Development 130, 1691-1700

doi:10.1242/dev.00392

DEVELOPMENT AND DISEASE

Conditional loss of PTEN leads to testicular teratoma and enhances embryonic germ cell production

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Accepted 14 January 2003

SUMMARY

The tumor suppressor gene *PTEN*, which is frequently mutated in human cancers, encodes a lipid phosphatase for phosphatidylinositol 3,4,5-triphosphate [PtdIns(3,4,5)P_3] and antagonizes phosphatidylinositol 3 kinase. Primordial germ cells (PGCs), which are the embryonic precursors of gametes, are the source of testicular teratoma. To elucidate the intracellular signaling mechanisms that underlie germ cell differentiation and proliferation, we have generated mice with a PGC-specific deletion of the *Pten* gene. Male mice that lacked PTEN exhibited bilateral testicular teratoma, which resulted from impaired mitotic arrest and outgrowth of cells with immature characters. Experiments with PTEN-null PGCs in culture revealed that these cells had greater proliferative capacity and enhanced pluripotent embryonic germ (EG) cell colony formation. PTEN appears to be essential for germ cell differentiation and an important factor in testicular germ cell tumor formation.

Key words: PTEN, Germ cells, Teratoma, EG cells, Mouse, Human

INTRODUCTION

The tumor suppressor PTEN (phosphatase and tensin homolog deleted from chromosome 10; also known as MMAC1 or TEP1) is a lipid phosphatase, which plays crucial roles in the regulation of cellular proliferation, differentiation, apoptosis, adhesion and migration (Cantley and Neel, 1999; Di Cristofano and Pandolfi, 2000; Simpson and Parsons, 2001; Yamada and Araki, 2001). Germline mutations of *PTEN* have been identified in hereditary disorders, such as Cowden disease, Lhermitte-Duclos disease and Bannayan-Zonna syndrome, which are characterized by multiple benign tumors (mostly hamartomas) and increased risk of cancers, such as breast and thyroid malignancies (Liaw et al., 1997; Marsh et al., 1997; Marsh et al., 1999; Nelen et al., 1997). Deletions and mutations in the *PTEN* gene are also found at high frequency in many sporadic human cancers, such as glioblastomas, endometrial, prostate and breast cancers (Dahia, 2000). *PTEN* is one of the most frequently mutated tumor suppressor genes in human cancer, and the overall frequency of *PTEN* mutations in sporadic human cancers is similar to that of the tumor suppressor p53 (Stokoe, 2001).

PTEN contains a protein tyrosine phosphatase domain that is similar to those of the dual-specificity phosphatases, which dephosphorylate both phosphotyrosine and serine/threonine residues (Li and Sun, 1997; Myers et al., 1997). In spite of the homology with dual-specificity phosphatases, the in vitro protein phosphatase activity of PTEN is not high, and PTEN is very active on a highly acidic substrate. Both in vitro and in vivo analyses indicate that the major substrate of PTEN is the lipid phosphatidylinositol (3,4,5)-triphosphate [PtdIns(3,4,5)P_3], which is a direct product of PI3-kinase (PI3K) (Maehama and Dixon, 1998; Myers et al., 1998; Stambolic et al., 1998). This substrate specificity has been confirmed by the crystal structure of PTEN (Lee et al., 1999). Loss of PTEN function in embryonic stem (ES) cells and human cancer cell lines results in PtdIns(3,4,5)P_3 accumulation and the activation of its downstream signaling molecule, Akt/PKB (Li and Sun, 1998; Li et al., 1998; Ramaswamy et al., 1999; Sun et al., 1999; Wu et al., 1998). Subsequently, activation of the PI3K/Akt pathway by the loss of PTEN stimulates various biological functions, such as cell cycle progression, cell survival and cell migration.

Several studies, including ours, have sought to elucidate the

in vivo role of PTEN in development and tumorigenesis. PTEN is crucial for normal development, as homozygous *Pten*-null mice show overgrowth of cells and disorganized cell layers in the cephalic and caudal regions, and die during early embryogenesis at E6.5-9.5 (Di Cristofano et al., 1998; Podsypanina et al., 1999; Suzuki et al., 1998). In addition, mice that are heterozygous for the *Pten*-null mutation develop a broad range of tumors. The Cre-LoxP conditional gene-targeting system was adopted to analyze the in vivo function of PTEN in development, differentiation and tumorigenesis (Backman et al., 2001; Groszer et al., 2001; Kwon et al., 2001; Li et al., 2002; Marino et al., 2002; Suzuki et al., 2001). Somatic cell lineage-specific *Pten*-null mice have shown distinct functions of PTEN in different organs. For example, T-cell-specific deficiencies lead to defects in immunological tolerance and subsequent autoimmune disease (Suzuki et al., 2001). Nerve cell-specific loss leads to increased proliferation and self-renewal of neural stem and/or progenitor cells (Groszer et al., 2001).

Primordial germ cells (PGCs) are germ cell precursors that emerge around E7.5 and exist transiently in embryos (Wylie, 1999). PGCs migrate into the genital ridges and eventually differentiate into eggs and sperm. Although PGCs are committed to the germ cell lineage, two lines of evidence suggest that mammalian PGCs can de-differentiate into cells that have broader developmental potential. First, PGCs can give rise to testicular teratomas with various types of differentiated cells when grafted to adult testis (Stevens, 1967; Stevens, 1984). Second, pluripotent embryonic germ (EG) cells can be established by culturing PGCs in vitro in the presence of SCF (Stem cell factor), LIF (leukemia inhibitory factor) and bFGF (basic fibroblast growth factor) (Matsui et al., 1992; Resnick et al., 1992). However, it is likely that unknown mechanisms operate to prevent PGC de-differentiation, as testicular teratoma is rare in normal mice (Stevens, 1967) and PGCs cannot be incorporated into normal development even if injected into blastocysts (Labosky et al., 1994a). In addition, clinical testicular teratomas are known to develop from PGCs (Jiang and Nadeau, 2001; Looijenga and Oosterhuis, 1999).

In order to analyze the role of PTEN in germ cell differentiation, we produced conditional *Pten* knockout mice by crossing $Pten^{flox/flox}$ females with $Pten^{+/-}$ males that carried a single *TNAP/Cre* locus, in which *Cre* was knocked into the PGC-specific *TNAP* (tissue-non-specific alkaline phosphatase) gene (Lomeli et al., 2000; Suzuki et al., 1998; Suzuki et al., 2001). We discovered that deletion of the *Pten* gene in PGCs caused testicular teratomas in all male newborn mice, and that EG cell production was increased in both sexes. These observations underscore the essential role of PTEN in germ cell differentiation.

MATERIALS AND METHODS

Mice

To inactivate *Pten* in the PGCs, male $Pten^{+/-}$:*TNAP/Cre* mice were crossed with female $Pten^{flox/flox}$ mice, as ectopic recombination may be avoided when the *TNAP/Cre* locus is transmitted from the male (Lomeli et al., 2000; Suzuki et al., 1998; Suzuki et al., 2001). The morning on which a copulation plug was found was defined as E0.5.

Histology, immunohistochemistry and the TUNEL assay

The testes, embryoid bodies and teratomas were fixed with 4% paraformaldehyde and embedded in either methyl methacrylate or OCT compound. The sections were stained with Hematoxylin and Eosin using standard procedures.

For immunohistochemistry, the sections were stained with the following primary antibodies: anti-PTEN (1:50; Neomarkers, Fremont, CA); anti-phosphorylated Akt (1:50; NEB, Beverly, MA); anti-mvh (1:1000) (Toyooka et al., 2000); and the monoclonal antibodies TRA98 (1:500) (Tanaka et al., 1997) and 4C9 (1: 500) (Yoshinaga et al., 1991). The Vectastain ABC kit (Vector Lab, Burlingame, CA) was used for the detection of PTEN. Signals for mvh and TRA98 were visualized using the appropriate Alexa-Fluor-conjugated secondary antibodies (Molecular Probes, Eugene, OR). The 4C9 and phosphorylated Akt proteins were detected using biotin-conjugated antibodies (Vector Lab), followed by streptavidin-peroxidase or streptavidin-Texas Red (Invitrogen, Carlsbad, CA).

For TNAP staining, whole embryos were fixed with 4% paraformaldehyde and embedded in OCT compound. Serial transverse sections were collected for every three sections, and stained using the Alkaline Phosphatase Staining Kit (Sigma Diagnostics, St Louis, MO). Ectopic PGCs were also stained for 4C9, as described above. The TUNEL assay was carried out using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany), according to the manufacturer's recommendations.

PGC culture

Genital ridges were obtained from E11.5 embryos and dissociated into single cells by incubation with 0.05% trypsin, 0.02% EDTA in PBS. Dispersed suspensions of PGC-containing tissues were cultured on Sl/Sl^4-m220 feeder cells in DMEM that was supplemented with 15% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate and non-essential amino acids, in the presence or absence of LIF and bFGF (Koshimizu et al., 1996; Matsui et al., 1992). The feeder cells were treated with 5 μg/ml mitomycin C for 2 hours and plated at 2×10^5 cells/well in 24-well plates 1 day before use. PGCs and EG cells were fixed with 4% paraformaldehyde and visualized by alkaline phosphatase staining, as described above. The number of adherent PGCs 8 hours after seeding was defined as the number of seeded PGCs. Multi-layered colonies that contained more than 20 cells were counted as EG cell colonies, as described previously (Koshimizu et al., 1996). The sex of the E11.5 embryos was determined using sex chromosome-specific PCR (Chuma and Nakatsuji, 2001).

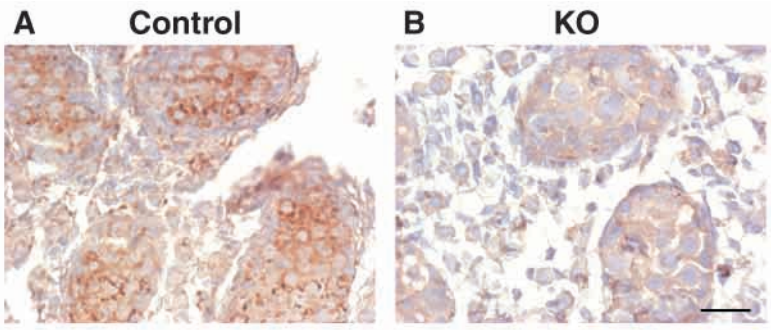

Fig. 1. Deletion of PTEN in PGCs. Immunohistochemical staining for PTEN was carried out on E15.5 control (A, $Pten^{flox/-}$: *TNAP/Cre*$^-$) and mutant (B, $Pten^{flox/-}$: *TNAP/Cre*$^+$) testes. In control mice, strong cytoplasmic staining was observed in germ cells but the expression level was low in somatic cells. In mutant mice, only background level of expression was observed both in germ and somatic cells. Scale bar: 20 μm.

Analysis of EG cells

EG cells (5×10^3 cells/ml) were plated as single cell suspensions in DMEM medium that contained 1.0% methylcellulose to induce the differentiation of embryoid bodies. At day 8 after induction, embryoid bodies were collected and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA). One microgram of total RNA was reverse-transcribed with the ThermoScript RT-PCR System (Invitrogen). Semi-quantitative PCR was performed for 1 cycle at 95°C for 1 minute, followed by amplification cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 40 seconds and, finally, 72°C for 7 minutes in a PCR System 9700 (PE Applied Biosystems, Foster City, CA). PCR amplification of the cDNA remained linear for 30 cycles (data not shown). The amplification cycles for each gene were as follows: *Wnt1*, 27 cycles; collagen IV, 21 cycles; *T*, 24 cycles; and β-actin, 17 cycles. The PCR products were analyzed by 2.0% agarose gel electrophoresis, and visualized using ethidium bromide staining. The following primer pairs were used for PCR amplification: *Wnt1*, 5′-GATTGCGAAGATGAACGCTGTTTC-3′ and 5′-TCCTCCACG-AACCTGTTGACGG-3′; collagen IV, 5′-CAAGCATAGTGGTCC-GAGTC-3′ and 5′-AGGCAGGTCAAGTTCTAGCG-3′; *T*, 5′-ATG-CCAAAGAAAGAAACGAC-3′ and 5′-AGAGGCTGTAGAACAT-GATT-3′; and β-actin, 5′-GTGACGAGGCCCAGAGCAAGAG-3′ and 5′-AGGGGCCGGACTCATCGTACTC-3′.

For teratoma induction, control and mutant EG cells (10^6 cells) were suspended in PBS and subcutaneously injected into the flanks of nude mice. After 2-4 weeks, the mice were sacrificed and the tumors were processed for pathological analysis.

RESULTS

Conditional deletion of *Pten* in PGCs

To study the roles of PTEN in germ cell differentiation, and to overcome the early embryonic lethality caused by conventional knockout strategies, we generated a conditional *Pten* knockout mouse by flanking exon 5, which encodes the phosphatase domain of PTEN, with *loxp* sequences ($Pten^{flox}$) (Suzuki et al., 2001). To avoid maternal expression of the Cre recombinase in oocytes, $Pten^{flox/flox}$ females were crossed with $Pten^{+/-}$ males that carried a single *TNAP/Cre* locus, in which *Cre* was knocked into the PGC-specific *TNAP* gene (Lomeli et al., 2000). Analysis of *TNAP/Cre* mice that carried the reporter transgene showed that the recombination efficiency was ~60% at E13.5, and increased thereafter (Lomeli et al., 2000). Semi-

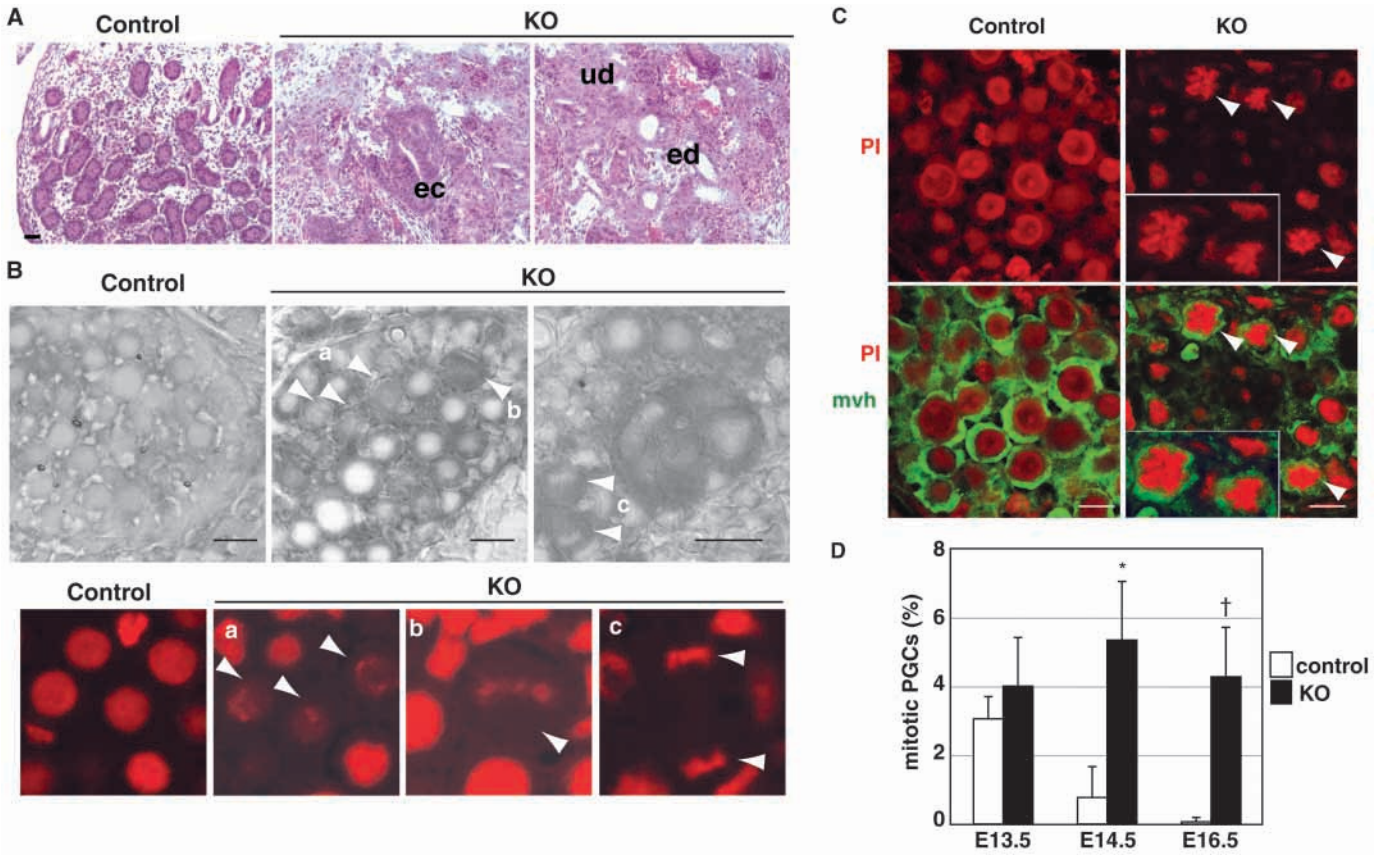

Fig. 2. (A) Hematoxylin and Eosin (HE)-stained sections of P0 testes. All *Pten* mutant mice developed bilateral teratomas with multiple foci (*n*=9). ec, ectodermal vesicle; ed, endodermal vesicle; ud, undifferentiated cells. Scale bar: 20 μm. (B) Immunostaining with anti-phospho-Akt antibody (top) showed hyper-phosphorylation of Akt in germ cells and early teratoma foci in E16.5 mutant mice. The same sections were stained with propidium iodide (PI) (bottom) and the morphology of nuclei was examined by confocal microscopy at 0.8 μm optical sections (arrowheads in a-c). Some of the hyper-phosphorylated cells had pyknotic nuclei (a) and mitotic figures (b,c). Scale bars: 10 μm. (C) Sections of E14.5 testes were double-stained with anti-Mvh antibody and PI, and analyzed by confocal microscopy at 0.8 μm optical sections. Mitotic figures were observed in *Pten* mutant PGCs (arrowheads). Scale bars: 5 μm. (D) The percentage of mitotic PGCs at E13.5, E14.5 and E16.5 in control and mutant mice. The sections were stained with PI and anti-Mvh antibody. The percentage of mitotic cells in Mvh-positive cells (mean±s.d.) was calculated and analyzed by Student's *t*-test (P=0.48 at E13.5, $^*P<0.01$ at E14.5 and $^\dagger P<0.002$ at E16.5). Three to six mice were examined at each stage.

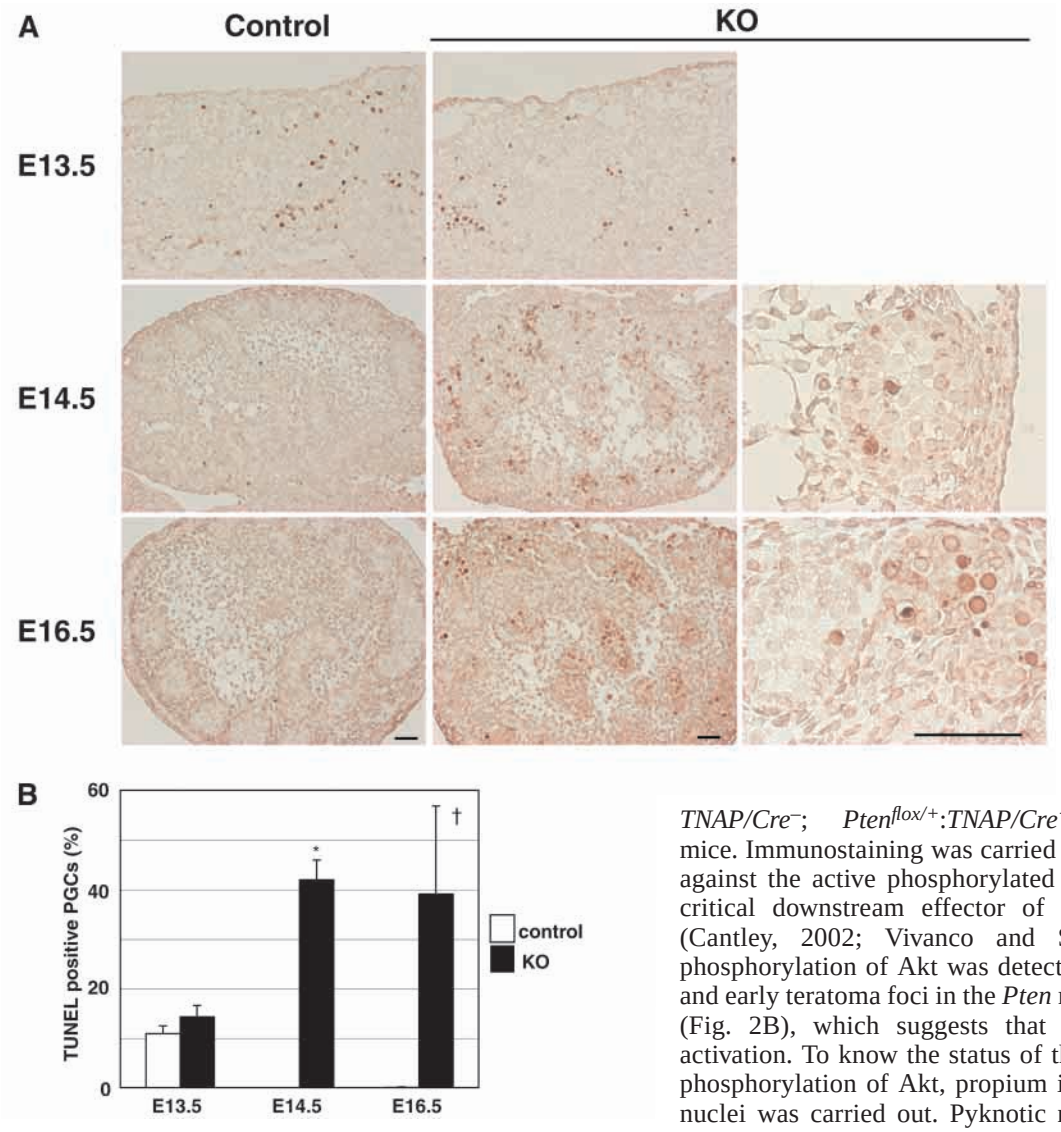

Fig. 3. Apoptosis in *Pten* mutant mice.
(A) Sections of embryo testes from E13.5, E14.5 and E15.5 control and mutant mice were analyzed by the TUNEL assay. Scale bars: 20 μm.
(B) The percentages of apoptotic PGCs at E13.5, E14.5 and E16.5. The percentage of apoptotic cells in Mvh-positive germ lineage cells (mean±s.d.) was calculated and analyzed using Student's *t*-test (P=0.22 at E13.5, $^{*}P$<0.01 at E14.5 and $^{\dagger}P$<0.001 at E16.5). Three to six mice were examined at each stage.

quantitative genomic PCR analysis of purified PGCs showed that Cre-mediated recombination at the $Pten^{flox}$ locus occurred with similar efficiency both in males and in females (data not shown). Immunohistochemical analysis of E15.5 testes was carried out to examine the efficiency of Cre-mediated deletion of the floxed PTEN allele. The expression of PTEN was high in germ cells but almost undetectable in somatic cells of the control mice (Fig. 1A). By contrast, only background level of expression was observed in the vast majority of germ cells in the mutant mice (Fig. 1B), which demonstrates that Cre-mediated deletion occurred very efficiently until E15.5.

Inevitable testicular teratoma formation in *Pten*-null male newborn mice

Examination of the *Pten* mutant ($Pten^{flox/-}$:*TNAP/Cre*$^{+}$) male mice at the newborn stage (P0) revealed that bilateral testicular teratomas with multiple foci had developed in all of the mutant mice. The teratomas consisted of various differentiated and undifferentiated cell types, and few germ cells were detected in the mutant testes (Fig. 2A, Fig. 4B). These abnormal phenotypes were not detected in the control ($Pten^{flox/-}$:*TNAP/Cre*$^{-}$; $Pten^{flox/+}$:*TNAP/Cre*$^{+}$; $Pten^{flox/+}$:*TNAP/Cre*$^{-}$) mice. Immunostaining was carried out with specific antibody against the active phosphorylated form of Akt, as Akt is a critical downstream effector of PtdIns(3,4,5)P_3 signaling (Cantley, 2002; Vivanco and Sawyers, 2002). Hyper-phosphorylation of Akt was detected in most gonadal PGCs and early teratoma foci in the *Pten* mutant male mice at E16.5 (Fig. 2B), which suggests that PTEN loss induces Akt activation. To know the status of the cells expressing hyper-phosphorylation of Akt, propium iodide (PI) staining of the nuclei was carried out. Pyknotic nuclei, typical features of apoptotic cells (Fig. 2B, part a), and mitotic figures (Fig. 2B, parts b,c) were observed in the significant proportion of the cells. Meanwhile, unilateral teratoma formation was observed occasionally in female *Pten* mutant mice a few weeks after birth (data not shown). It has been believed that ovarian teratomas originate from the parthenogenetically activated oocytes (Colledge et al., 1994; Eppig et al., 1977; Hashimoto et al., 1994). The mechanism of ovarian teratoma formation is under investigation by crossing floxed-*Pten* mice and oocyte-specific ZP3-Cre mice.

During normal male germ cell development, PGCs enter mitotic arrest at E13.5 and resume mitosis after birth (Stevens, 1967; Wylie, 1999). The percentage of mitotic PGCs in control mice was about 3% at E13.5; this percentage gradually declined, and was undetectable after E15.5. Although the percentage of mitotic PGCs in mutant mice at E13.5 was similar, a significant proportion of the PGCs in mutant mice exhibited mitotic figures between E14.5 and E16.5 (Fig. 2C,D).

The apoptosis of germ cells was examined by the TUNEL method (Fig. 3A). The percentages of apoptotic cells in mvh (mouse vasa homologue)-positive germ cells were calculated

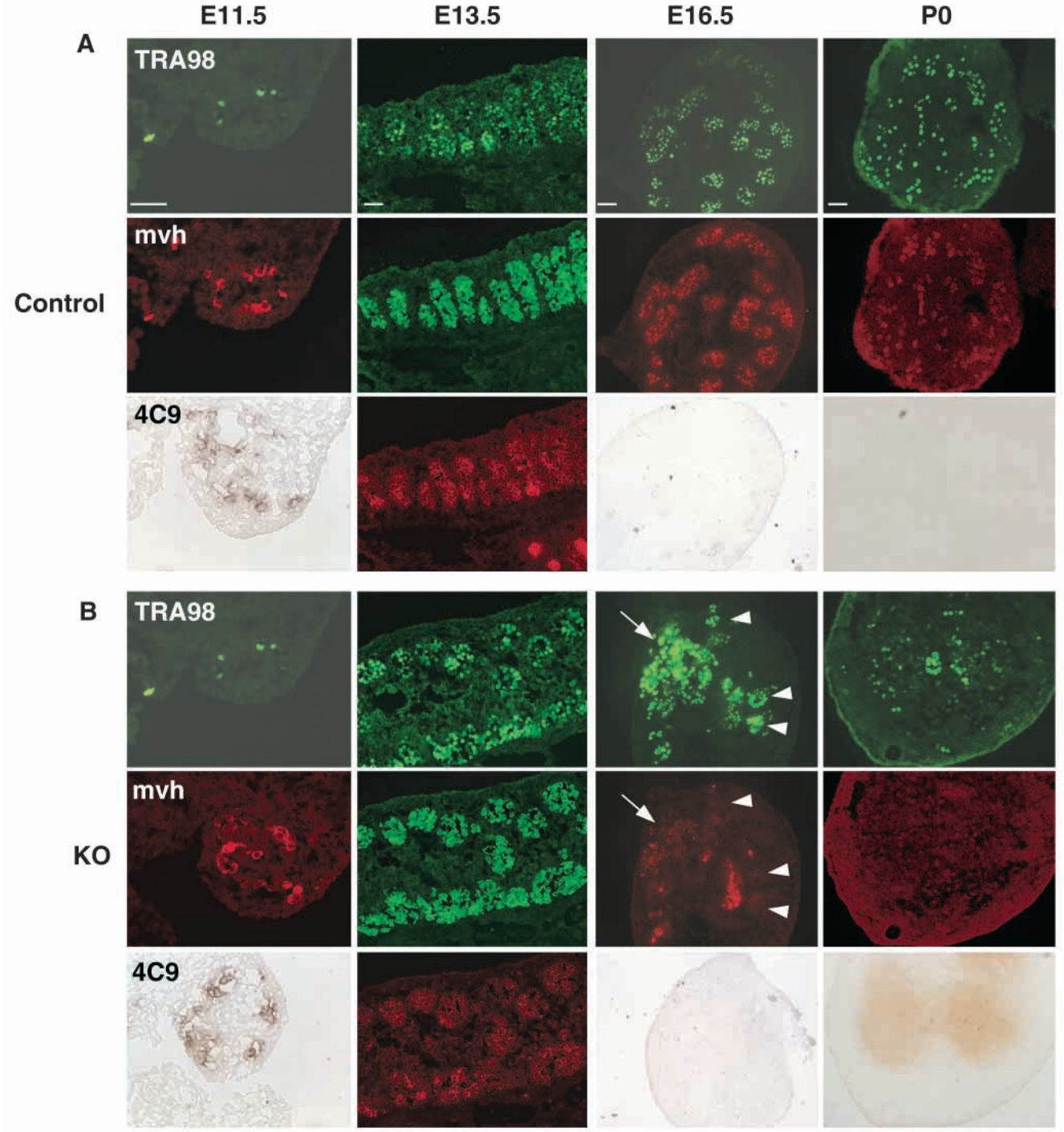

Fig. 4. Expression of the germ cell markers (Mvh and TRA98) and the immature cell marker (4C9) in the testes of control (A) and *Pten* mutant (B) mice during embryogenesis. Sections of testes at E11.5, E16.5 and P0 were double-stained with TRA98 (green) and anti-Mvh (red) antibodies. Adjacent or neighboring sections of the same samples were stained with 4C9 (brown) antibody. Similarly, sections of E13.5 gonads were double-stained with anti-Mvh (green) and 4C9 (red) antibodies, and adjacent sections were stained with TRA98 (green) antibody. At E13.5, all 4C9-positive PGCs expressed the germ-lineage-specific marker Mvh. In E16.5 mutant gonads, TRA98-positive teratoma cells, which were Mvh negative or weakly positive for Mvh (arrowheads), and outgrowth of these cells (arrow) were observed. In P0, large teratomas that were Mvh negative, weakly positive for TRA98 and 4C9 positive were observed. Scale bars: 50 μm. At each developmental stage, at least three mice were examined and essentially similar results were obtained.

(Toyooka et al., 2000). As shown in Fig. 3B, no significant differences were observed between the control and *Pten*-null mice at E13.5. No apoptotic germ cells were observed in the control male gonads after E14.5. However, a significant percentage of the germ cells in the gonads of *Pten*-null mice showed apoptosis after E14.5.

Expression of pluripotent cell and germ cell markers in *Pten*-null testes

To determine the profile of PGC development in control and mutant mice, we examined the expression patterns of germ cell and pluripotent cell markers (Fig. 4). The germ-lineage-specific marker mvh is expressed in post-migratory PGCs in gonads (Toyooka et al., 2000). The other germ cell marker, a nuclear antigen recognized by the TRA98 monoclonal antibody, is expressed in gonadal PGCs and EG cells (Tanaka et al., 1997). The 4C9 monoclonal antibody, which reacts with carbohydrate groups on the surfaces of pluripotent cells from two-cell embryos to epiblast cells and in immature PGCs, was used as a stem cell marker (Yoshinaga et al., 1991).

PGCs in control mice began to express mvh and TRA98 at E11.5, and expression was sustained until birth (Fig. 4A). By contrast, expression of the pluripotent cell marker 4C9 was undetectable by E16.5. Similarly, all of the 4C9-positive PGCs in the *Pten* mutant embryos were mvh-positive at E13.5, which indicates that the PGCs in the mutant mice acquired the characteristics of germ lineage cells (Fig. 4B). TRA98 expression was also activated in the PGCs of the mutants. Taken together, the results show that all immature PGCs differentiated into relatively mature PGCs, even in the PTEN-null condition (Fig. 4B). However, at around E16.5, TRA98-positive cells, which were either mvh-negative or weakly positive for mvh, emerged (Fig. 4B, arrowheads) and outgrowth of these cells was observed (Fig. 4B, arrow). Finally, large teratomas were observed at P0. Those teratomas consisted of the cells with the marker expression similar to EG cells, namely, mvh-negative, weakly positive for TRA98 and 4C9-positive. PTEN null teratomas would emerge by proliferation of relatively immature PGCs or de-differentiation from germ cells. In either case, loss of PTEN caused significant effects on the differentiation state of germ lineage cells.

Increased numbers of ectopic PGCs in the *Pten*-null mice

PGCs first appear as a TNAP-positive population posterior to the primitive streak at the gastrula stage (E7.5), in the base of the allantoic diverticulum (Gomperts et al., 1994; Wylie, 1999). The cells become incorporated into the developing hindgut and then migrate out of the hindgut into the surrounding connective tissue at E9.5. The PGCs leave the gut endoderm and transverse the dorsal mesentery toward the coelomic angles. Finally, the first PGCs reach the genital ridges through the mesonephros region by E11.0-11.5. The vast majority of PGCs colonize the gonad primordia by E13.

PGC distribution at E12.5 was examined by TNAP staining (Fig. 5). The number of PGCs in the genital ridges of normal mice was comparable with that of *Pten*-null mice. By contrast, the numbers of ectopic PGCs that were localized outside the genital ridges in control and *Pten*-null mice differed significantly. The ectopic PGCs were positioned mainly along the path of PGC migration. However, low numbers of ectopic PGCs were detected in an irrelevant region (designated as 'out of route' in Fig. 5A,C). Thereafter, the ectopic PGCs disappeared in both the control and *Pten*-null mice by E15.5. Although the cell adhesion activities of the *Pten*-null PGCs were extensively examined, they were not significantly different from those of control PGCs (data not shown). In addition, the percentage of PGCs with motile morphology in vitro was not increased (data not shown). These observations imply that the increased numbers of ectopic PGCs could be caused by the increased survival rather than the altered migratory capacity, but further analysis is necessary to discriminate these two possibilities.

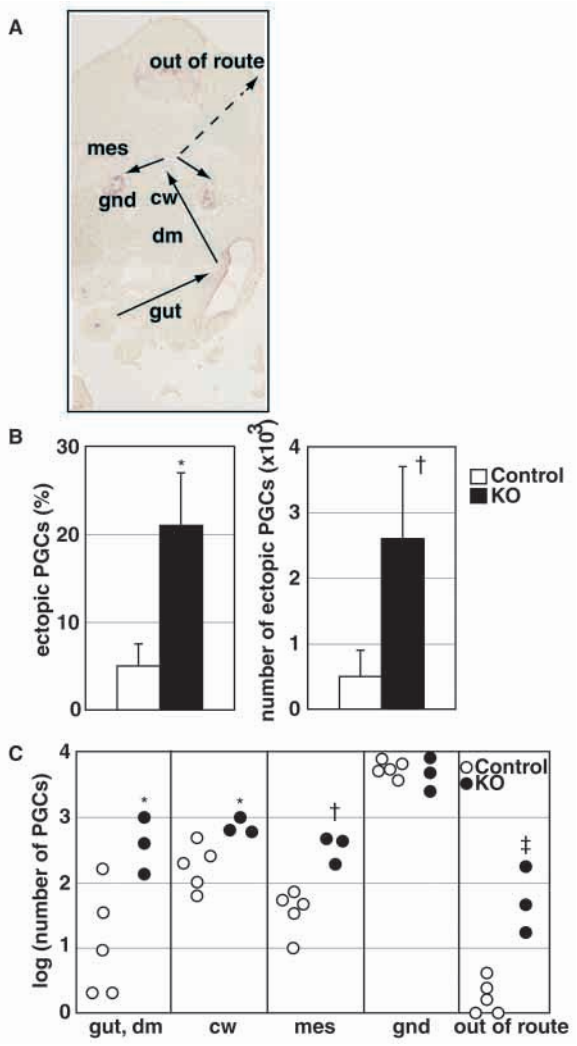

Fig. 5. Extra-gonadal PGCs in *Pten* mutant mice. (A) Route of PGC migration. Transverse section of E12.5 embryos stained for alkaline phosphatase activity. Alkaline phosphatase-positive cells were stained red. The normal migration pathway is indicated by arrows. PGCs become incorporated into the wall of the developing hindgut (gut; E8.5), migrate actively into the dorsal mesentery (dm; E9.5) and then to the genital ridges (E10.5-12.5). The path of 'out of route' PGCs is shown by a broken arrow. dm, dorsal mesentery; cw, coelomic wall; gnd, gonad; mes, mesonephros. (B) The percentage and the number of extra-gonadal PGCs at E12.5. The serial transverse sections of whole embryos were stained with alkaline phosphatase substrate as described in A. The number of PGCs in three sections was counted in every case. Ectopic PGCs were also stained with 4C9 antibody for confirmation. The results were analyzed by Student's *t*-test (**P*<0.0025, †*P*<0.01). (C) The distribution of PGCs at E12.5. The values for individual mice are plotted (Student's *t*-test; **P*<0.05, †*P*<0.01, and ‡*P*<0.005).

EG cell formation in PGC-specific *Pten*-null mice

We also tested whether the *Pten* deletion affected the

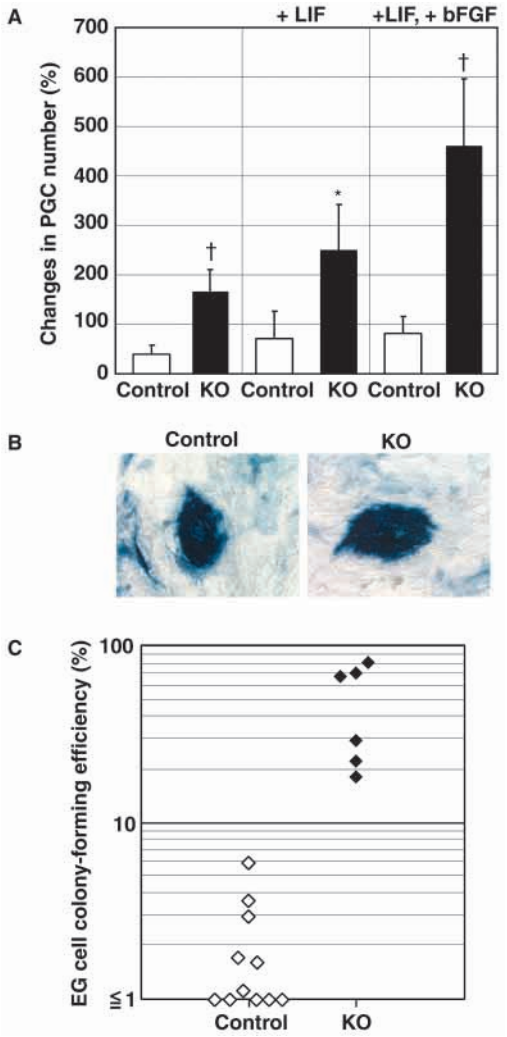

Fig. 6. In vitro culture of PGCs from *Pten* mutant mice. (A) Increased proliferation of E11.5 PGCs from *Pten* mutant mice. Cell suspensions of E11.5 gonads were seeded on Sl/Sl^4-m220 feeder cells that expressed the membrane-bound form of SCF, and cultured in the presence or absence of LIF and bFGF. Changes in PGC number (%) were calculated by dividing the number of PGCs at day 3 by the number of seeded PGCs. Data are mean±s.d. (*P<0.005, $^{\dagger}P$<0.05). (B) EG cell colonies formed in the presence of LIF and bFGF at day 6 after seeding. EG cells were visualized by alkaline phosphatase staining. (C) Increased EG cell production in E11.5 PGCs from *Pten* mutant mice. Multi-layered colonies that contained more than 20 cells were counted as EG cell colonies, as described previously (Koshimizu et al., 1996). The EG colony-forming efficiency (%) was calculated by dividing the number of EG cell colonies at day 6 by the number of seeded PGCs. The values for individual mice are plotted (P<0.01).

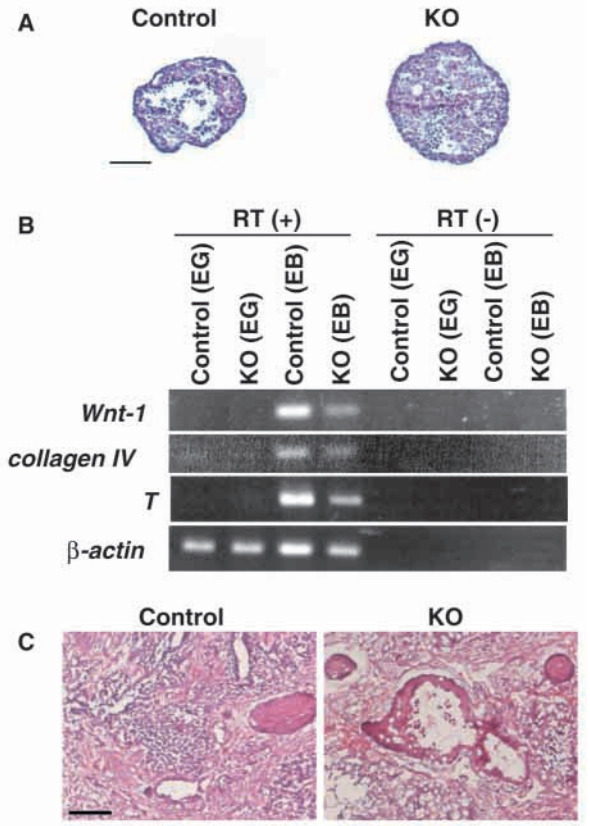

Fig. 7. Differentiation capacity of *Pten* mutant-derived EG cells. (A) Embryoid bodies with various cell types formed when differentiation was induced in methylcellulose cultures for 8 days. Scale bars: 50 μm. (B) RNA from embryoid bodies was subjected to semi-quantitative RT-PCR analysis to examine the expression of ectodermal (*Wnt1*), endodermal (collagen IV) and mesodermal (*T*) markers. β-Actin was used as a loading control. Expression of all three markers was induced both in control and in mutant EG cell-derived embryoid bodies. (C) Teratomas with differentiated cells that formed when EG cells were transplanted into nude mice. Scale bars:50 μm.

proliferative capacity and EG cell-forming efficiency of PGCs in vitro. SCF, LIF and bFGF act as survival and growth factors for PGCs in vitro, and EG cells can be formed when all these growth factors are added to PGC cultures (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1992; Matsui et al., 1990; Resnick et al., 1992). When E11.5 gonadal PGCs from control embryos were cultured on feeder cells that expressed the membrane-bound form of SCF, most of the E11.5 PGCs rapidly underwent apoptosis, as previously reported (Fig. 6A) (Koshimizu et al., 1996; Matsui et al., 1992). By contrast, PGC numbers increased when cells from mutant mice were used. In the presence of LIF alone, or LIF and bFGF, significantly more mutant PGCs than controls were observed after three days of culture (Fig. 6A). Significant differences were observed in PGCs from both male and female mice.

The efficiency of EG cell colony formation was remarkably high (48.2±11.5%, mean±s.e.m.) when the E11.5 PTEN-mutant PGCs were cultured (Fig. 6B). EG colonies were defined by TNAP staining (Fig. 6C) and immunostaining with anti-SSEA-1 antibody (data not shown). The EG cell-cloning efficiency was significantly higher (P<0.01; Student's t-test) in mutant PGCs than in control PGCs (1.5±0.6%). Once again, significant differences were observed regardless of mouse gender. EG cells that were established from mutant PGCs had a degree of normal differentiation capacity, as revealed by in vitro embryoid body formation and in vivo teratoma formation. Embryoid body formation occurred at the same frequency in mutant EG cells and in controls (data not shown), and RT-PCR analysis revealed that the mutant embryoid bodies consisted of three germ layers (Fig. 7A,B). Both *Pten* mutant and control

EG cells produced teratomas after transplantation into nude mice (Fig. 7C).

DISCUSSION

In this study, we demonstrate that PTEN is critical for the correct differentiation of PGCs to mature germ cells. Increased mitotic levels, higher percentages of apoptotic cells, and teratoma formation were observed in vivo for mutant male gonads (Figs 2-4). Moreover, in vitro experiments showed that the proliferative activities of PGCs and the efficiency of EG cell colony formation were enhanced by the loss of PEN function (Figs 6, 7).

Our observations indicate deregulated maintenance of PGCs in *Pten*-null male mice. Two main abnormalities were detected in *Pten*-null male gonads after E14.5, i.e. increased numbers of mitotic figures and higher apoptotic ratios (Figs 2, 3). One of the important roles of PTEN is to induce G1 arrest through the suppression of the PI3K/Akt pathway (Li and Sun, 1998; Ramaswamy et al., 1999; Sun et al., 1999). It seems reasonable that a significantly higher proportion of the *Pten*-null germ cells show mitotic figures. Although PTEN is known to cause apoptosis in many cell types (Li et al., 1998; Myers et al., 1998; Stambolic et al., 1998; Sun et al., 1999), loss of PTEN induced apoptosis of germ cells. This phenomenon seems ostensibly paradoxical, but might be explained as follows. Isolated activation of the PI3K/Akt pathway without concomitant activation of the other pathways may be perceived as abnormal by the cells and thereby trigger apoptosis. The mechanism by which some proportion of PTEN null germ cells escaped apoptosis and continued to grow remains unknown at present. Meanwhile, it seems unlikely that additional genetic alteration took place for teratoma formation, because the onset of teratoma was quite early. Selection by local environmental factors or epigenetic events of germ lineage cells might be involved in the survival and subsequent teratoma formation.

Overall, significant numbers of male germ cells die by apoptosis; some cells survive and keep growing, and eventually, teratoma develops from the surviving cells in the *Pten*-null mice. These phenotypes are similar to those of 129/Sv-*ter* mutant mice, in which both testicular teratoma and germ cell loss occur (Noguchi et al., 1996; Noguchi and Stevens, 1982; Stevens, 1984). PGC mitotic activity correlates strongly with the incidence of teratomas in 129/Sv-*ter* mice (Noguchi and Stevens, 1982). *Pten* mutant mice show impaired mitotic arrest, as do 129/Sv-*ter* mice, which suggests that mitotic quiescence is the critical event in the establishment of male germ cell commitment. The *Pten* and *ter* loci are on chromosomes 19 and 18, respectively (Asada et al., 1994; Hansen and Justice, 1998; Sakurai et al., 1994); however, common molecular mechanisms for teratoma formation and loss of germ cells may exist.

The in vitro survival and proliferation properties of PGCs were enhanced by the *Pten*-null mutation, which resembles testicular teratoma formation, and EG cell colony formation was also increased significantly (Figs 6, 7). The potential of EG cell formation of normal mice is very high in migratory phase PGCs at E8.5, then declined gradually, and eventually disappeared in E13.5 gonadal PGCs (Labosky et al., 1994a; Labosky et al., 1994b; Matsui et al., 1992). The high level of EG cell formation in the E11.5 *Pten* mutants resembles that of immature PGCs, and may be attributed partly to the high proliferative activities of PGCs. However, it is unlikely that the abnormalities are caused simply by increased proliferation, as cells that are derived from PTEN-null PGCs attain much broader differentiation potential as teratomas or EG cells. It is reasonable to assume that PTEN loss induces the de-differentiation of germ lineage-committed cells or sustains the immature state of PGCs longer than in control mice.

Two lines of evidence support this hypothesis. The first comes from the study of cell lines, in which PI3K signaling is involved in de-differentiation (Kobayashi et al., 1999; Singh et al., 2002). The second is derived from tissue-specific PTEN-deficient mice. PTEN-deficient neural stem cells exhibit increased proliferation and self-renewal capacities (Groszer et al., 2001). In addition, B-cell-specific PTEN-null mice have significantly increased numbers of B1 B cells, i.e. a subpopulation of B cells that resides mainly in the pleural and peritoneal cavities, and possess self-renewing activities (Suzuki et al., 2003). Taken together with our present data, the tumor suppressor PTEN negatively regulates self-renewal and proliferation in various stem cell systems. In addition to self-renewal and proliferation, our results suggest that PTEN is involved in the normal differentiation of PGCs. Mutant PTEN is frequently involved in tumor proliferation, and may enable tumor cells to regain pluripotency (Cantley and Neel, 1999; Di Cristofano and Pandolfi, 2000; Simpson and Parsons, 2001; Yamada and Araki, 2001). Tumors often originate through the transformation of stem cells, and it has been hypothesized that the physiological and pathological properties of stem cells are regulated by the same signaling pathway (Penninger and Woodgett, 2001; Reya et al., 2001). It seems likely that PTEN negatively regulates not only self-renewal and proliferation, but also the maintenance of immaturity of both tumor cells and stem cells.

The receptors for various growth factors that activate the PI3K signaling pathway, e.g. SCF, LIF and bFGF, are expressed in gonadal PGCs (Cheng et al., 1994; Pesce et al., 1997; Resnick et al., 1998). As Akt was hyper-activated in *Pten* mutant PGCs, it is likely that the loss of PTEN enhanced the PI3K signaling pathway, which then resulted in the deregulated differentiation of PGCs. Although EG cell formation was enhanced by the null mutation of *Pten*, no EG cell formation was observed without the addition of LIF or bFGF. Accordingly, the loss of PTEN function itself is not sufficient for enhanced EG colony formation, but probably increases the signals for PI3K, which is one of the downstream molecules for signaling via SCF, LIF and bFGF.

In conclusion, PTEN (PI3K) signaling plays a critical role in germ cell differentiation. Teratoma formation and increased EG colony formation suggest that PtdIns(3,4,5)P_3 signaling is involved in the maintenance of stem cell characteristics. Further analysis of PtdIns(3,4,5)P_3 signaling may provide new insights into stem cell biology.

We thank Drs T. Muramatsu, T. Noce, Y. Nishimune, Y. Matsui and D. Williams for providing reagents, Dr M. Noguchi, S. Chuma and N. Nakatsuji for valuable discussions, and Ms A. Mizokami for secretarial assistance. This work was supported in part by grants from the Ministry of Education, Science, Sports and Culture of Japan, the Japanese Society for Promotion of Science (JSPS-RFTF98L01101),

Ono Medical Research Foundation, Senri Life Science Foundation and the Osaka Cancer Research Foundation.

REFERENCES

Asada, Y., Varnum, D. S., Frankel, W. N. and Nadeau, J. H. (1994). A mutation in the Ter gene causing increased susceptibility to testicular teratomas maps to mouse chromosome 18. *Nat. Genet.* **6**, 363-368.

Backman, S. A., Stambolic, V., Suzuki, A., Haight, J., Elia, A., Pretorius, J., Tsao, M. S., Shannon, P., Bolon, B., Ivy, G. O. et al. (2001). Deletion of Pten in mouse brain causes seizures, ataxia and defects in soma size resembling Lhermitte-Duclos disease. *Nat. Genet.* **29**, 396-403.

Cantley, L. C. (2002). The phosphoinositide 3-kinase pathway. *Science* **296**, 1655-1657.

Cantley, L. C. and Neel, B. G. (1999). New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. *Proc. Natl. Acad. Sci. USA* **96**, 4240-4245.

Cheng, L., Gearing, D. P., White, L. S., Compton, D. L., Schooley, K. and Donovan, P. J. (1994). Role of leukemia inhibitory factor and its receptor in mouse primordial germ cell growth. *Development* **120**, 3145-3153.

Chuma, S. and Nakatsuji, N. (2001). Autonomous transition into meiosis of mouse fetal germ cells in vitro and its inhibition by gp130-mediated signaling. *Dev. Biol.* **229**, 468-479.

Colledge, W. H., Carlton, M. B., Udy, G. B. and Evans, M. J. (1994). Disruption of c-mos causes parthenogenetic development of unfertilized mouse eggs. *Nature* **370**, 65-68.

Dahia, P. L. (2000). PTEN, a unique tumor suppressor gene. *Endocr. Relat. Cancer* **7**, 115-129.

Di Cristofano, A. and Pandolfi, P. P. (2000). The multiple roles of PTEN in tumor suppression. *Cell* **100**, 387-390.

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

Dolci, S., Williams, D. E., Ernst, M. K., Resnick, J. L., Brannan, C. I., Lock, L. F., Lyman, S. D., Boswell, H. S. and Donovan, P. J. (1991). Requirement for mast cell growth factor for primordial germ cell survival in culture. *Nature* **352**, 809-811.

Eppig, J. J., Kozak, L. P., Eicher, E. M. and Stevens, L. C. (1977). Ovarian teratomas in mice are derived from oocytes that have completed the first meiotic division. *Nature* **269**, 517-518.

Godin, I., Deed, R., Cooke, J., Zsebo, K., Dexter, M. and Wylie, C. C. (1991). Effects of the steel gene product on mouse primordial germ cells in culture. *Nature* **352**, 807-809.

Gomperts, M., Wylie, C. and Heasman, J. (1994). Germline development. *Ciba Found. Symp.* **182**, 121-139.

Groszer, M., Erickson, R., Scripture-Adams, D. D., Lesche, R., Trumpp, A., Zack, J. A., Kornblum, H. I., 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.

Hansen, G. M. and Justice, M. J. (1998). Pten, a candidate tumor suppressor gene, maps to mouse chromosome 19. *Mamm. Genome* **9**, 88-90.

Hashimoto, N., Watanabe, N., Furuta, Y., Tamemoto, H., Sagata, N., Yokoyama, M., Okazaki, K., Nagayoshi, M., Takeda, N., Ikawa, Y. et al. (1994). Parthenogenetic activation of oocytes in c-mos-deficient mice. *Nature* **370**, 68-71.

Jiang, L. I. and Nadeau, J. H. (2001). 129/Sv mice–a model system for studying germ cell biology and testicular cancer. *Mamm. Genome* **12**, 89-94.

Kobayashi, M., Nagata, S., Iwasaki, T., Yanagihara, K., Saitoh, I., Karouji, Y., Ihara, S. and Fukui, Y. (1999). Dedifferentiation of adenocarcinomas by activation of phosphatidylinositol 3-kinase. *Proc. Natl. Acad. Sci. USA* **96**, 4874-4879.

Koshimizu, U., Taga, T., Watanabe, M., Saito, M., Shirayoshi, Y., Kishimoto, T. and Nakatsuji, N. (1996). Functional requirement of gp130-mediated signaling for growth and survival of mouse primordial germ cells in vitro and derivation of embryonic germ (EG) cells. *Development* **122**, 1235-1242.

Kwon, C. H., Zhu, X., Zhang, J., Knoop, L. L., Tharp, R., Smeyne, R. J., Eberhart, C. G., Burger, P. C. and Baker, S. J. (2001). Pten regulates neuronal soma size: a mouse model of Lhermitte-Duclos disease. *Nat. Genet.* **29**, 404-411.

Labosky, P. A., Barlow, D. P. and Hogan, B. L. (1994a). Embryonic germ cell lines and their derivation from mouse primordial germ cells. *Ciba Found. Symp.* **182**, 157-178.

Labosky, P. A., Barlow, D. P. and Hogan, B. L. (1994b). Mouse embryonic germ (EG) cell lines: transmission through the germline and differences in the methylation imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. *Development* **120**, 3197-3204.

Lee, J. O., Yang, H., Georgescu, M. M., di Cristofano, A., Maehama, T., Shi, Y., Dixon, J. E., Pandolfi, P. and Pavletich, N. P. (1999). Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association. *Cell* **99**, 323-334.

Li, D. M. and Sun, H. (1997). TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. *Cancer Res.* **57**, 2124-2129.

Li, D. M. and Sun, H. (1998). PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells. *Proc. Natl. Acad. Sci. USA* **95**, 15406-15411.

Li, G., Robinson, G. W., Lesche, R., Martinez-Diaz, H., Jiang, Z., Rozengurt, N., Wagner, K. U., Wu, D. C., Lane, T. F., Liu, X. et al. (2002). Conditional loss of PTEN leads to precocious development and neoplasia in the mammary gland. *Development* **129**, 4159-4170.

Li, J., Simpson, L., Takahashi, M., Miliaresis, C., Myers, M. P., Tonks, N. and Parsons, R. (1998). The PTEN/MMAC1 tumor suppressor induces cell death that is rescued by the AKT/protein kinase B oncogene. *Cancer Res.* **58**, 5667-5672.

Liaw, D., Marsh, D. J., Li, J., Dahia, P. L., Wang, S. I., Zheng, Z., Bose, S., Call, K. M., Tsou, H. C., Peacocke, M. et al. (1997). Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. *Nat. Genet.* **16**, 64-67.

Lomeli, H., Ramos-Mejia, V., Gertsenstein, M., Lobe, C. G. and Nagy, A. (2000). Targeted insertion of Cre recombinase into the TNAP gene: excision in primordial germ cells. *Genesis* **26**, 116-117.

Looijenga, L. H. and Oosterhuis, J. W. (1999). Pathogenesis of testicular germ cell tumours. *Rev. Reprod.* **4**, 90-100.

Maehama, T. and Dixon, J. E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J. Biol. Chem.* **273**, 13375-13378.

Marino, S., Krimpenfort, P., Leung, C., van der Korput, H. A., Trapman, J., Camenisch, I., Berns, A. and Brandner, S. (2002). PTEN is essential for cell migration but not for fate determination and tumourigenesis in the cerebellum. *Development* **129**, 3513-3522.

Marsh, D. J., Dahia, P. L., Zheng, Z., Liaw, D., Parsons, R., Gorlin, R. J. and Eng, C. (1997). Germline mutations in PTEN are present in Bannayan-Zonana syndrome. *Nat. Genet.* **16**, 333-334.

Marsh, D. J., Kum, J. B., Lunetta, K. L., Bennett, M. J., Gorlin, R. J., Ahmed, S. F., Bodurtha, J., Crowe, C., Curtis, M. A., Dasouki, M. et al. (1999). PTEN mutation spectrum and genotype-phenotype correlations in Bannayan-Riley-Ruvalcaba syndrome suggest a single entity with Cowden syndrome. *Hum. Mol. Genet.* **8**, 1461-1472.

Matsui, Y., Zsebo, K. and Hogan, B. L. (1992). Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. *Cell* **70**, 841-847.

Matsui, Y., Zsebo, K. M. and Hogan, B. L. (1990). Embryonic expression of a haematopoietic growth factor encoded by the Sl locus and the ligand for c-kit. *Nature* **347**, 667-669.

Myers, M. P., Stolarov, J. P., Eng, C., Li, J., Wang, S. I., Wigler, M. H., Parsons, R. and Tonks, N. K. (1997). P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. *Proc. Natl. Acad. Sci. USA* **94**, 9052-9057.

Myers, M. P., Pass, I., Batty, I. H., van der Kaay, J., Stolarov, J. P., Hemmings, B. A., Wigler, M. H., Downes, C. P. and Tonks, N. K. (1998). The lipid phosphatase activity of PTEN is critical for its tumor supressor function. *Proc. Natl. Acad. Sci. USA* **95**, 13513-13518.

Nelen, M. R., van Staveren, W. C., Peeters, E. A., Hassel, M. B., Gorlin, R. J., Hamm, H., Lindboe, C. F., Fryns, J. P., Sijmons, R. H., Woods, D. G. et al. (1997). Germline mutations in the PTEN/MMAC1 gene in patients with Cowden disease. *Hum. Mol. Genet.* **6**, 1383-1387.

Noguchi, M., Watanabe, C., Kobayashi, T., Kuwashima, M., Sakurai, T., Katoh, H. and Moriwaki, K. (1996). The ter mutation responsive for germ cell deficiency but not testicular nor ovarian teratocarcinogenesis in ter/ter congenic mice. *Develop. Growth Differ.* **38**, 59-69.

Noguchi, T. and Stevens, L. C. (1982). Primordial germ cell proliferation in

fetal testes in mouse strains with high and low incidences of congenital testicular teratomas. *J. Natl. Cancer Inst.* **69**, 907-913.

Penninger, J. M. and Woodgett, J. (2001). Stem cells: pten–coupling tumor suppression to stem cells? *Science* **294**, 2116-2118.

Pesce, M., di Carlo, A. and de Felici, M. (1997). The c-kit receptor is involved in the adhesion of mouse primordial germ cells to somatic cells in culture. *Mech. Dev.* **68**, 37-44.

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. *Proc. Natl. Acad. Sci. USA* **96**, 1563-1568.

Ramaswamy, S., Nakamura, N., Vazquez, F., Batt, D. B., Perera, S., Roberts, T. M. and Sellers, W. R. (1999). Regulation of G1 progression by the PTEN tumor suppressor protein is linked to inhibition of the phosphatidylinositol 3-kinase/Akt pathway. *Proc. Natl. Acad. Sci. USA* **96**, 2110-2115.

Resnick, J. L., Bixler, L. S., Cheng, L. and Donovan, P. J. (1992). Long-term proliferation of mouse primordial germ cells in culture. *Nature* **359**, 550-551.

Resnick, J. L., Ortiz, M., Keller, J. R. and Donovan, P. J. (1998). Role of fibroblast growth factors and their receptors in mouse primordial germ cell growth. *Biol. Reprod.* **59**, 1224-1249.

Reya, T., Morrison, S. J., Clarke, M. F. and Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. *Nature* **414**, 105-111.

Sakurai, T., Katoh, H., Moriwaki, K., Noguchi, T. and Noguchi, M. (1994). The ter primordial germ cell deficiency mutation maps near Grl-1 on mouse chromosome 18. *Mamm. Genome* **5**, 333-336.

Simpson, L. and Parsons, R. (2001). PTEN: life as a tumor suppressor. *Exp. Cell Res.* **264**, 29-41.

Singh, B., Reddy, P. G., Goberdhan, A., Walsh, C., Dao, S., Ngai, I., Chou, T. C., O-Charoenrat, P., Levine, A. J., Rao, P. H. et al. (2002). p53 regulates cell survival by inhibiting PIK3CA in squamous cell carcinomas. *Genes Dev.* **16**, 984-993.

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.

Stevens, L. C. (1967). Origin of testicular teratomas from primordial germ cells in mice. *J. Natl. Cancer Inst.* **38**, 549-552.

Stevens, L. C. (1984). Spontaneous and experimentally induced testicular teratomas in mice. *Cell Differ.* **15**, 69-74.

Stokoe, D. (2001). Pten. *Curr. Biol.* **11**, R502.

Sun, H., Lesche, R., Li, D. M., Liliental, J., Zhang, H., Gao, J., Gavrilova, N., Mueller, B., Liu, X. and Wu, H. (1999). PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. *Proc. Natl. Acad. Sci. USA* **96**, 6199-6204.

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. *Curr. Biol.* **8**, 1169-1178.

Suzuki, A., Yamaguchi, M. T., Ohteki, T., Sasaki, T., Kaisho, T., Kimura, Y., Yoshida, R., Wakeham, A., Higuchi, T., Fukumoto, M. et al. (2001). T cell-specific loss of Pten leads to defects in central and peripheral tolerance. *Immunity* **14**, 523-534.

Suzuki, A., Kaisho, T., Ohishi, M., Tsukio-Yamaguchi, M., Tsubata, T., Koni, P. A., Sasaki, T., Mak, T. W. and Nakano, T. (2003). Critical roles of Pten in B cell homeostasis and immunoglobulin class switch recombination. *J. Exp. Med.* (in press).

Tanaka, H., Pereira, L. A., Nozaki, M., Tsuchida, J., Sawada, K., Mori, H. and Nishimune, Y. (1997). A germ cell-specific nuclear antigen recognized by a monoclonal antibody raised against mouse testicular germ cells. *Int. J. Androl.* **20**, 361-366.

Toyooka, Y., Tsunekawa, N., Takahashi, Y., Matsui, Y., Satoh, M. and Noce, T. (2000). Expression and intracellular localization of mouse Vasa-homologue protein during germ cell development. *Mech. Dev.* **93**, 139-149.

Vivanco, I. and Sawyers, C. L. (2002). The phosphatidylinositol 3-Kinase AKT pathway in human cancer. *Nat. Rev. Cancer* **2**, 489-501.

Wu, X., Senechal, K., Neshat, M. S., Whang, Y. E. and Sawyers, C. L. (1998). The PTEN/MMAC1 tumor suppressor phosphatase functions as a negative regulator of the phosphoinositide 3-kinase/Akt pathway. *Proc. Natl. Acad. Sci. USA* **95**, 15587-15591.

Wylie, C. (1999). Germ cells. *Cell* **96**, 165-174.

Yamada, K. M. and Araki, M. (2001). Tumor suppressor PTEN: modulator of cell signaling, growth, migration and apoptosis. *J. Cell Sci.* **114**, 2375-2382.

Yoshinaga, K., Muramatsu, H. and Muramatsu, T. (1991). Immunohistochemical localization of the carbohydrate antigen 4C9 in the mouse embryo: a reliable marker of mouse primordial germ cells. *Differentiation* **48**, 75-82.