DEVELOPMENT AND DISEASE

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

Tohru Kimura1, Akira Suzuki2, Yukiko Fujita1, Kentaro Yomogida3, Hilda Lomeli4, Noriko Asada1, Megumi Ikeuchi1, Andras Nagy4, Tak W. Mak5 and Toru Nakano1

1Department of Molecular Cell Biology, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan
2Department of Biochemistry, Akita University School of Medicine, Akita 010-8543, Japan
3Department of Laboratory Sciences for Animal Experimentation, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan
4Samuel Lunenfeld Research Institute, Toronto, Ontario M5G 1X5, Canada
5Amgen Research Institute, Ontario Cancer Institute, and University of Toronto, Toronto, Ontario M5G 2C1, Canada

*Author for correspondence (e-mail: tnakano@biken.osaka-u.ac.jp)

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)P3] 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)P3], 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)P3 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; Grosse 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 (Grosz 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; Loosijenga and Oosterhuis, 1999).

In order to analyze the role of PTEN in germ cell differentiation, we produced conditional Pten knockout mice by crossing Ptenfloxflo females with Ptenfloxflo 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 Ptenfloxflo;TNAP/Cre mice were crossed with female Ptenfloxflo 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 TUNEL staining, whole embryos were fixed with 4% paraformaldehyde and embedded in OCT compound. 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 Si/Si-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 (Kosimizu et al., 1996; Matsui et al., 1992). The feeder cells were treated with 5 µg/ml mitomycin C for 2 hours and plated at 2×10⁵ 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 (Kosimizu 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, Ptenfloxflo; TNAP/Cre) and mutant (B, Ptenfloxflo; 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.](image-url)
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¢-GA TTGCGAAGA TGAACGCTGTTTC-3¢ and 5¢-TCCTCCACG- AACCTGTTGACGG-3¢; collagen IV, 5¢-CAAGCATAGTGTCG- GAGTC-3¢ and 5¢-AGGCAGGTCAAGTTCTAGCG-3¢; T, 5¢-ATG- CCAAAGAAAGAAACGAC-3¢ and 5¢-AGAGGCTGTAGAACA T- GA TT-3¢; and β-actin, 5¢-GTGACGAGGCCCAGAGCAAGAG-3¢ and 5¢-AGGGGCCGGACTCA TCGTACTC-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^floxtm^) (Suzuki et al., 2001). To avoid maternal expression of the Cre recombinase in oocytes, Pten^floxtm^ 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.](image)

(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 †P<0.002 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 \textit{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 \textit{Pten}-null male newborn mice**

Examination of the \textit{Pten} mutant (\textit{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 (\textit{Pten} flox/-; TNAP/Cre+; \textit{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)P3 signaling (Cantley, 2002; Vivanco and Sawyers, 2002). Hyper-phosphorylation of Akt was detected in most gonadal PGCs and early teratoma foci in the \textit{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 \textit{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-\textit{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
PTEN and germ cell development

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.

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).
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-lineage-specific marker mvh 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.

EG cell formation in PGC-specific Pten-null mice

We also tested whether the Pten deletion affected the
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; Matsu 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 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)P3 signaling is involved in the maintenance of stem cell characteristics. Further analysis of PtdIns(3,4,5)P3 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),

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 methylating imprint of insulin-like growth factor 2 receptor (Igf2r) gene compared with embryonic stem (ES) cell lines. Development 120, 3197-3204.


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


