function of PTEN in prostate cancer, but will generate a novel animal model for possible treatment.

14. SUBJECT TERMS
prostate cancer, PTEN tumor suppressor, animal model

15. NUMBER OF PAGES
18

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

INTRODUCTION

PTEN is a tumor suppressor gene frequently deleted in many human cancers, including prostate cancers. The goal of this funded proposal is to study the function of PTEN in prostate cancer development in vivo. Since loss of PTEN function causes embryonic lethality, we proposed to specifically inactivate Pten in the prostate.

Three specific tasks should be accomplished in this study:

I. To characterize $Pten^{loxp/loxp}$ mouse strain;
II. To inactivate Pten in the secretory epithelium of the prostate gland by intercrossing $Pten^{loxp/loxp}$ and probasin Cre transgenic mice;
III. To focally inactivate Pten through injection of CMV-Cre-T adenovirus into the prostate of $Pten^{loxp/loxp}$ mice.

BODY: STUDIES AND RESULTS

I. To characterize Pten$^{loxp/loxp}$ mouse strain

I-1. To generate Pten$^{loxp/loxp}$ mouse strain on 129svJ/C57Bl/6 and 129svJ/Balb/c backgrounds (accomplished during the first funding period).

I-2. To generate isogenic 129svJ Pten$^{loxp/loxp}$ mouse strain (accomplished during the first funding period).

I-3. To characterize Pten$^{loxp/loxp}$ mouse strain (accomplished during the past funding period).

As a prerequisite for subsequent inactivation, it is crucial to prove that introduction of loxp sites into the introns on either side of exon 5 has no effect on the function of PTEN in Pten$^{loxp/loxp}$ mice. In contrast to the embryonic lethal phenotype observed in *Pten*$^{-/-}$ mice, *Pten*$^{loxp/loxp}$ animals were viable. Normal PTEN level and function were detected in *Pten*$^{loxp/loxp}$ MEF cells and no spontaneous tumor formations were observed up to two years, suggesting that introducing the loxp sites into the *Pten* locus does not perturb the normal function of PTEN.

Since this is an exon 5-specific deletion and exon 4 may splice to exon 6, we tested whether a truncated protein could be generated. No PTEN protein could be detected using either anti-N terminal or anti-C terminal antibodies (Figure 1b), suggesting that exon 5 deletion leads to either a complete null mutation or a truncated protein which is very unstable.

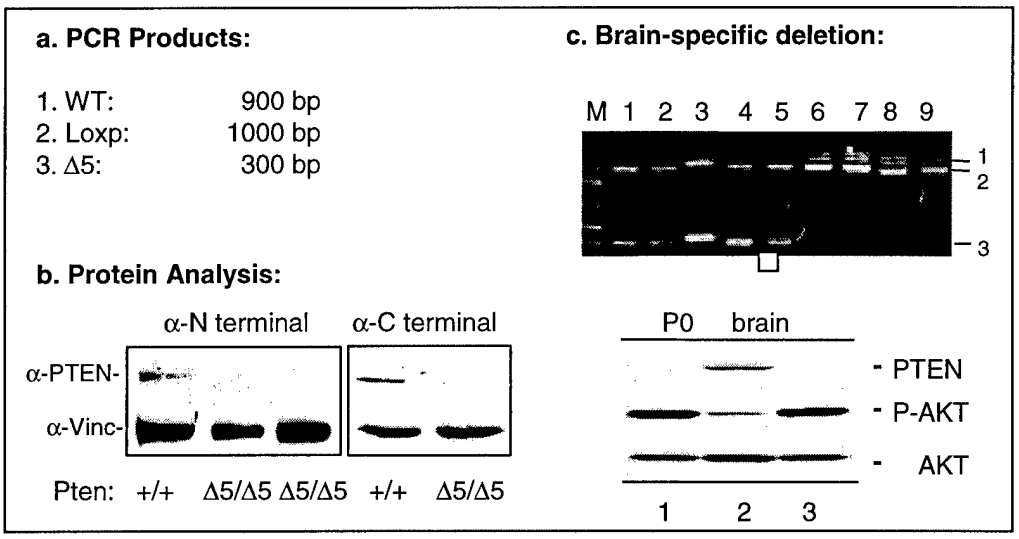

Figure 1: Conditional inactivation of *Pten* gene.
(a). Predicted PCR products. Primers used are: P1 5'-ACTCAAGGCAGGGATGAGC-3', P2 5'-AATCTAGGGCCTCTTGTG CC-3', and P3 5'-GCTTGATATCGAATTCCTGCAGC-3'.
(b). Western blot analysis using α-N terminal (left) and α-C terminal antibodies.
(c). GFAP-Cre–mediated *Pten* deletion in *Pten*$^{loxp/+}$;*Cre*$^{+/-}$ mice (upper panel). Lanes 1-5, neural tissues: cortex, hippocampus, cerebellum, brain stem, and spinal cord, respectively; lanes 6-9, non-neural tissues: thymus, heart, kidney, skin, respectively. Western blot analysis (lower panels) using P0 *Pten*$^{loxp/loxp}$;Cre$^{+/-}$

brain samples. Lanes 1 and 3, mutant; lane 2, WT control. Antibodies used: α-PTEN (C terminal) NEB; α-P-AKT and α-AKT (Santa Cruz).

To demonstrate Cre-mediated exon 5 deletion, we crossed *Pten*$^{loxp/loxp}$ animals with the GFAP-Cre transgenic mice (Zhuo et al., 2001) aimed for brain-specific deletion. As shown in Figure 1C, Cre expression in the *Pten*$^{loxp/+}$;*GFAP-Cre*$^{+/-}$ mice resulted in neural-specific excision of exon 5 (lanes 1-5). In contrast, very low or no excision could be detected in other non-neural tissues (lanes 6-9). Finally, we showed that no PTEN protein could be detected in conditional deleted tissue and the known downstream signaling molecule AKT/PKB was hyperphosphorylated (Figure 1C). Thus, the *Pten*$^{loxp/loxp}$ mouse line generated can be used for studying the function of PTEN in animal development and tumorigenesis.

II. To inactivate *Pten* in the prostate secretory epithelium by intercrossing *Pten*$^{loxp/loxp}$ mice and *probasin(PB)-Cre* transgenic mice (Partially accomplished during the last funding period).

As stated in the last report, we have obtained *PB-Cre* transgenic mice from Dr. Norm Greenberg. Unfortunately, the mice shipped to us could not serve our purpose. We have since got the Cre line with modified PB promoter from Dr. Roy-Burman at USC. Animal breeding is in progress and we have produced a cohort of *Pten*$^{loxp/loxp}$; *Cre*$^{+/-}$ and *Pten*$^{loxp/d5}$;*Cre*$^{+/-}$ mice. The oldest animals are two month-old now and we will start systematic analysis in two months.

III. To focally inactivate *Pten* through injection of CMV-Cre-T adenovirus into the prostate of *Pten*$^{loxp/loxp}$ mice.

As reported in last year's summary, the original plan of injecting first generation of CMV-Cre-T adenovirus into the prostate of *Pten*$^{loxp/loxp}$ mice turned out to be problematic due to the host immune response. We have since explored two independent system to circumvent this problem:

III-1. To generate a "gut-less" adenovirus vector carrying the Cre recombinase.

The host immune response to the first generation of adenovirus vector is mainly due to the viral envelope proteins. Dr. Arnold Berk's laboratory has recently developed a gut-less adenovirus vector carrying the Cre recombinase. We have set up a formal collaboration to test this virus on prostate-specific *Pten* deletion. $1x10^9$ viral particles (approximately 10μl) will be surgically injected into the prostate gland of *Pten*$^{loxp/loxp}$ mice. Two mice will be sacrificed 7 days post injection to survey 1) possible host immune response; and 2) Cre-mediated *Pten* deletion.

III-2. To generate a TAT-Cre fusion protein for protein transduction (accomplished during the last funding period).

Recently, a novel technique has been discovered that might allow one to focally deliver Cre recombinase in vivo. The novel technology co-opts the interesting ability of the HIV TAT protein to cross cell membranes in a receptor-independent and endocytosis-independent manner. Although its

mechanism of action is unclear, a 36 amino acid domain of TAT has been defined to mediate this phenomenon (Nagahara, 1998). By fusing this TAT domain to Cre recombinase, we have generated TAT-Cre fusion protein and proved its function in tissue cultured cells and in the brain of $Pten^{loxp/+}$ mouse. We have injected the TAT-Cre fusion protein directly into the prostate glands of $Pten^{loxp/lox}$ mice. Four out of five injected animals developed prostate hyperplasia at four month-of –age, at least six months earlier than that of $Pten^{loxp/+}$ mice.

SUMMARY:

We have finished characterization of the $Pten^{loxp/lox}$ mice during the second funding period as original planned. Generation of $Pten^{loxp/loxp}$; $Cre^{+/-}$ and $Pten^{loxp/d5}$;$Cre^{+/-}$ mice was delayed due to the unexpected problem of PB-Cre line. However, we have quickly acquired another PB-Cre line and generated a cohort of mice ready to be tested soon. We also developed two alternative methodologies for focal inactivation *Pten* gene, which are not only important for our research, but will benefit other investigators as well.

KEY RESEARCH ACCOMPLISHMENTS

- Established and characterized *Pten* conditional knock-out mouse strain: $Pten^{loxp/loxp}$
- Generated $Pten^{loxp/loxp}$; $Cre^{+/-}$ and $Pten^{loxp/d5}$;$Cre^{+/-}$ mice for prostate epithelium-specific deletion of Pten
- Generated TAT-Cre fusion protein for focal deletion of *Pten* gene in the prostate glands

REPORTABLE OUTCOMES

- Publication:
  1. Dubey, P., **Wu, H.,** Reiter, R. and Witte, O. (2001) Alternative Pathways to Prostate Carcinoma Activated PSCA Expression, Cancer Research (in press).
  2. Neshat, M., Mellinghoff, I., Tran, C., Stiles, B., Thomas, G., **Wu, H.**, and Sawyers, C. (2001) Enhanced sensitively of PTEN-Deficient Tumors to Inhibition of FRAP/mTOR *Proc. Natl. Acad. Sci. USA*. 98: 10314-10319.
  3. Groszer, M., Erickson, R., Scripture-Adams, D., Lesche, R., Trumpp, A., Zack, J., Kornblum, H., Liu, X., and **Wu, H**. (2001) Negative Regulation of Neural Stem/Progenitor Cell Proliferation by The Pten Tumor Suppressor Gene In Vivo. *Science* 294: 2186-2189.
  4. Lesche, R., Groszer, M., Gao, J., Wang, Y., Messing, A., Liu, X., and **Wu, H.** (2002) Cre/loxp-Mediated Inactivation of The Murine Pten Tumor Suppressor Gene. *Genesis* (in press).
  5. Stiles, B., Gilman, V., Khnzenzon, N., Lesche, R., Li, A., Qiao, R., Liu, X., and **Wu, H.** (2002) The Essential Role of AKT-1/PKB in PTEN Controlled Tumorigenesis. *Mol. Cell. Biology* (in press).
  6. Li, G., Robinson, G.W., Lesche, R., Martinez-Diaz, H., Jiang, Z.R., Rozengurt, N., Wagner, K., Wu, D.C., Lane, T.F., Liu, X., Hennighausen, L., and **Wu, H.** (2002) Conditional Deletion of Pten Leads to Precocious Development and Neoplasia in The Mammary Gland (submitted).

- Presentation: CapCure Annual Meeting, Sept. 2000, Lake Tahoe, Nevada

- 10/200 Seminar, Department of Biochemistry, University of California, Riverside, CA
  7/2001 Invited speaker, PTEN/AKT Summit meeting, Boston, MA
  9/2001 Seminar, Harbor-UCLA medical Center, CA
  10/2001 Invited speaker, Modeling prostate caner in mice, Bar Harbor, Main
  1/2002 Invited speaker, Somatic and Embryonic Stem Cell Research Symposium, NIH, Bethesda, MD
  2/2002 Invited speaker, M.D. Anderson Cancer Center, Houston, TX
  2/2002 Invited speaker, Norris Cancer Center, University of South California, School of Medicine
  3/2002 Invited speaker, Department of Chemistry and Biochemistry, University of Colorado-Boulder

- Repositories: NCI Animal Models for Human Cancers Consortium.

CONCLUSION

Our study on *Pten* knock-out mice suggest that PTEN is a crucial tumor suppressor gene in controlling the prostate cancer development. Thus, prostate-specific deletion of *Pten* will provide a valuable model for studying prostate cancer, especially signaling pathways involved in prostate cancer formation. Results derived from this study will provide molecular insight to tumorigenesis in the prostate glands and possible therapeutic intervention for treatment of prostate cancers.

REFERENCES

1. Di Cristofano, A., Pesce, B., Cordon-Cardo, C., and Pandolfi, P. P. (1998). Pten is essential for embryonic development and tumour suppression, Nature Genetics *19*, 348-55.
2. Podsypanina, K., Ellenson, L. H., Nemes, A., Gu, J., Tamura, M., Yamada, K. M., Cordon-Cardo, C.,Catoretti, G., Fisher, P. E., and Parsons, R. (1999). Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems, Proceedings of the National Academy of Sciences of the United States of America *96*, 1563-8.
3. Stambolic, V., Suzuki, A., de la Pompa, J. L., Brothers, G. M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J. M., Siderovski, D. P., and Mak, T. W. (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN, Cell *95*, 29-39.
4. Suzuki, A., de la Pompa, J. L., Stambolic, V., Elia, A. J., Sasaki, T., del Barco Barrantes, I., Ho, A., Wakeham, A., Itie, A., Khoo, W., *et al.* (1998). High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice, Current Biology *8*, 1169-78.
5. Nagahara, H., Vocero-Akbani, A., Snyder, E.L., Ho, A., Latham, D.G., Lissy, N.A., Becker-Hapak, M., Ezhevsky, S.A., Dowdy, S.F. (1998) Transduction of full length TAT fusion proteins into mammalian cells: p27kip1 mediates cell migration. Nature Medicine 4:1449-1452.
6. Zhuo L, Theis M, Alvarez-Maya I, Brenner M, Willecke K, Messing A. 2001. hGFAP-cre transgenic mice for manipulation of glial and neuronal function in vivo. Genesis. 31(2):85-94.

APPENDICES

Reprints of two recent publications.

Science

Negative Regulation of Neural Stem/Progenitor Cell Proliferation by the *Pten* Tumor Suppressor Gene in Vivo

Matthias Groszer, Rebecca Erickson, Deirdre D. Scripture-Adams, Ralf Lesche, Andreas Trumpp, Jerome A. Zack, Harley I. Kornblum, Xin Liu, Hong Wu

7 December 2001, Volume 294, pp.2186–2189

Negative Regulation of Neural Stem/Progenitor Cell Proliferation by the *Pten* Tumor Suppressor Gene in Vivo

Matthias Groszer,[1,2,4] Rebecca Erickson,[2] Deirdre D. Scripture-Adams,[5] Ralf Lesche,[1,2*] Andreas Trumpp,[7] Jerome A. Zack,[5,6] Harley I. Kornblum,[2,3] Xin Liu,[2,4†] Hong Wu[1,2†]

The mechanisms controlling neural stem cell proliferation are poorly understood. Here we demonstrate that the PTEN tumor suppressor plays an important role in regulating neural stem/progenitor cells in vivo and in vitro. Mice lacking PTEN exhibited enlarged, histoarchitecturally abnormal brains, which resulted from increased cell proliferation, decreased cell death, and enlarged cell size. Neurosphere cultures revealed a greater proliferation capacity for tripotent $Pten^{-/-}$ central nervous system stem/progenitor cells, which can be attributed, at least in part, to a shortened cell cycle. However, cell fate commitments of the progenitors were largely undisturbed. Our results suggest that PTEN negatively regulates neural stem cell proliferation.

The *Pten* tumor suppressor gene encodes the first phosphatase frequently mutated somatically in various human cancers, including glioblastoma (*1*). Besides carcinogenesis, *Pten* may play important roles in brain development, as suggested by its ubiquitous central nervous system (CNS) expression pattern in embryos (*2, 3*) as well as by neurological disorders associated with PTEN germ-line mutations in humans (*4*). However, the early embryonic lethality of conventional $Pten^{-/-}$ mice (*5, 6*) has precluded further studies of PTEN function during brain development.

To explore PTEN's role in early brain development, we generated a conditional *Pten* knockout mouse by flanking exon 5, encoding the phosphatase domain of PTEN, with *loxp* sequences ($Pten^{loxp}$) (Fig. 1A). $Pten^{loxp/loxp}$ females were crossed with males carrying a nestin promoter-driven *Cre* transgene ($Cre^{+/-}$) that is activated in CNS stem/progenitor cells at embryonic day (E) 9 or 10, resulting in almost complete gene deletion in the CNS by midgestation (*7, 8*). In $Pten^{loxp/+};Cre^{+/-}$ mice, *Cre*-mediated deletion of the *loxp* allele (Δ5) was detectable in all neural tissues examined (Fig. 1B, lanes 1 to 5). To ensure complete deletion of *Pten*, we generated $Pten^{loxp/\Delta 5};Cre^{+/-}$ mice carrying a conventional exon 5 deleted allele ($Pten^{\Delta 5}$) and a $Pten^{loxp}$ allele. No PTEN protein could be detected in the mutant brain (Fig. 1C), indicating nearly complete *Pten* deletion. *Pten* deletion leads to hyperphosphorylation of Akt and S6 kinase, known downstream effectors of phosphatidylinositol 3-kinase (Fig. 1C) that have been implicated in neuron survival and cell cycle control (*9*).

Examination of the $Pten^{loxp/\Delta 5};Cre^{+/-}$ brain revealed a marked increase in brain size (Fig. 1D). Measurements taken at E14, E18, and P0 (birth) demonstrated continuous increases in brain weight and in the ratio of brain weight to body weight (Fig. 1E, upper panels). P0 mutant brain weight and cell number were double those of wild-type controls (Fig. 1E, upper and lower left), a difference much greater than that seen in mice overexpressing BCL-2 (*10*) or in mice lacking p27 (*11*). Because deletion of *Drosophila PTEN* led to increased S6 kinase activity and enlarged cell size, we measured the cell size distribution in mutant brains by flow cytometry. Cells from $Pten^{-/-}$ brains were larger than those of controls (Fig. 1E, lower right), providing evidence that PTEN regulates cell size in mammals.

Mutant mice were born with open eyes (Fig. 1D) and died soon after birth. Histological analyses of newborn mutant brains showed a proportional increase in overall brain structures, with no signs of hydrocephalus (Fig. 2A). In the brainstem, nuclei in mutant animals were not easily identifiable (Fig. 2B). It is unclear whether specific nuclei, such as CN7n (arrow), were missing or

[1]Howard Hughes Medical Institute, [2]Department of Molecular and Medical Pharmacology, [3]Department of Pediatrics, [4]Department of Pathology and Laboratory Medicine, [5]Department of Medicine and AIDS Institute, [6]Department of Microbiology and Molecular Genetics, UCLA School of Medicine, Los Angeles, CA 90095, USA. [7]Institut Suisse de Recherche Expérimentale sur le Cancer, Ch. de Boveresses 155, 1066 Epalinges/Lausanne, Switzerland.

*Present address: Epigenomics AG, Kastanienallee 24, 10435 Berlin, Germany.

†To whom correspondence should be addressed. E-mail: xliu@mednet.ucla.edu, hwu@mednet.ucla.edu

disorganized beyond histological recognition. In addition, a severe disturbance of the laminar patterns in the cortex, hippocampus (*12*), and cerebellum was evident (Fig. 2C). Because PTEN controls both cell cycle and cell adhesion or migration, the observed layering defects might be due to uncontrolled progenitor proliferation and/or altered cell adhesion or migration (*13–15*).

We next tested whether *Pten* deletion affected the cell fate determination of neural progenitors. Immunohistochemical analyses of P0 brains were conducted using antibodies specific to the GluR1 subunit of postsynaptic AMPA receptors (Fig. 2D). GluR1 staining was present throughout the brain, indicating that neuronal differentiation had taken place. However, the staining pattern was abnormal, as predicted by the disorganization observed in hematoxylin- and eosin-stained sections (Fig. 2C). To provide more quantitative measurement, we compared the in vitro differentiation potentials of cortical cells from E14.5, the peak of neurogenesis, and E16.5, the onset of gliogenesis. No significant difference between mutant and control was observed in the number of TuJ-1–positive neurons (in green, Fig. 2E) or in the number of glial fibrillary acidic protein (GFAP)–positive astrocytes (Fig. 2F). The degree of induction of astrocyte differentiation in response to leukemia inhibiting factor (LIF) stimulation (*16*) was also not altered by *Pten* deletion (Fig. 2F, right). Thus, the programmed developmental sequence of cell fate determination from neurogenesis to gliogenesis (*17, 18*) is not overtly disturbed by *Pten* deletion. However, neurogenesis is the major event during embryonic brain development, whereas gliogenesis occurs primarily during the postnatal stage (*18, 19*). Thus, we cannot rule out the possibility that postnatally, *Pten* deletion will affect cell fate determination.

To address the mechanism of increased cell number in the mutant, we analyzed progenitor cell proliferation at E14.5 using the nucleotide substitution method. E14.5 was chosen because (i) *Cre*-mediated *Pten* deletion is complete (*12*); (ii) brain weight is not significantly increased relative to control littermates (Fig. 1E), which allowed us to focus on neural stem/progenitor cells rather than more differentiated progenies; and (iii) intensive neural stem/progenitor cell proliferation takes place (*13, 14*). A significant increase in bromodeoxyuridine (BrdU)-labeled nuclei was observed in the ventricular zone (VZ) of mutant animals (Fig. 3, upper panels) (*12*). The increased BrdU labeling in the mutant brain could be due to a shortened cell cycle time (see below), a decrease of apoptosis, or both. Using the TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling) assay (*20*), we were able to detect a significant decrease

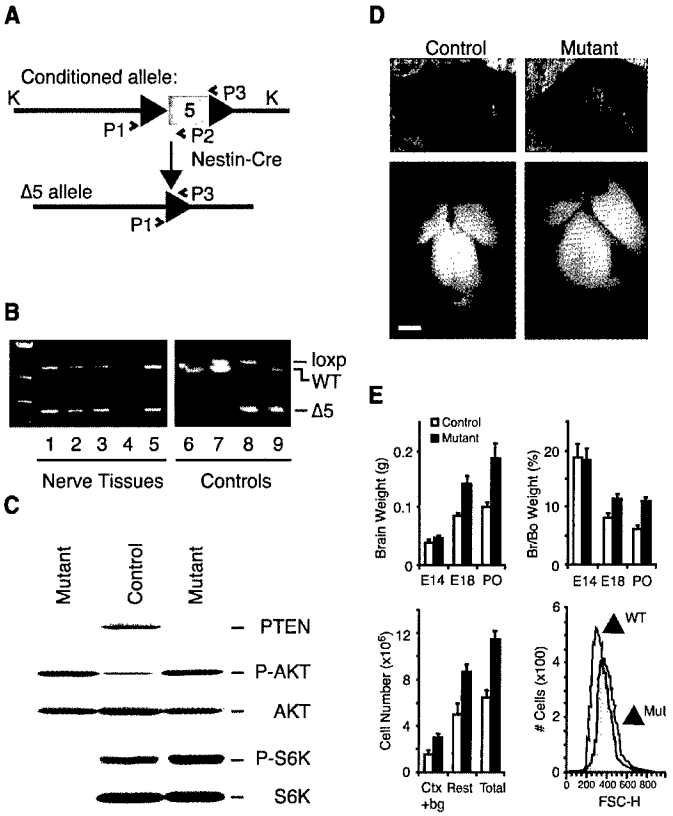

Fig. 1. Genotype and phenotype of mice lacking PTEN in the brain. **(A)** Schematic representation of *Pten* conditional knockout allele. P1, P2, and P3 localize polymerase chain reaction (PCR) primers used for genotyping. *Cre*-mediated recombination deletes exon 5 and creates a $Pten^{\Delta 5}$ allele. K, Kpn I site. **(B)** PCR screen for exon 5 deletion in adult $Pten^{loxp/+}$;Nestin-$Cre^{+/-}$ mice. Lanes 1 to 5, DNAs prepared from cortex, hippocampus, cerebellum, spinal cord, and retina, respectively; lanes 6 to 9, DNAs isolated from wild-type (WT), $Pten^{loxp/+}$, $Pten^{loxp/\Delta 5}$, and $Pten^{+}$ mice, respectively. **(C)** Western blot analysis of whole brain lysates from control and $Pten^{loxp/\Delta 5}$;$Cre^{+/-}$ (mutant) newborn mice for PTEN levels, and Akt and S6 kinase phosphorylation using phospho-specific antibodies. **(D)** Macrocephaly (no exencephaly) and open-eye phenotypes in the mutant animals. Scale bar, 3 mm. **(E)** Increased brain weight (upper left and right), cell number (lower left), and cell size (lower right) in mutant mice. Ctx+bg, cortex and striatum; Rest, rest of the brain structures; Total, whole brain. Data are means ± SD; $n = 6$ to 15, $P < 0.01$.

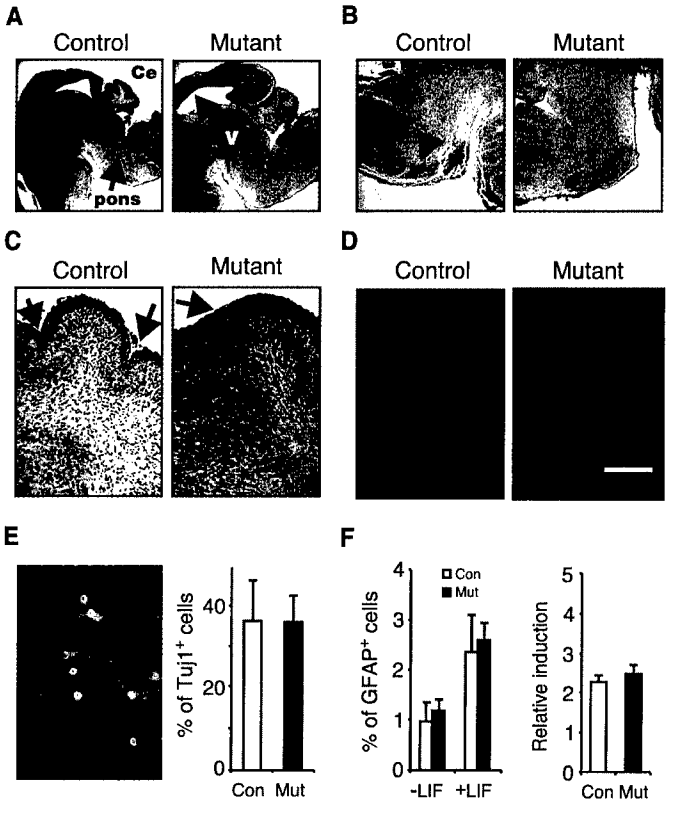

Fig. 2. *Pten* deletion causes enlarged, histoarchitecturally abnormal brains. **(A)** Sagittal section shows an increase in overall brain size in mutant animals. V, midbrain vesicle; rostral is to the left. **(B)** Perturbed nuclear structure in the mutant brainstem. Arrow, CN7n facial nerve nucleus; arrowhead, anterior perioliviary nuclei; asterisk, pontine gray nucleus; rostral is to the right. **(C)** Disturbed lamination and near-absence of foliation (arrow) of the mutant cerebellum. **(D)** GluR1 immunostaining revealed differentiation despite severely disturbed histoarchitecture. Scale bar, 100 μm. **(E)** In vitro neuronal cultures from E14.5 cortex. **(F)** Cells from E16.5 cortex were cultured for 3 days without or with LIF (50 ng/ml). GFAP-positive astrocytes were counted; data are presented as relative induction in response to LIF stimulation ($n = 4$).

of apoptosis in E14.5 mutant telencephalon (Fig. 3, middle panels) (*12*). Thus, our results indicate that both increased cell proliferation and decreased cell death in the neural stem/progenitor cells probably account for the increased cell number in the mutant animals. We did not observe extensive BrdU labeling in ectopic CNS regions outside the proliferation zone, as has been reported for Rb-deficient mice (*21*). Staining of E14.5 brain sections for the neuronal marker TuJ-1 (in green) showed no sign of disturbed layering and no prominent enlargement of the proliferative zone (dark area) in mutants (Fig. 3, lower panels), as reported for caspase-9–deficient mice (*20*). Taken together, these results suggest that PTEN controls neural stem/progenitor cells by negatively regulating their cell cycle progression rather than preventing postmitotic neurons from reentering the cell cycle. Because nestin is expressed in stem cells and more restricted progenitors in the brain as well as other cell types during development, we cannot rule out the possibility that effects on neural stem cell populations are mediated indirectly by *Pten* deletion in these cell types.

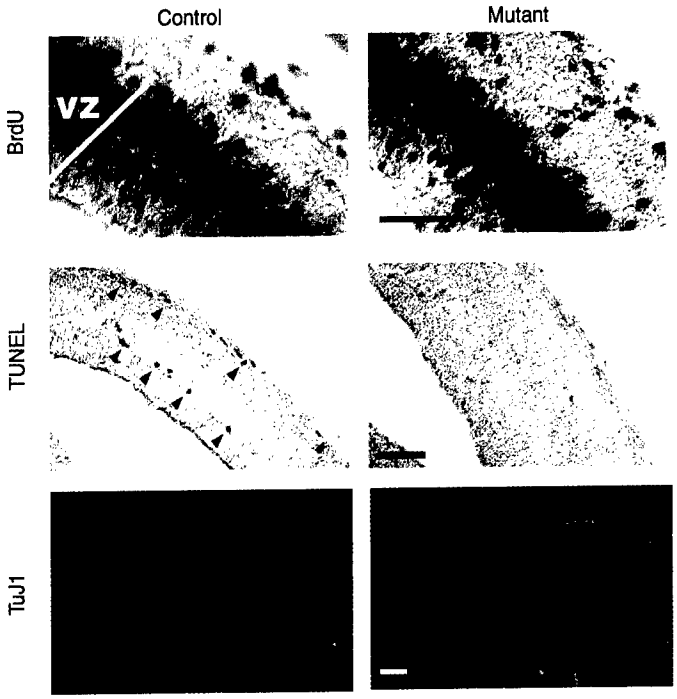

Fig. 3. Increased cell proliferation and decreased cell death in the ventricular zone (VZ) of E14.5 mutant telencephalons. Increased numbers of BrdU$^+$ cells (upper panels) but decreased numbers of TUNEL$^+$ cells (middle panels) were observed in mutant cortex. TuJ-1 staining (green) revealed neuronal differentiation (lower panels). Scale bars, 100 μm.

Using the in vitro neurosphere system (*22*), we further studied how PTEN controls neural stem/progenitor cell proliferation. Neurospheres were cultured from E14.5 and P0 (*12*) cortex as described (*19, 23*). Western blot analyses confirmed that no PTEN could be detected in E14.5 neural tissue or in cultured neurospheres (*12*). When cells of E14.5 brains were cultured at moderate density [40,000 cells/ml (*12*)] or clonal density [1000 cells/ml (*12*)], the number of spheres was significantly greater in *Pten* mutants (Fig. 4A), indicating that there were more CNS stem/progenitor cells in mutant animals. Mutant neurospheres propagated more readily, as indicated by a greater average diameter of the spheres (*12*), an increased number of cells per sphere, and a greater number of cells incorporating BrdU (Fig. 4B). Cells in mutant neurosphere cultures were also significantly larger than those in controls (*12*), which suggests that increases in sphere size can be attributed to both in-

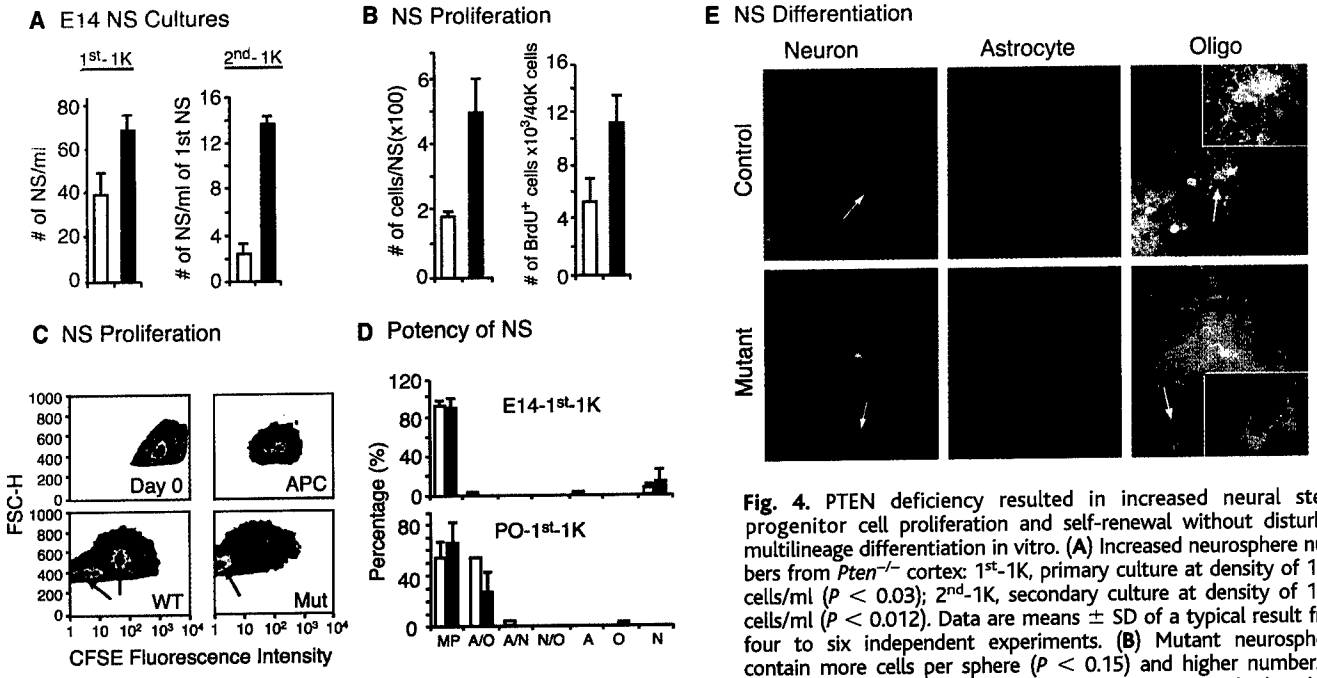

Fig. 4. PTEN deficiency resulted in increased neural stem/progenitor cell proliferation and self-renewal without disturbing multilineage differentiation in vitro. **(A)** Increased neurosphere numbers from *Pten*$^{-/-}$ cortex: 1st-1K, primary culture at density of 1000 cells/ml ($P < 0.03$); 2nd-1K, secondary culture at density of 1000 cells/ml ($P < 0.012$). Data are means ± SD of a typical result from four to six independent experiments. **(B)** Mutant neurospheres contain more cells per sphere ($P < 0.15$) and higher numbers of BrdU$^+$ cells ($P < 0.01$). **(C)** Mutant stem/progenitor cells display increased cell divisions, as indicated by decreased CFSE labeling. Cells are displayed in a pseudo-colored density plot, where red represents the highest and blue the lowest cell numbers. APC, aphidicolin-treated. **(D)** Neurosphere potency. Histograms: differentiation potential of E14.5 (upper) and P0 (lower) mutant and control neurospheres cultured at 1000 cells/ml. Data represent three independent experiments. Markers used: αTuJ1 for neurons (N), αGFAP for astrocytes (A), and αO4 for oligodendrocytes (O). MP, multipotent (positive for all three markers). **(E)** A representative of neurosphere differentiation from 2nd-1K cultures. Scale bar, 50 μm. Enlarged photo inserts show oligodendrocytes denoted by the arrows.

creased cell proliferation and enlarged cell size.

To directly test whether PTEN controls cell cycle time, we conducted a CFSE washout experiment. CFSE [5(6)-carboxyfluorescein diacetate succininyl ester] is a fluorescent dye that penetrates cell membranes and is metabolized and trapped within the cell. The dye is evenly distributed to daughter cells, so fluorescence intensity decreases by half with each cell division (*24*). Cells from E14.5 cortex were pulse-labeled with CFSE and cultured in neurosphere medium. After 6 days, neurospheres were dissociated and subjected to flow cytometry. The majority of mutant cells had shifted to the dimmer side of the fluorescence scale, in contrast to wild-type or aphidicolin-arrested control cells (Fig. 4C, arrows), which indicates that most *Pten*$^{-/-}$ cells had progressed through more cell divisions during the same culture period. These studies suggest that PTEN inhibits neural stem cell proliferation in vitro by controlling cell cycle progression.

A key characteristic of stem cells is their self-renewal ability. We compared self-renewal in mutant and control stem/progenitor cells by propagating neurosphere cultures. When neurospheres from primary cultures were dissociated and repropagated at 1000 cells/ml, mutant cultures contained 6.5 times as many spheres per milliliter of the initial culture (Fig. 4A, 2nd-1K), a greater difference than for the primary cultures (1st-1K); this finding is consistent with the hypothesis that PTEN-null neural stem cells undergo more self-renewing divisions in vitro than do wild-type counterparts. The secondary mutant spheres also proliferated more readily than control spheres, as revealed by their larger diameters (*12*).

Similar to our in vivo studies (Figs. 2 and 3), *Pten* deletion did not alter the differentiation potential of stem cells in neurosphere cultures. Upon differentiation, the spheres from E14.5 and P0 mutant and control animals were generally tripotent, generating neurons, astrocytes, and oligodendrocytes (Fig. 4D); no significant differences in overall sphere potentiality were apparent. The tripotency of clonal secondary spheres was also similar between mutant and control cultures (Fig. 4D). To determine whether *Pten* deletion may affect cell fate specification, we calculated the percentage of neurons within individual spheres. Neurons constituted about 1 to 2%, whereas astrocytes constituted the vast majority of cells present in differentiated neurospheres (Fig. 4E). The percentages of neurons per secondary clonal neurosphere were similar between mutant and controls: 1.54% and 1.34%, respectively.

Taken together, our in vivo and in vitro observations suggest that PTEN negatively controls proliferation of neural stem cells. Caution must be taken in interpreting these results, because a loss of PTEN in neural stem cells may not be responsible for the entire phenotype observed. Our mutant animals are expected to lose PTEN not only in nestin-expressing neural stem cells, but also in all their progeny. Thus, if PTEN is required for normal migration of postmitotic neurons, this effect would be observed in our mutants. Furthermore, nestin is expressed by uncommitted stem cells and committed glial progenitors. Therefore, a direct effect of *Pten* deletion on glial development is not precluded by our results, although an effect on the stem cell population could explain many of our findings. Recent reports indicate that glioblastoma formation in vivo is promoted by transformation of neural progenitor cells, but not differentiated astrocytes, with oncogenic Ras and Akt (*25*). In line with this idea, homozygous loss of PTEN on its own is sufficient to promote proliferation of neural stem/progenitor cells. Thus, this mouse model may help to further understand the biology of this tumor entity.

References and Notes

1. J. Li *et al.*, *Science* **275**, 1943 (1997).
2. O. Gimm *et al.*, *Hum. Mol. Genet.* **9**, 1633 (2000).
3. K. Luukko *et al.*, *Mech. Dev.* **83**, 187 (1999).
4. D. J. Marsh *et al.*, *Nature Genet.* **16**, 333 (1997).
5. A. Di Cristofano *et al.*, *Nature Genet.* **19**, 348 (1998).
6. V. Stambolic *et al.*, *Cell* **95**, 29 (1998).
7. B. Bates *et al.*, *Nature Neurosci.* **2**, 115 (1999).
8. G. Fan *et al.*, *J. Neurosci.* **21**, 788 (2001).
9. S. R. Datta, A. Brunet, M. E. Greenberg, *Genes Dev.* **13**, 2905 (1999).
10. J. C. Martinou *et al.*, *Neuron* **13**, 1017 (1994).
11. H. Kiyokawa *et al.*, *Cell* **85**, 721 (1996).
12. For supplemental figures and details of methods, see *Science* Online (www.sciencemag.org/cgi/content/full/1065518/DC1).
13. T. Takahashi *et al.*, *J. Neurosci.* **19**, 10357 (1999).
14. T. Takahashi *et al.*, *J. Neurocytol.* **21**, 185 (1992).
15. J. Liliental *et al.*, *Curr. Biol.* **10**, 401 (2000).
16. A. Bonni *et al.*, *Science* **278**, 477 (1997).
17. S. J. Morrison, *Neuron* **28**, 1 (2000).
18. X. Qian *et al.*, *Neuron* **28**, 69 (2000).
19. V. Tropepe, C. G. Craig, C. M. Morshead, D. van der Kooy, *J. Neurosci.* **17**, 7850 (1997).
20. K. Kuida *et al.*, *Cell* **94**, 325 (1998).
21. E. Y. Lee *et al.*, *Genes Dev.* **8**, 2008 (1994).
22. B. A. Reynolds, S. Weiss, *Science* **255**, 1707 (1992).
23. D. H. Geschwind *et al.*, *Neuron* **29**, 325 (2001).
24. B. F. de St. Groth, *Immunol. Cell Biol.* **77**, 530 (1999).
25. E. C. Holland *et al.*, *Nature Genet.* **25**, 55 (2000).

26. We thank K. Tatsukawa, Y. Wang, and D. Irvin for valuable technical assistance and D. van der Kooy, S. Morrison, H. Herschman, Y. Sun, G. Fan, J. Yuan, and members of our laboratories for helpful comments on the manuscript. H.W. is an Assistant Investigator of the Howard Hughes Medical Institute (HHMI). M.G. is supported by the Swiss National Science Foundation. R.L. was partially supported by HHMI and the Deutsche Forschungsgemeinschaft. Supported by NIH grants NS38489 (X.L.) and MH062800-01 (H.I.K.) and by U.S. Department of Defense grants PC991538 (H.W.) and A136059 (J.A.Z.).

20 August 2001; accepted 11 October 2001
Published online 1 November 2001;
10.1126/science.1065518
Include this information when citing this paper.

genesis 32:000-000 (2002)
DOI 10.1002/gene.00000

Cre/*loxP*-Mediated Inactivation of the Murine *Pten* Tumor Suppressor Gene

Ralf Lesche,[1] Matthias Groszer,[2] Jing Gao,[1] Ying Wang,[1] Albee Messing,[3] Hong Sun,[4] Xin Liu,[2] and Hong Wu[1]*

[1]Howard Hughes Medical Institute and Department of Molecular and Medical Pharmacology, UCLA School of Medicine, Los Angeles, California
[2]Department of Pathology and Laboratory Medicine, UCLA School of Medicine, Los Angeles, California
[3]Department of Pathobiological Sciences, Waisman Center and School of Veterinary Medicine, University of Wisconsin, Madison, Wisconsin
[4]Department of Genetics, Yale University School of Medicine, New Haven, Connecticut

Received 14 December 2001; Accepted 16 December 2001
Published online 00 Month 2002

PTEN (or *MMAC1/TEP1*) tumor suppressor gene is frequently mutated in a variety of human cancers and in three cancer predisposition syndromes (Eng and Peacocke, 1998; Dahia, 2000). PTEN negatively regulates the phosphatidylinositol 3-kinase (PI3 kinase) signaling pathway by dephosphorylating PIP3, the product of PI3 kinase (for review, see Cantley and Neel, 1999). Inactivation of *Pten* (chromosome 19) in mouse models confirmed PTEN to be a bona fide tumor suppressor (Di Cristofano *et al.*, 1998; Podsypanina *et al.*, 1998; Suzuki *et al.*, 1998; Lesche *et al.*, submitted): $Pten^{+/-}$ mice developed tumors in multiple organs and $Pten^{-/-}$ mice died during embryogenesis before midgestation. To overcome the early lethal phenotype in $Pten^{-/-}$ mice and to study the roles of PTEN in embryonic development, adult tissue function, and tumorigenesis, we have generated a conditional *Pten* knockout mouse strain.

LoxP sequences were inserted into the endogenous *Pten* locus flanking exon 5 as illustrated in Figure 1. Exon 5 encodes the phosphatase domain of PTEN in which many tumor-associated mutations have been detected. $Pten^{loxP/+}$ ES cells were injected into either C57/B6 or Balb/c blastocysts. Chimeric mice were backcrossed to either C57/B6 or Balb/c mice and germ-line transmission of the $Pten^{loxP/+}$ allele was confirmed by Southern blot and PCR genotyping (not shown). In contrast to the embryonic lethal phenotype observed in $Pten^{-/-}$ mice, $Pten^{loxP/loxP}$ animals were viable. Normal PTEN level and function were detected in $Pten^{loxP/loxP}$ MEF cells and no spontaneous tumor formations were observed up to two years, suggesting that introducing the *loxP* sites into the *Pten* locus does not perturb the normal function of PTEN.

To demonstrate Cre-mediated exon 5 deletion, we crossed $Pten^{loxP/loxP}$ animals with the GFAP-Cre transgenic mice (Zhuo *et al.*, 2001) aimed for brain-specific deletion. As shown in Figure 2c, Cre expression in the $Pten^{loxP/+}$; $GFAP\text{-}Cre^{+/-}$ mice resulted in neural-specific excision of exon 5 (lanes 1-5). In contrast, very low or no excision could be detected in other nonneural tissues (lanes 6-9). Finally, we showed that no PTEN protein could be detected in conditional deleted tissue and the known downstream signaling molecule AKT/PKB was hyperphosphorylated (Fig. 2c). Thus, the $Pten^{loxP/loxP}$ mouse line generated will be valuable for studying the function of PTEN in animal development and tumorigenesis.

ACKNOWLEDGMENTS

H.W. is an Assistant Investigator of the Howard Hughes Medical Institute (HHMI). M.G. is supported by The Swiss National Science Foundation. R.L. was partially supported by HHMI and the Deutsche Forschungsgemeinschaft. This work was supported by the following grants from the NIH: NS38489 (X.L.), NS22475 (A.M.), CA77695 (H.S.), CapCure Foundation (H.W.); NCI: CA-98-013 (C.S./H.W.); and DOD: PC991538 (H.W.).

LITERATURE CITED

Cantley LC, Neel BG. 1999. New insight into tumor suppression: PTEN suppress tumor formation by restraining the phosphoiniside 3-kinase/AKT pathway. Proc Natl Acad Sci USA 96:4240-4245.

Dahia P. 2000. Pten, a unique tumor suppressor gene. Endocrine-Related Cancer 7:115-129.

Di Cristofano A, Pesce B, Cordon-Cardo C, Pandolfi PP. 1998. Pten is essential for embryonic development and tumour suppression. Nat Genet 19:348-355.

Eng C, Peacocke M. 1998. Genetics of cowden syndrome: Through the looking glass of oncology. Int J Oncol 12:701-710.

Podsypanina K, Ellenson LH, Nemes A, Gu J, Tamura M, Yamada KM, Cordon-Cardo C, Catoretti G, Fisher PE, Parsons R. 1998. Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. Proc Natl Acad Sci USA 96:1563-1568.

Suzuki A, de la Pompa JL, Stambolic V, Elia AJ, Sasaki T, del Barco Barrantes I, Ho A, Wakeham A, Itie A, Khoo W, et al. 1998. High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. Curr Biol 8:1169-1178.

Zhuo L, Theis M, Alvarez-Maya I, Brenner M, Willecke K, Messing A. 2001. hGFAP-cre transgenic mice for manipulation of glial and neuronal function in vivo. Genesis 31(2):85-94.

Dr. Lesche's current address is: Epigenomics AG, Kastanienallee 24, 10435 Berlin, Germany.

*Correspondence to: Dr. Hong Wu, Howard Hughes Medical Institute and Department of Molecular and Medical Pharmacology, UCLA School of Medicine, Los Angeles, CA 90095.
E-mail: hwu@mednet.ucla.edu

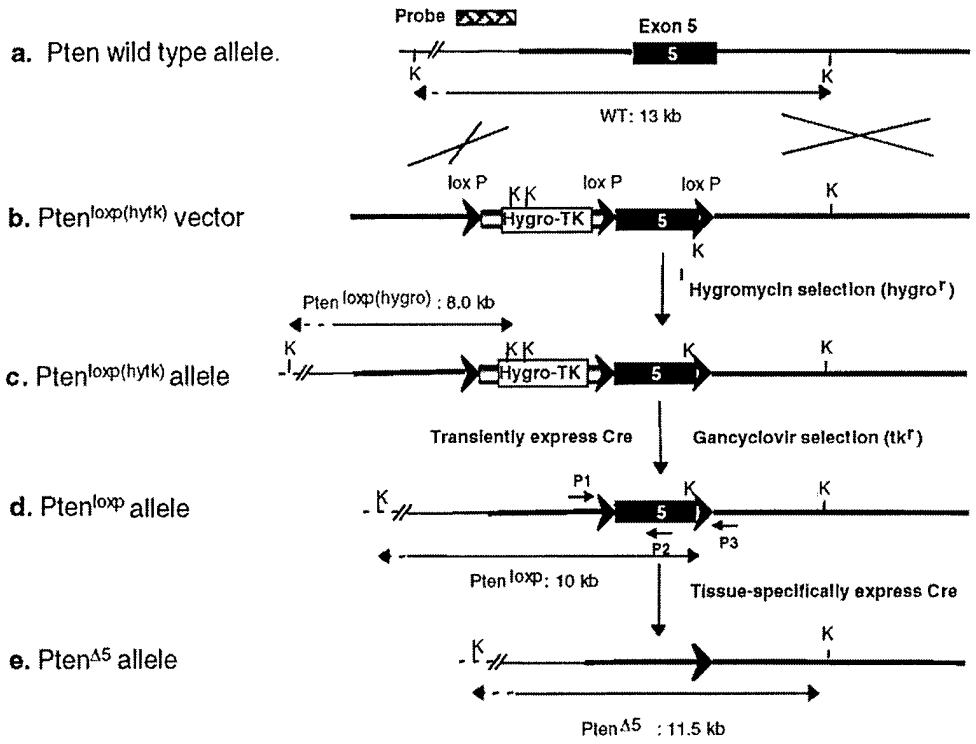

FIG. 1. Generation of *Pten*$^{loxP/+}$ allele. **(a)** Genomic structure of *Pten* locus with exon 5 boxed. **(b)** *Pten*$^{loxP(hytk)}$ targeting vector. **(c)** After electroporation (50 μg linearized DNA/10^7 LW1 ES cells; 400 V/25 μF) and hygromycin (80 μg/ml) selection, homologous recombinants were identified by Southern blot analysis using an external probe indicated in (a) (not shown). **(d)** Targeted ES cells were transiently transfected with Cre-expressing vector and selected with gancyclovir (1 μM/ml). Surviving clones with flanked-exon 5 were used to generate *Pten*$^{loxP/+}$ mouse strains according to standard procedure. P1–P3, primers used for PCR genotyping. **(e)** Exon 5 flanked by the *loxP* sites can be deleted upon Cre expression. This event can be monitored by Southern blot or PCR analysis.

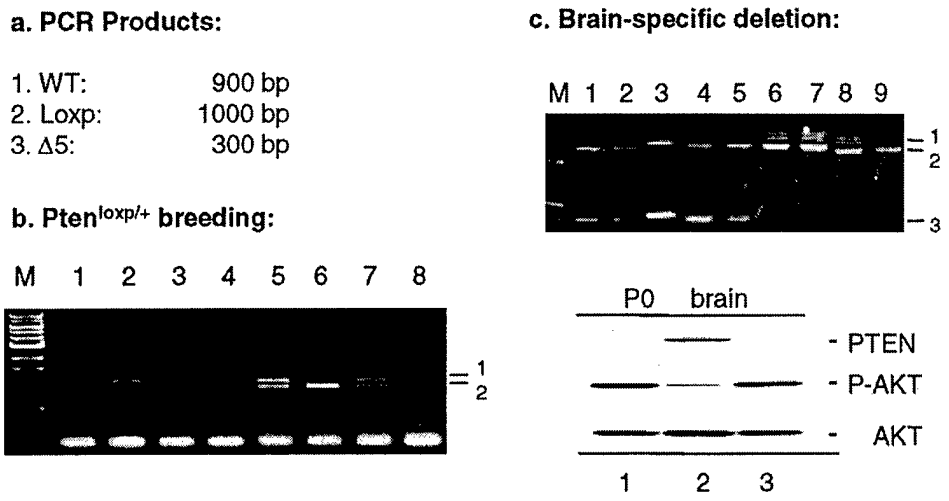

FIG. 2. Conditional inactivation of *Pten* gene. **(a)** Predicted PCR products. Primers used are forward primer P1, 5′-ACTCAAGGCAGG-GATGAGC-3′, and two reverse primers, P2 5′-AATCTAGGGCCTCTTGTG CC-3′ and P3 5′-GCTTGATATCGAATTCCTGCAGC-3′. **(b)** An example of PCR genotyping. Lanes 1 and 6, WT; lanes 5 and 7, heterozygous; lanes 2–4, homozygous for *loxP* alleles; lane 8, no DNA added. (c) GFAP-Cre-mediated *Pten* deletion in *Pten*$^{loxP/+}$;*Cre*$^{+/-}$ mice (upper panel). Lanes 1–5, neural tissues: cortex, hippocampus, cerebellum, brain stem, and spinal cord, respectively; lanes 6–9, nonneural tissues: thymus, heart, kidney, skin, respectively. Western blot analysis (lower panels) using P0 *Pten*$^{loxP/loxP}$;*Cre*$^{+/-}$ brain samples. Lanes 1 and 3, mutant; lane 2, WT control. Antibodies used were α-PTEN, NEB; α-P-AKT, and α-AKT (Santa Cruz).