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TESI DI DOTTORATO IN BIOLOGIA E PATOLOGIA CELLULARE E

MOLECOLARE

XVI° CICLO

ROLE OF PHOSPHATASE TENSIN HOMOLOGUE (PTEN) IN TESTICULAR TUMORS

COORDINATORE

CH.MO PROF.

VITTORIO ENRICO AVVEDIMENTO

RELATORE
Ch.mo Prof.
Alfredo Fusco

CANDIDATA
Dott.ssa
Letizia Cito

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INTRODUCTION

Cancer cells escape normal growth control mechanisms as a consequence of activating (i.e., gain-of-function) mutations and/or increased expression of one or more cellular proto-oncogenes and/or inactivating (i.e., loss-of function) mutations and/or decreased expression of one or more tumor suppressor genes. Most oncogene and tumor suppressor gene products are components of signal transduction pathways that control cell cycle entry or exit, promote differentiation, sense DNA damage and initiate repair mechanisms, and/or regulate cell death programs. Several oncogenes and tumor suppressor genes belong to the same signaling pathway. Nearly all tumors have mutations in multiple oncogenes and tumor suppressor genes, indicating that cells employ multiple parallel mechanisms to regulate cell growth, differentiation, DNA damage control, and death.

The *PTEN* gene was discovered only in 1997 as a new tumor suppressor, and yet it is now known to play major roles not only in suppressing cancer but also in embryonic development, cell migration and apoptosis (reviews include Maehama and Dixon, 1999; Cantley and Neel, 1999; Besson et al., 1999; Tamura et al., 1999c; Ali et al., 1999; Di Cristofano and Pandolfi, 2000; Vazquez and Sellers, 2000; Bonneau and Longy, 2000; Simpson and Parsons, 2001). Altough an increasing numbers of biologically important phosphatases are being characterized (Li and Dixon, 2000; Tonks and Neel, 1996), but PTEN has been the focus of particularly intense interest because of its central role in suppressing malignancy.

CLONING OF PTEN

The *PTEN* (phosphatase and tensin homolog deleted on chromosometen)/*MMAC* (mutated in multiple advanced cancers) was identified virtually simultaneously by two groups (Ling al., 1992; Webster et al., 1998) as a candidate tumor suppressor gene located at 10q23; another group (Moscatello et al.1998) identified the same gene in a search for new dual-specificity phosphatases and named it TEP-1 (TGF-β-regulated and epithelial cell-enriched phosphatase). The PTEN protein sequence suggested that is was a member of the protein-tyrosine phosphatase (PTP) gene superfamily . PTEN catalytic domains contain the canonical sequence HCXXGXXRSyT, known as the PTP ‘‘signature motif’’ (Rasheed et al. 1997) (see figure 1A); the presence of this motif within any protein makes it a virtual certainty that it has PTP activity. In particular, the PTEN sequence suggested that it was a dual-specificity phosphatase, an enzyme that, as its name implies, typically dephosphorylate phosphotyrosine, phosphoserine, and/or phosphothreonine *in vitro*. However, PTEN is an unusual phosphatase in the sense that it dephosphorylate both lipids and proteins. PTEN protein contains 403 amino acids and can be divided into three domains: a phosphatase domain (1–185), a C2 domain (186–352), and a tail domain (353–403) (Lee et al. 1999) (see figure 2).

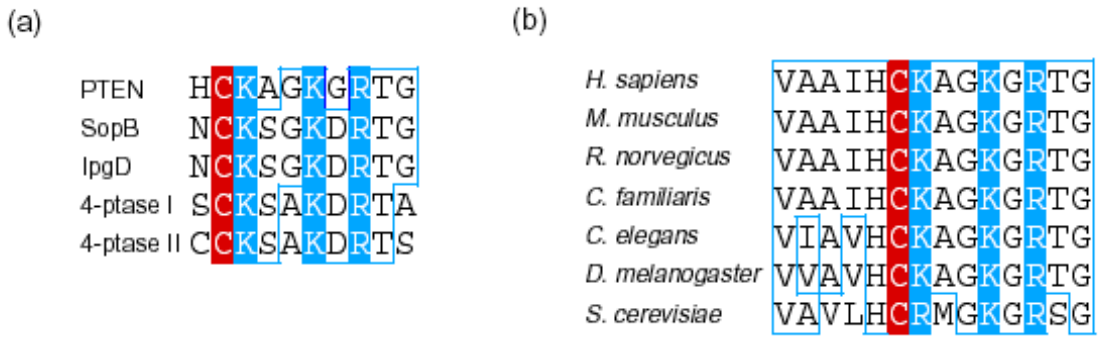

Figure1. A. Conserved amino acid sequence motif present in inositol phosphatases. The amino acid sequence of human PTEN, *Salmonella dublin* SopB, *Salmonella flexneri* IpgD, human inositol polyphosphatase type I and type II are indicated. **B.**Alignment of PTEN signature motifs. The amino acid sequences of human (*Homo sapiens*), mouse (*Mus musculus*), rat (*Rattus norvegicus*), dog (*Canis familiaris*), worm (*Caenorhabditis elegans*), fly (*Drosophila melanogaster*) and yeast (*Saccharomyces cerevisiae*) PTEN are shown. The crucial catalytic cysteine and the invariant basic residues are highlighted in red and blue, respectively.

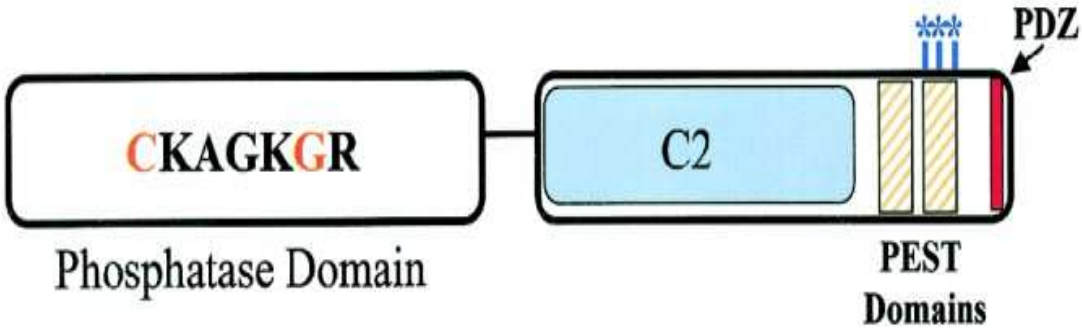

Figure 2. The N-terminal-phosphatase domain (amino acids 1 185) is shown with the catalytic core. The C-terminal domain (amino acids 186 403) C2 domain ¯lipid-bindin PEST domains-regulate protein stability PDZ domain-is important in protein-protein interactions. CK2 phosphorylation sites -are important for stability

The phosphatase and C2 domains are required for efficient membrane binding (Das et al. 2003). Mutations of the phosphatase motifof PTEN do not perturb membrane binding but inhibit PIP3 catalysis. Interestingly, amino acids 1–14 encode a PIP2 binding domain and recent evidence demonstrates that PIP2 binding at this site enhances PTEN catalytic function (Campbell et al. 2003). The tail domain is an important region for negative regulation of PTEN. Deletion of the tail activates PTEN's ability to inhibit AKT while reducing its half-life (Vazquez et al. 2000). Several groups have reported that the tail region is a site of constitutive serine and threonine phosphorylation on multiple sites (Vazquez et al. 2000). Casein kinase II appears to be responsible for PTEN phosphorylation. It should be noted that the stoichiometry and sites of phosphorylation varied from group to group. Alanine mutations that block phosphorylation at three or more sites behave like tail deletions in that they have increased potency and reduced stability. The mechanism through which the tail inhibits PTEN function appears to be through the regulation of access to the plasma membrane. In fact mutants that remove the tail or its phosphorylation sites (serine 380, threonine 383) are found on the plasma membrane. Interestingly, stable expression of these mutants requires that the catalytic site be inactivated (C124A). Therefore, it appears that increasing phosphorylation of the PTEN tail is likely to have oncogenic consequences by sequestering PTEN away from the plasma membrane.

MOLECULAR TARGETS OF PTEN

As stated above, PTEN phosphatase activity has been observed against both lipid and protein substrates. Overall, the primary physiological substrate of PTEN appears to be the signaling lipid Phosphatidylinositol, (3, 4, 5) P3 (since now PIP3) (see figure 3A) (Maehama and Dixon, 1998; Myers et al., 1998). PIP3 is a major product of PI 3-kinase, which is activated by cell receptors including various tyrosine kinase growth factor receptors and integrins (Rameh and Cantley, 1999; Leevers et al., 1999). PTEN cleaves the 3'phosphate from PIP3 to generate PIP2 (Maehama and Dixon, 1998), which lacks the activities of PIP3 but has its own actions on cytoskeleta lfunction. By antagonizing the action of PI 3-kinase (see figure 3B), PTEN affects a number of cell biological processes (see below). In addition, it can dephosphorylate the signaling molecule inositol (1,3,4,5)-tetrakisphosphate (Maehama and Dixon, 1998), although the biological importance of this activity is not yet clear.

In vitro, PTEN can also remove phosphate residues from phosphotyrosine-containing peptides and proteins (Li and Sun, 1997; Myers et al., 1997; Tamura et al., 1998; Gu et al., 1998; Gu et al., 1999), although the relative importance of this enzymatic function in vivo compared with its lipid phosphatase activity has been controversial.

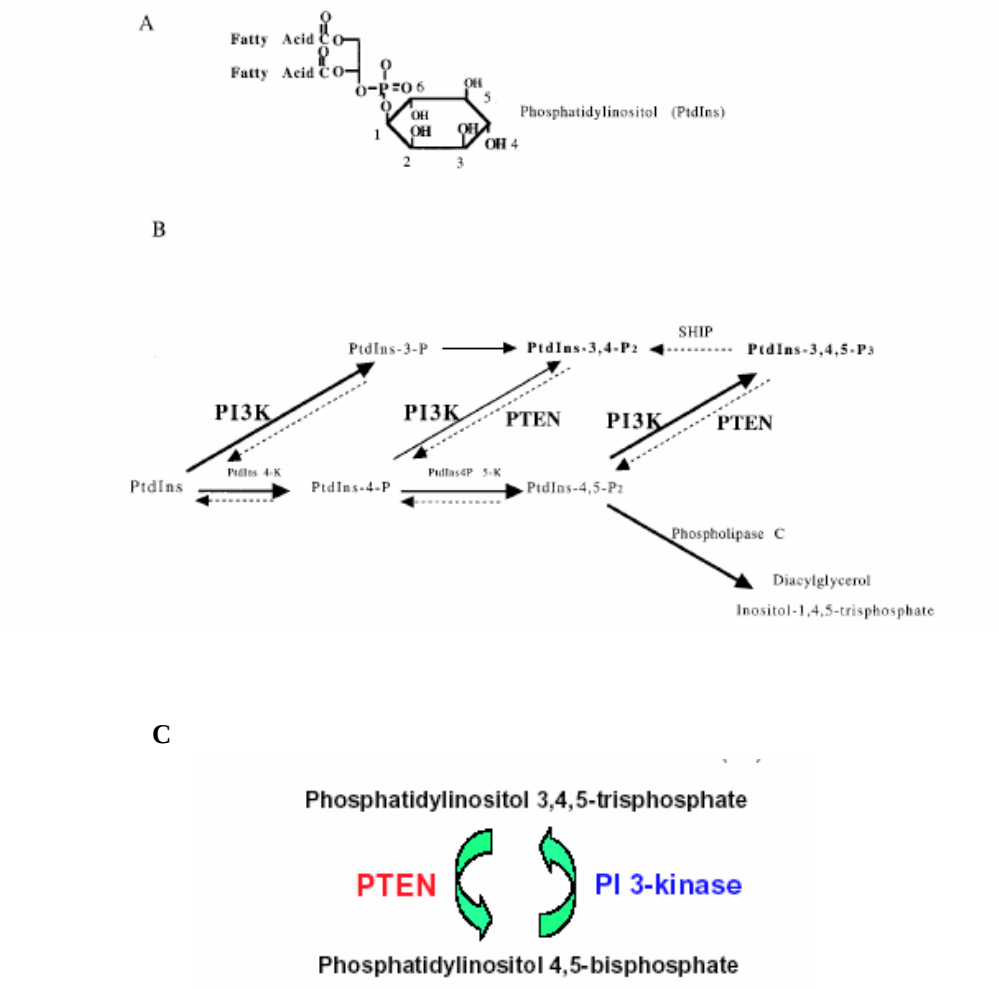

Figure 3. A. PtdIns contains a *myo*-inositol headgroup connected to diacylglycerol by a phosphodiester linkage. The numbering system of the inositol ring is indicated. **B**.The class I PI3K enzymes can phosphorylate the 3 position of PtdIns, PtdIns-4-P, or PtdIns-4,5-P2 to produce PtdIns-3-P, PtdIns-3,4-P2, or PtdIns-3,4,5-P3, respectively. PtdIns-3,4-P2 can also be produced by dephosphorylating the 5 position of PtdIns-3,4,5-P3, and one enzyme that does this is an SH2-containing 5-phosphatase called SHIP. In addition, PtdIns-3,4-P2 can be produced by phosphorylating the 4 position of PtdIns-3-P [reviewed by Fruman *et al*. (76)]. PTEN has been shown to dephosphorylate the 3 position of both PtdIns-3,4,5-P3 (26, 43) and PtdIns-3,4-P2 (44) to reverse the reactions catalyzed by PI3K. **C**. Major enzymatic function of PTEN. The tumor suppressor PTEN opposes the action of phosphoinositide 3-kinase (PI 3-kinase) by dephosphorylating the signaling lipid phosphatidylinositol (3,4,5)-trisphosphate.

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Two cytoplasmic phosphoprotein substrates of PTEN are focal adhesion kinase (FAK) and the adapter protein Shc (see figure 4), whereas a number of other cellular tyrosine-phosphorylated proteins appear unaffected by PTEN (Tamura et al., 1998; Gu et al., 1999). FAK and Shc are central components of distinct signaling pathways . The FAK signaling pathway is activated by integrins and other receptors and is linked to cell migration and other cellular activities. The Shc pathway is activated by receptors that include various tyrosine kinase receptors and integrins, and is part of a pathway that leads to activation of ERK MAP kinases. Although these in vitro effects have proven valuable for dissecting pathways that regulate cell migration (see below), analyses of cells from *PTEN*-knockout embryos fail to show changes in basal FAK phosphorylation or ERK activity (Stambolic et al., 1998; Liliental et al., 2000). These findings indicate that the major target of PTEN under steady-state conditions is PIP3 and not FAK, although transient changes in PTEN levels might nevertheless still have physiological effects on FAK/Shc activity. Furthermore, the G129E PTEN mutation abrogates most PTEN activity against PIP3, but it retains activity against peptide and protein substrates (Myers et al., 1998); this mutation is found in some cancers.

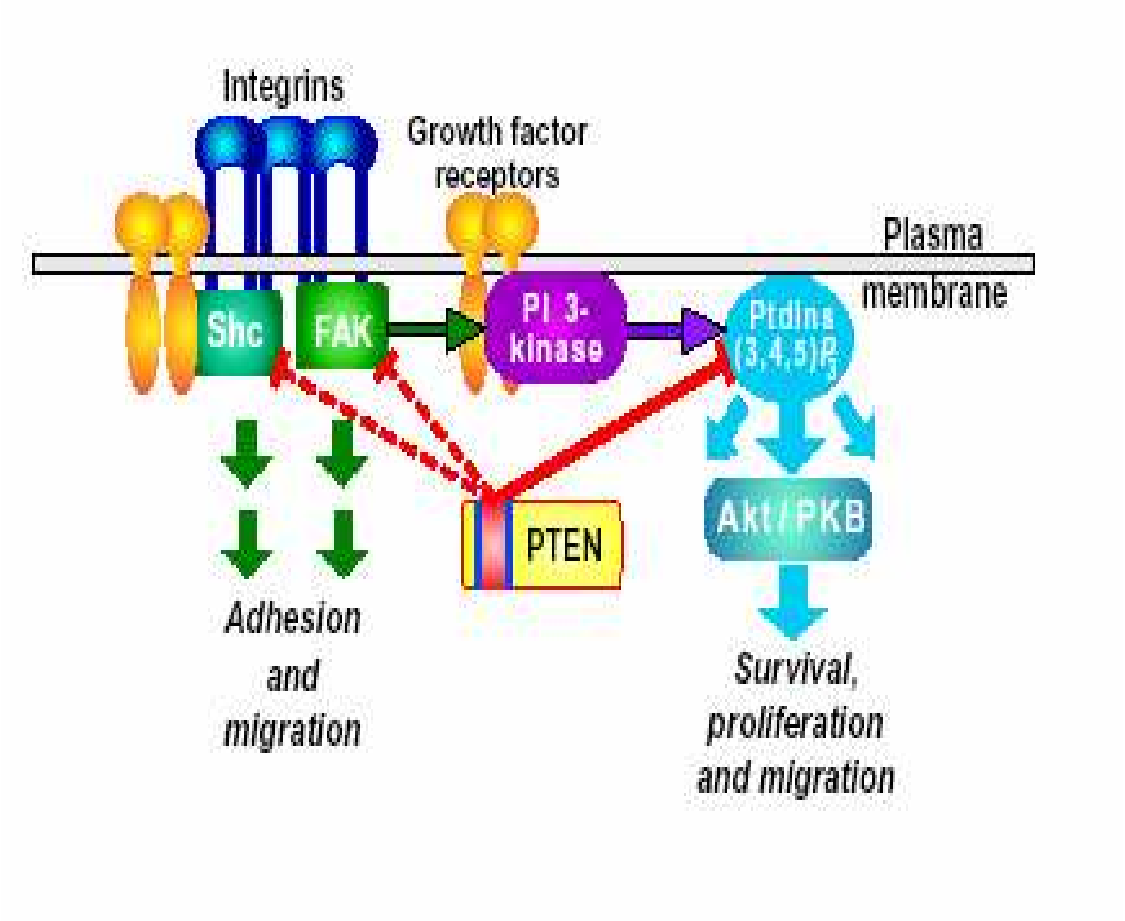


Figure 4. Reported sites of action of PTEN. Extracellular interactions trigger signaling from integrins and growth factor receptors. The majorfunction of PTEN appears to be downregulationof the PI 3-kinase product PtdIns(3,4,5)*P*3, which regulates Akt and complex downstream pathways affecting cell growth, survival and migration. In addition, PTEN has weak proteintyrosine phosphatase activity, which may target focal adhesion kinase (FAK) and Shc, and thereby modulate other complex pathways. The phosphatase domain of PTEN (red) dephosphorylates and downregulates (red lines) substrate molecules.

MECHANISM OF PTEN TUMOR SUPPRESSION

PTEN regulates the PI-3 kinase pathway by removing the third phosphate from the inositol ring of the PIP3 second messenger (Sulis et al. 2003; Vivanco and Sawyers , 2002). Interestingly PTEN has conserved this function in many animal species including mammals, *Drosophila*, and *C. elegans*. (see figure 1 B). Although PIP3 is able to bind to over one hundred cellular proteins, genetic studies have demonstrated that a major output of PIP3 is AKT/protein kinase B. Lack of PTEN in a cell leads to increased PIP3 levels and AKT kinase activity. Mammalian cells lacking PTEN have increased proliferation, reduced apoptosis, altered migration, and increased size—all phenotypes that favor tumorigenesis. Downstream substrates of AKT that are altered genetically in malignancy include TSC2, a tumor suppressor mutated in the hamartoma syndrome tuberous sclerosis that regulates mTOR, and MDM2, an oncogene amplified in sarcomas that suppresses p53 function. Other AKT substrates include the FOXO transcription factors, p27, p21, GSK3, and BAD, which have important roles in the regulation of the cell cycle and apoptosis (see figure 5). Re-expression of PTEN in tumor cell lines lacking the gene led to inhibition of AKT and a variety of outputs that included inhibition of the cell cycle, activation of apoptosis, rearrangement of the cytoskeleton, altered cellular migration, and suppression of angiogenesis—phenotypes that varied depending upon the cell line and dose of PTEN. While most PTEN phenotypes require that PTEN be in a catalytically active state, observations on the control of

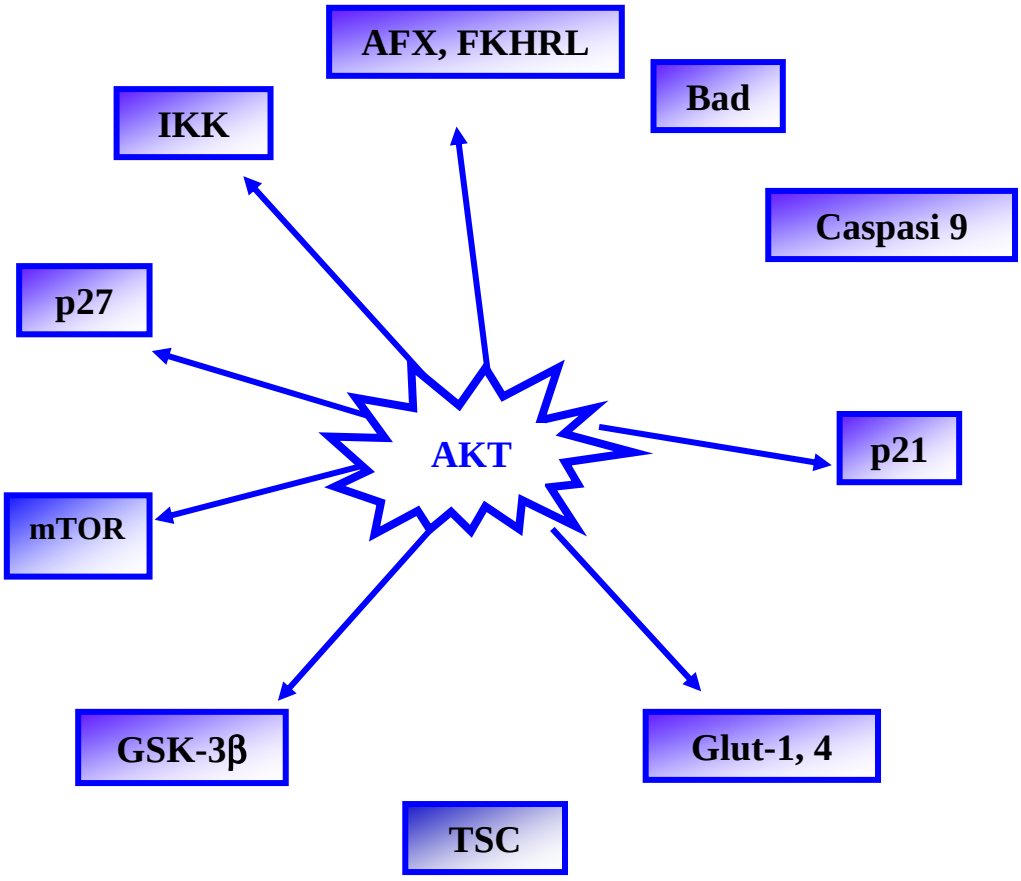


Figure 5. Akt, principal effector of PTEN , interacts with several substrates that control various phenomena of cell life, such as for example progression of cell cycle, (i.e. $p27^{kip1}$ and $p21^{cip1}$) and apoptosis (i.e.Bad and Caspase 9).

migration, the cytoskeleton, and the p53 pathway suggest that PTEN exerts PIP3-independent phenotypes (Tamura et al. 1998, Freeman et al. 2003).

PTEN IS DEVELOPMENTALLY REGULATED AND NECESSARY FOR EMBRYONIC DEVELOPMENT

Although substantial progress has been made in understanding the role of PTEN in tumor suppression, much less is known about its role in normal embryonic development (except that PTEN-knockout mice die early in development) or about its regulation during normal tissue function.

Expression levels of PTEN protein are low in development until approximately day 11, when levels rise substantially in multiple tissues (Podsypanina et al., 1999), and the protein becomes widely distributed (Gimm et al., 2000). Soon after its discovery, several laboratories generated null mutations of the *PTEN* gene in mice to assess its function in vivo. The phenotypes differed considerably, presumably because of genetic differences between the mice used by each group. Nevertheless, all *PTEN*-knockout mice die before birth, demonstrating a requirement for PTEN in embryogenesis (Di Cristofano et al., 1998; Suzuki et al., 1998; Podsypanina et al., 1999). The phenotypes differed substantially: one study identified major defects in proper differentiation and organization of the ectoderm, mesoderm and endoderm (Di Cristofano et al., 1998) and observed death by embryonic day (E) 7.5; another found severe malformations

of cephalic and caudal regions, which suggested an imbalance of growth and patterning, and observed death by E9.5 (Suzuki et al., 1998); a third study found severe defects by approximately E6.5, indicating that even the low level of PTEN protein present in early embryos is needed for successful embryonic development (Podsypanina et al., 1999). These differing results suggest a major role for context in PTEN functions. For example, PTEN may play important roles in germ layer organization or differentiation in one genetic background, but not in another; it may instead play crucial roles in regulating local apoptosis or proliferation in another setting.

PTEN IN GROWTH, APOPTOSIS AND ANOIKIS

Because a tumor suppressor might be expected to suppress cell proliferation, several research groups have tested whether restoration of PTEN expression to cells that have mutated *PTEN* alleles suppresses growth. Transient expression using plasmid or adenoviral PTEN vectors suppresses proliferation. However, results are not always consistent, even in the same cell line. Most studies have shown suppression of proliferation due to arrest in G_1 phase of the cell cycle and corresponding increases in the levels of cell cycle inhibitors such as $p27^{KIP1}$ and decreased levels of retinoblastoma (Rb) protein phosphorylation (Furnari et al., 1998; Li and Sun, 1998; reviewed by Tamura et al., 1999c and Simpson and Parsons, 2001). The G_1 phase cell cycle arrest is due to the lipid phosphatase activity of PTEN against PIP3 (Ramaswamy et al., 1999). In fact, cell cycle effects of PTEN can be mimicked by SHIP-2, an enzyme that hydrolyzes

another phosphate group on PIP3 (Taylor et al., 2000). However, results using the G129E mutant provide evidence for phosphatase activity in G_1 cell cycle arrest (Hlobilkova et al., 2000). It should be emphasized that in these experiments PTEN has not been shown to be a physiological regulator of the normal cell cycle, since the above studies involved sudden restoration (and possibly overexpression) of an enzyme in cells adapted to proliferate in its absence. Moreover, normal cells expressing PTEN can undergo rapid proliferation. In fact, one group found that rates of cell proliferation and levels of $p27^{KIP1}$ were normal in *PTEN*-null fibroblasts, despite being abnormal in *PTEN*-null embryonic stem cells (Liliental et al., 2000; Sun et al., 1999). Thus, even though sudden reconstitution of PTEN can suppress proliferation, the long-term role of this activity in cancer progression remains unclear.

The role of PTEN in apoptosis is clearer. Re-expression of PTEN in several carcinoma cell lines can induce apoptosis directly (Li et al., 1998), even though an apoptotic stimulus is often needed (Stambolic et al., 1998; reviewed by Tamura et al., 1999c; Simpson and Parsons, 2001). A particularly important role of PTEN is in the anoikis, a form of apoptosis characterized by loss of contact with the extracellular matrix (Frisch and Ruoslahti, 1997). This property may be a central feature of normal epithelial cell function (and perhaps certain other cell types) that prevents growth at abnormal sites, especially in suspension. This anchorage dependence of survival is defective in many transformed and malignant cells. Reconstitution of PTEN in cells that have *PTEN* mutations restores anoikis (Davies et al., 1998;

Tamura et al., 1999a; Davies et al., 1999; Lu et al., 1999). Anoikis has been linked to the signaling and scaffold protein FAK (Frisch et al., 1996). PTEN modulates apoptosis by reducing levels of PIP3. This signaling lipid regulates activation of Akt Newton, 2000; Persad et al., 2000), a well known regulator of apoptosis. Re-expression of PTEN in various tumor cell lines decreases PIP3 levels and reduces Akt activation (Stambolic et al., 1998; Haas-Kogan et al., 1998; Myers et al., 1998; Davies et al., 1998). The role of FAK in anoikis may involve at least in part its ability to increase levels of PIP3 by enhancing PI 3-kinase activity (Tamura et al., 1999a). Dephosphorylation of FAK by PTEN would enhance the effects of PTEN on PIP3, since FAK phosphorylation enhances PI3-kinase activity (Reiske et al., 1999); the combined effects of reduced PI 3-kinase activity and direct reductions in PIP3 levels by PTEN would block Akt activation and enhance apoptosis.

PTEN IN CELL ADHESION, MIGRATION AND INVASION

PTEN reconstitution or overexpression inhibits cell migration (Tamura et al., 1998; Liliental et al., 2000). This inhibition can be accompanied by transient effects on cell adhesion and spreading: the number of focal contacts specialized contacts mediating cell-substrate adhesion - is reduced, and the actin cytoskeleton is altered (Tamura et al., 1998), although the remaining focal contacts often appear to be larger. The mechanisms by which focal contacts can be modulated by PTEN include effects on the FAK-p130-signaling pathway (Gu et al., 1999) and selective effects on focal contact constituents caused by

changes in PIP3 levels, as suggested by unrelated studies showing that PDGF can modulate focal contacts through PIP3 (Greenwood et al., 2000). PTEN suppresses migration of a variety of cell types, including primary human fibroblasts, non-transformed mouse fibroblasts, and tumor cells (Tamura et al., 1998; Tamura et al., 1999b). *PTEN*-null mouse fibroblasts also show enhanced rates of migration, which are reduced by reintroduction of PTEN (Liliental et al., 2000). PTEN also suppresses tumor cell invasion, as measured by in vitro assays of invasion across barriers of basement membrane extract (Tamura et al., 1999b). It reduces rates of migration through several mechanisms.

THE ROLE OF PTEN IN SPORADIC TUMOUR

Several studies confirmed that PTEN was mutated in a wide variety of human cancer. Mutation of PTEN could occur early in tumor development as seen in Cowden disease and endometrial tumors (see figure 6) (Levine et al. 1998).

In most cases, however, mutation of PTEN occurrs in advanced cancers. Such is the case for tumors of the brain, prostate, colon, and cervix (Rasheed et al. 1997). Early studies indicated that 10q, where PTEN maps (see figure 7), abnormalities are more common in advanced tumors (hence the appellation *MMAC*). In fact the initial cloning studies reported *PTEN*/*MMAC*/*TEP-1* (hereafter, *PTEN*) mutations in a large fraction of glioblastoma multiforme cell lines, xenografts, and primary tumors, as well as in smaller samples of

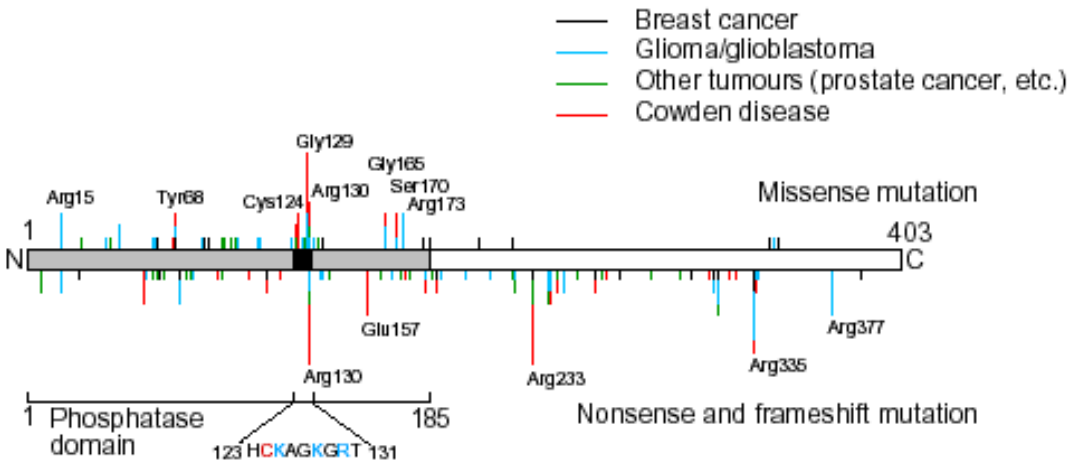


Figure 6. *PTEN* mutations found in human tumours and Cowden disease. The mutations in breast cancer (black), glioma/glioblastoma (blue), other tumours (green) and Cowden disease (red) are represented by vertical lines. Vertical lines above (or below) represent the frequency at which missense mutations (or nonsense and frameshift mutations) are found at each particular residue. The grey and black boxes represent the phosphatase domain and the catalytic core motif, respectively.

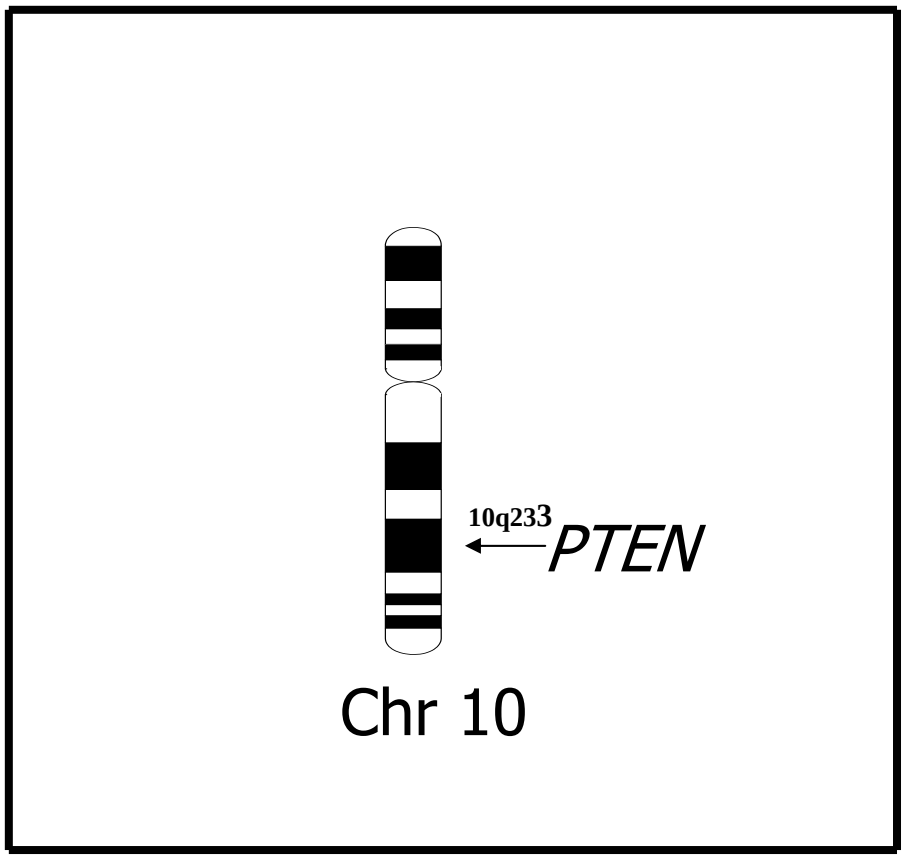


Figure 7. Localization of *PTEN* on the longe arm of chromosome 10.

breast and prostate cancers (Liu et al 1997, Steck et al. 1997)), and subsequent analyses confirmed that homozygotic inactivation of *PTEN* occurs in a large fraction of glioblastomas (at least 30% of primary tumors and 50–60% of cell lines) but not in lower-grade (i.e., less advanced) glial tumors (Liu et al. 1997). *PTEN* mutations also are extremely common in melanoma cell lines (50%) (9), advanced prostate cancers (Risinger et al. 1997)), and endometrial carcinomas (30–50%) (Tashiro et al.1997). Although *PTEN* mutations are found predominantly in advanced glial and prostate tumors, mutations occur with equal frequency at all stages of endometrial cancer (Tashiro et al. 1997), suggesting that *PTEN* activation is an early event in endometrial carcinogenesis. Whereas germ-line *PTEN* mutations lead to increased breast cancer incidence, *PTEN* mutations are not a frequent cause of familial breast cancer (Chen et al. 1998). Occasional *PTEN* mutations are reported in head and neck (Okami et al. 1998) and thyroid (Dahia et al. 1998) cancers, but not in other tumors associated with 10q abnormalities, including meningioma (8) and lung cancer (Okami et al. 1998). Regarding hematological tumors, mutation of *PTEN* occurs but is uncommon in multiple myeloma and non-Hodgkin's lymphoma. Moreover, PTEN protein expression is reduced in many types of cancer. To highlight the common cancers, reduced PTEN expression has been documented in 66% of glioblastoma, 61% of endometrial cancer, 24% of non-small cell lung cancer, 38% of breast cancer, 27% of ovarian cancer, 20% of prostate cancer, 41% of colorectal cancer with microsatellite instability, and

TUMORS	Reduced expression of PTEN (%)
GLIOBLASTOMA	66%
ENDOMETRIAL CANCER	61%
COLORECTAL CANCER	41% with microsatellite Instability and 17% without Microsatellite instability
BREAST CANCER	38%
OVARIAN CANCER	27%
NON-SMALL CELL LUNG CANCER	24%
PROSTATE CANCER	20%

Table I. Percenteage of reduced expression of PTEN in various type of tumors.

17% of colorectal cancer without microsatellite instability (see table I). As has been seen for mutations, reduced expression was associated with advanced disease. Studies of PTEN expression in breast, brain, tongue, gastric, esophageal, and endometrial cancer also indicated that reduced PTEN protein was associated with a poor prognosis for patients (Depowoski et al. 2001). Examination of leukemia and lymphoma has demonstrated that half of B cell chronic lymphocytic leukemias have reduced PTEN protein and that most acute myeloid leukemias have activated AKT associated with inactivated, hyperphosphorylated PTEN (Leupin et al. 2003) The weight of the evidence indicates that PTEN is a powerful tumor suppressor that is inactivated late in the course of development for most kinds of human cancer.

Many reductions of PTEN protein are not due to genomic mutation and are of undetermined origin. In addition, reduced PTEN protein expression comes in many flavors. PTEN may be absent or merely reduced relative to normal cells; loss of expression can be seen in the cytoplasm, the nucleus or both.

THE ROLE OF PTEN IN FAMILIAL TUMOURS

Germ-line mutations in *PTEN* cause three rare autosomal dominant inherited cancer syndromes with overlapping clinical features: Cowden disease (Liaw et al. 1997;Nelen et al. 1997), Lhermitte–Duclos disease (Liaw et al. 1997), and Bannayan–Zonana syndrome (Nelen et al. 1997). These syndromes are notable for hamartomas, benign tumors in which differentiation is normal, but cells are highly

disorganized.

Cowden disease is characterized by hamartomas in multiple sites, including the skin, thyroid, breast, oral mucosa, and intestine. In addition, about a third of patients will have macrocephaly. Affected females have a 30–50% incidence of breast cancer, and Cowden disease patients have increased risk of thyroid carcinoma ('10% incidence) and meningiomas (Liaw et al. 1997). Lhermitte–Duclos patients have multiple hamartomas, together with macrocephaly, ataxia, and seizures, caused by cerebellar glial tumors. Besides their hamartomas, Bannayan–Zonana patients exhibit macrocephaly, retardation, and unusual pigmentation of the penis (Nelen et al. 1997). Hamartomas from Cowden disease patients exhibit loss of heterozygosity around the *PTEN* locus, indicating that homozygotic loss of PTEN function probably is required for hamartoma formation. Whether the type of mutation in *PTEN* contributes to the distinct features of these three hamartomatous syndromes remains unclear, but other (i.e., modifying) loci probably play the primary role in determining the spectrum of abnormalities evoked by a given mutation. Indeed, recent analyses of mutant mice suggest that genetic background can significantly affect the PTEN-deficient phenotype. However, in Cowden disease patients, the type of *PTEN* mutation may affect the number of affected sites andyor the presence of breast disease (Nelen et al. 1997). These genetic data strongly suggest that PTEN function is required for normal development and that loss of PTEN function contributes to carcinogenesis. Gene transfer and knockout studies have confirmed these ideas.

TESTICULAR TUMOURS

Mice heterozygous for one null *Pten* allele (*Pten*$^{+/-}$) are prone to develop different types of tumors, including teratocarcinomas (Suzuki *et al*., 1998; Di Cristofano *et al*., 1998; Podsypanina *et al*., 1999). Furthermore, the conditional knockout of the *pten* gene in primordial germ cells causes the development of bilateral testicular teratomas, which resulted from impaired mitotic arrest and outgrowth of cells with immature characteristics (Kimura *et al*., 2003). However, the question as to whether *PTEN* is involved in human germ cell tumors has not yet been addressed.

Germ cell tumors of the testis (GCT) are a heterogeneous group of neoplasms seen mainly in young men (ages 20–40 years; Schottenfeld et al. 1982). Over the past several decades, the incidence of GCTs has been steadily increasing in the Western world (Bergstrom et al. 1996). Several risk factors for GCT development have been identified, which include cryptorchidism, spermatogenic or testicular dysgenesis, Klinefelter's syndrome, prior history of a GCT, and a positive family history . Positive family history indicates the involvement of inherited predisposing factors and hence is of importance in identifying novel genes that may play a role in GCT development.

GCTs are classified as seminomatous (SE-GCT) and non-seminomatous (NSE-GCT) tumors, both of which appear to arise from intratubular germ cell neoplasias (ITGCN) (Ulbright, 1998; Chaganti & Houldsworth, 2000). SE-GCTs retain the morphology of spermatogonial GCs and are exquisitely sensitive to treatment by

radiation as well as chemotherapy (Ulbright et al. 1993). NSE-GCTs display embryonal and extra-embryonal differentiation patterns which include primitive zygotic (Embryonal Carcinoma), embryonal-like somatically differentiated (Teratomas), and extra-embryonally differentiated (Choriocarcinomas, Yolk Sac Tumours) phenotypes (see figure 8) Ulbright et al. 1993). They are, as a group, sensitive to chemotherapy, although they are less sensitive to radiation treatment than are SE-GCTs (Bosl et al. 1997). NSE-GCTs usually occur as mixed tumors, with both differentiated and undifferentiated elements (Ulbright et al. 1993). Among tumors with differentiated elements, mature teratomas exhibit the most complete differentiation, often presenting such cell types as cartilage, neural tissue, and mucinous and nonmucinous glands. These tissue elements within a teratoma, however, develop in an unorganized fashion. On occasion, mature cell types in teratoma lesions undergo malignant transformation into neoplastic elements that show histological features characteristic of *de novo* tumors affecting multiple cell lineages (Motzer et al. 1998). GCTs of all types are frequently associated with ITGCN that, often, progresses to invasive cancer (Vos *et al*., 1990; Houldsworth, 1997). In nearly all cases, ITGCN lesions progress to invasive lesions. Both SE- and NSE-GCTs are suggested to arise from cytologically identical ITGCN lesions, indicating a common cell of origin of all GCTs.

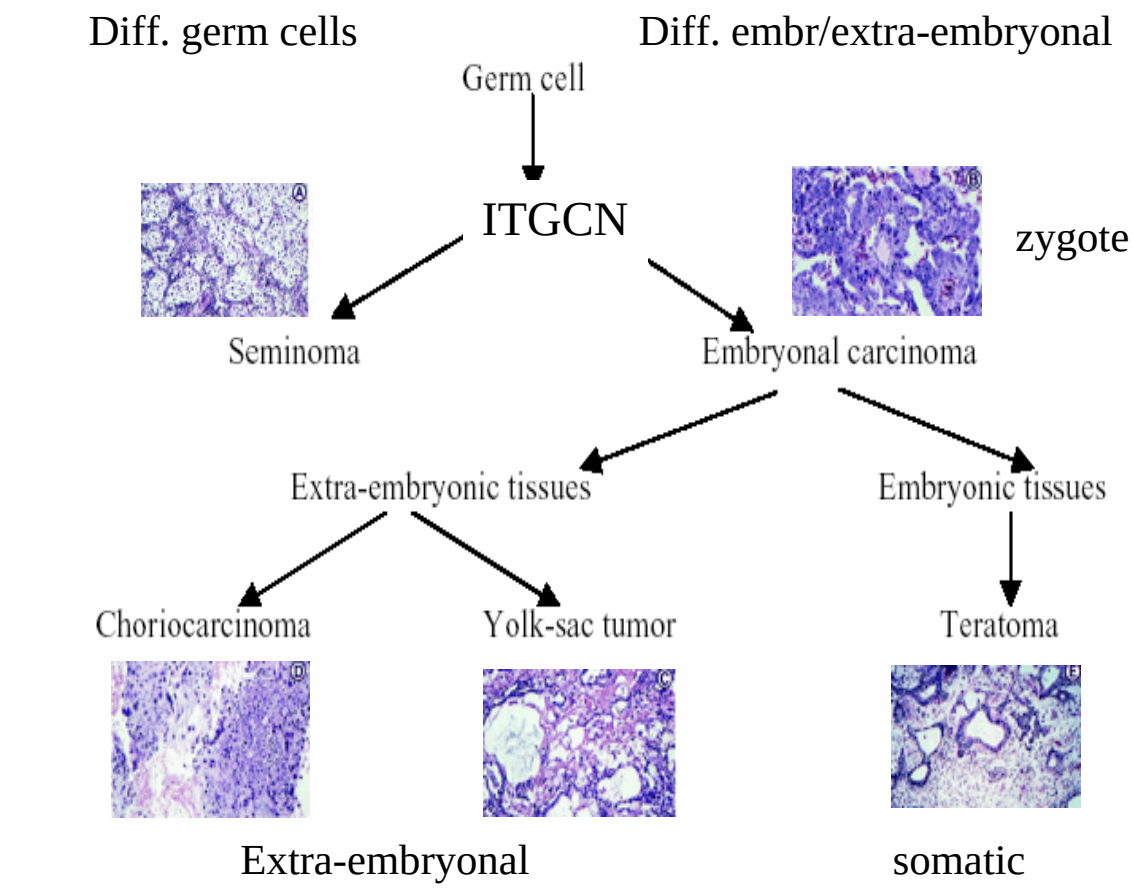


Figure 8. Different morphology of Non Seminomatous Germ Cell Tumors (NSGCT). embryonal and extra-embryonal differentiation patterns which include primitive zygotic (Embryonal Carcinoma), embryonal-like somatically differentiated (Teratomas), and extra-embryonally differentiated (Choriocarcinomas, Yolk Sac Tumours) phenotypes

MATURATION OF PRIMORDIAL GERM CELLS

Primordial germ cells (PGCs) are first recognized in the epiblast of the mammalian gastrulating embryo. They migrate to the primitive streak mesoderm, move on to the endoderm via the allantois, and passing through the hindgut, reach the genital ridges. In the human, they are incorporated in the developing gonad by the seventh or eighth week of fetal life, when they are sometimes termed the gonocytes, which differentiate into spermatogonia during the second and third trimesters of pregnancy (Figure 9). In the postnatal testis, the spermatogonial cells in the seminiferous tubules undergo a series of mitotic divisions leading to the development, successively of type A, intermediate, and type B spermatogonia. The type B spermatogonium undergoes premeiotic replication and enters meiosis as the primary spermatocyte. A protracted prophase comprising the leptotene, zygotene, pachytene, diplotene, and diakinesis stages is followed by mitoses I and II, culminating in four haploid cells that develop into spermatids and spermatozoa. Extensive cell death is a striking feature of spermatogenesis (Matsui et al. 1998). Apoptotic cell death plays an important role during development by regulating the size of a lineage in relation to it’s local environment, survival itself being dependent upon availability of growth factors and their regulatory stimuli (Conlon et al. 1999). In the postnatal murine testis, apoptosis is detected in type A spermatogonia through to meiotic spermatocytes

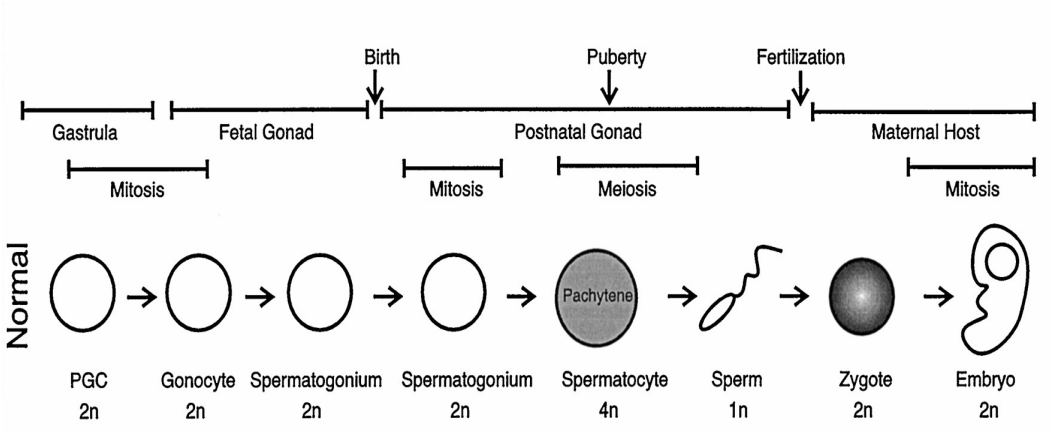


Figure 9. Pathway of development of a normal spermatozoo, from the stage of Primordial Germ Cell (PGC) in the gastrula to mature sperm in adult male. Here is underlined the changing of ploidy following mitosis and meiosis that led from PGC, to mature sperm and finally to the generation of a new embryo.

The spermatogenous cells in the adult human testis similarly undergo apoptosis.

For a GCT to develop, transformation has to occur in a PGC at some point during these highly complex proliferation and differentiation programs regulated by apoptosis. The fact that adult male GCTs display pluripotentiality for embryonal and somatic differentiation suggest that, to initiate a pluripotential tumor, a PGC committed to a differentiation path that leads to gametogenesis must overcome a restriction on proliferation and initiate differentiation cascades normally associated with embryogenesis. The transformed PGC must accomplish this differentiation program without the benefit of reciprocal parental (genetic) contributions from fertilization, which is an obligate prerequisite for normal embryonal differentiation of the totipotential zygote. Therefore, an understanding of the mechanisms of human male GCT development has considerable relevance for the understanding of normal GC development, mechanisms of GC transformation, as well as the regulation of embryonal differentiation pathways in mammals.

MOLECULAR GENETICS OF GCTS

The molecular basis of germ cell malignant transformation is poorly understood. The most common genetic alterations detected in GCT and ITGCN are a triploid/tetraploid chromosomal complement and an increased copy number of 12p, which results in the hyper-expression of the product of the CCND2 gene, i.e.G1 cyclin D2 (Houldsworth *et*

al., 1997). On the other hand, GCTs are often accompanied by hyper-expression of autocrine and/or paracrine growth and angiogenic factors (Viglietto *et al*., 1996; Baldassarre *et al*, 1997).

Although the ITGCN cell is generally regarded as the precursor of all adult male GCTs, the target stage of GC development at which transformation occurs is not known. Two models of origin of ITGCN cells have been put forward. One was proposed by Skakkebaek *et al.* (see figure 10) (Skakkebaek et al. 1997; Skakkebaek et al. 1998). This model suggested that fetal gonocytes, which have escaped normal development into spermatogonia, may undergo abnormal cell division mediated by a kit receptor/SCF paracrine loop, leading to the origin of ITGCN cells. The kit receptor is normally expressed by GCs during the first trimester and postnatally during meiosis, whereas SCF is expressed by the Sertoli cells (Loveland et al. 1997) . Gonocytes derailed in their normal development have been postulated to be susceptible to subsequent invasive growth through the mediation of postnatal and pubertal gonadotrophin stimulation.. A second model proposed by Ulbright and Chaganti (Figure 11) took into account four established genetic properties of GCTs, *i.e.*, increased 12p copy number, expression of cyclin D2 in CIS, consistent near triploid-tetraploid chromosome numbers, and abundant expression of wild-type p53 (Chaganti et al. 1997) . They have postulated that the most likely target cell for transformation during GC development may be one with replicated chromosomes that expresses wild-type p53, harbors DNA breaks, and may be prone to apoptosis. Such a stage is represented by the zygotene-pachytene spermatocyte, at which a "recombination

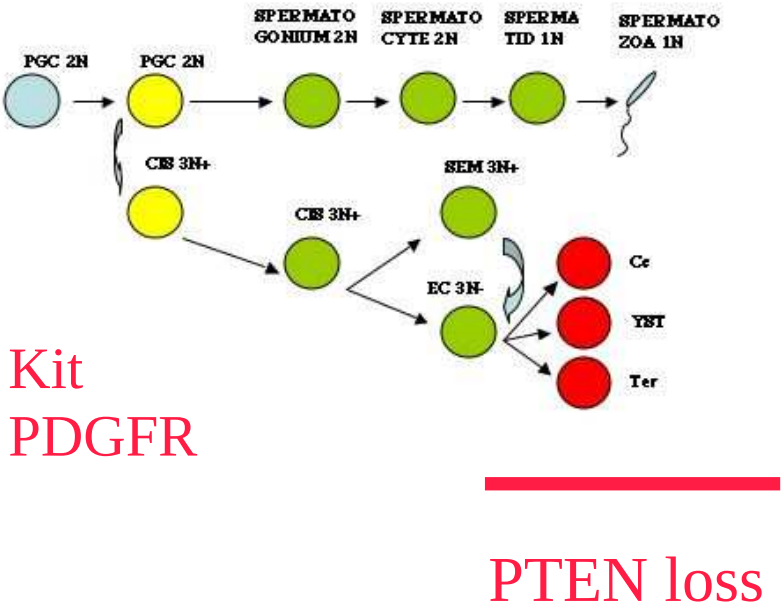


Figure 10. Model of genesis of ITGCN by Skakkebaek. A normal PCG escaped normal development and undergoes abnormal cell division mediated by a kit receptor/SCF paracrine loop, leading to the origin of ITGCN cells . Then, loss of PTEN contributes to enforce neoplastic-phenotype.

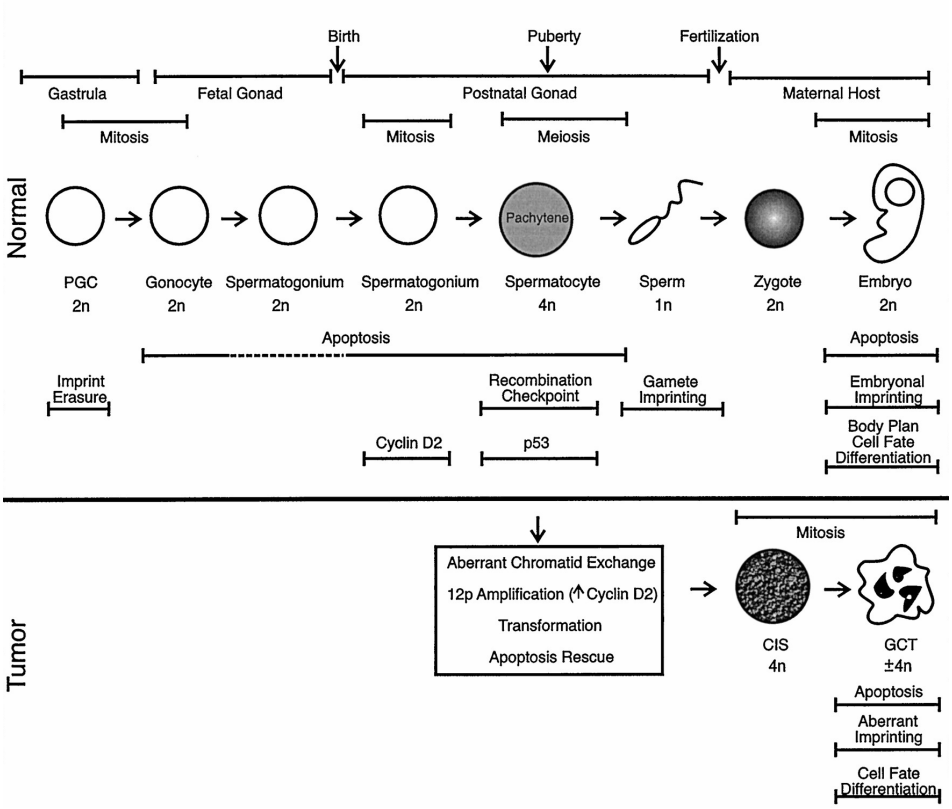


Figure 11. A diagrammatic representation of male GC development during a normal life span and the proposed model of GC transformation. The key genetic events that underlie normal male GC fate and embryonal development are shown with respect to their spatial and temporal relationships. GCT development is depicted in the context of normal GC biology as discussed in the text**.**

checkpoint" appears to operate (Schwartz et al. 1999) , which can provide an apoptotic trigger in the presence of unresolved DNA double-strand breaks. This stage is temporally the longest phase during spermatogenesis with the cell cycle machinery halted to permit recombinational events to complete. It also contains replicated DNA, and based on murine data, wild-type p53 protein is temporally expressed at this stage (Schwartz et al. 1999) . According to this model, aberrant chromatid exchange events associated with crossing-over during zygotene-pachytene may lead to increased 12p copy number and overexpression of cyclin D2. Such a cell may escape recombination checkpoint-associated apoptotic response through the oncogenic effect of cyclin D2, leading to aberrant reinitiation of cell cycle and genomic instability (Chaganti et al. 1997).

AIM OF THE WORK

During my work of thesis, we sought to determine whether the tumor suppressor lipid and protein phosphatase PTEN plays a role in the pathogenesis of germ cell tumors. We investigated: (1) PTEN expression in 60 male germ cell tumors (32 seminomas and 22 embryonal carcinomas and 6 teratomas); (2) PTEN expression in intratubular germ cell neoplasia; and (3) the effects of PTEN re-expression in an embryonal carcinoma cell line.

We have investigated PTEN expression in 60 bioptic specimens of germ cell tumors (32 seminomas, 22 embryonal carcinomas and 6 teratomas) and 22 intratubular germ cell neoplasias adjacent to the tumors for PTEN protein and mRNA expression. Ten testicular biopsies were used as controls. In the testis, PTEN was abundantly expressed in germ cells whereas it was virtually absent from 56% of seminomas as well as from 86% of embryonal carcinomas and virtually all teratomas. On the contrary, intratubular germ cell neoplasias (ITGCN) intensely expressed PTEN, indicating that loss of PTEN expression is not an early event in testicular tumor development. The loss of PTEN expression occurs mainly at the RNA level as determined by in situ hybridization of cellular mRNA (17/22) but also it may involve some kind of post-transcriptional mechanisms in the remaining 25% of cases. Analysis of microsatellites D10S551, D10S541 and D10S1765 in GCTs (n=22) showed LOH at the *PTEN*

locus at 10q23 in at least 36% of GCTs (3 embryonal carcinoma, 3 seminoma, 2 teratoma); two seminomas and one embryonal (13%) carcinoma presented an inactivating mutation in the *PTEN* gene (3/22). Finally, we demonstrated that the phosphatidylinositol 3'-kinase/AKT pathway, which is regulated by the PTEN phosphatase, is crucial in regulating the proliferation of the NT2/D1 embryonal carcinoma cells, and that the cyclin-dependent kinase inhibitor $p27^{kip1}$ is a key downstream target of this pathway.

The findings reported herein indicate that loss of PTEN expression may play a role in the development of testicular germ cell tumors and that the cyclin-dependent kinase inhibitor $p27^{kip1}$ is a key PTEN target in embryonal carcinoma cells.

RESULTS

A. *PTEN* EXPRESSION IN NORMAL TESTIS

Normal germ cell epithelium showed positive cytoplasmic staining for PTEN, as observed in prostatic and endometrial epithelium (Mutter *et al*., 2000; McMenamin, *et al*., 1999). Intense nuclear staining also occurred in several cells, as reported for thyroid and endocrine pancreatic tumor cells (Gimm *et al*., 2000; Perren *et al*., 2000), though the functional meaning of nuclear PTEN staining remains unclear. See Figure 12 for a representative experiment. In normal testis, PTEN expression was heterogeneous: the outer layer of cells (spermatogonia, spg) stained irregularly, with several cells showing positivity for PTEN expression; spermatocytes (spc) and spermatids (spt) also stained positive for PTEN antibodies (Figure12 and 14 A). Endothelial cells and Sertoli cells stained positive for PTEN (Figure 12 and 14A).

B. *PTEN* EXPRESSION IN MOUSE TESTICULAR CELLS.

To better define the cells in which PTEN is expressed in normal testis, immunohistochemical analysis was performed on serial sections of mouse testis using antibody against PTEN protein. PTEN protein was widely expressed in the germinal epithelium (spermatogonia, spermatocytes, spermatids) and Sertoli cells, while it was not detectable in spermatozoa (Fig 13A). The antiserum used in this study fulfils the criteria of specificity. In particular, immunoadsorption tests revealed that the labeling was totally blocked by preincubation of the antibody with 10^{-6} M of the cognate peptide (data not shown).

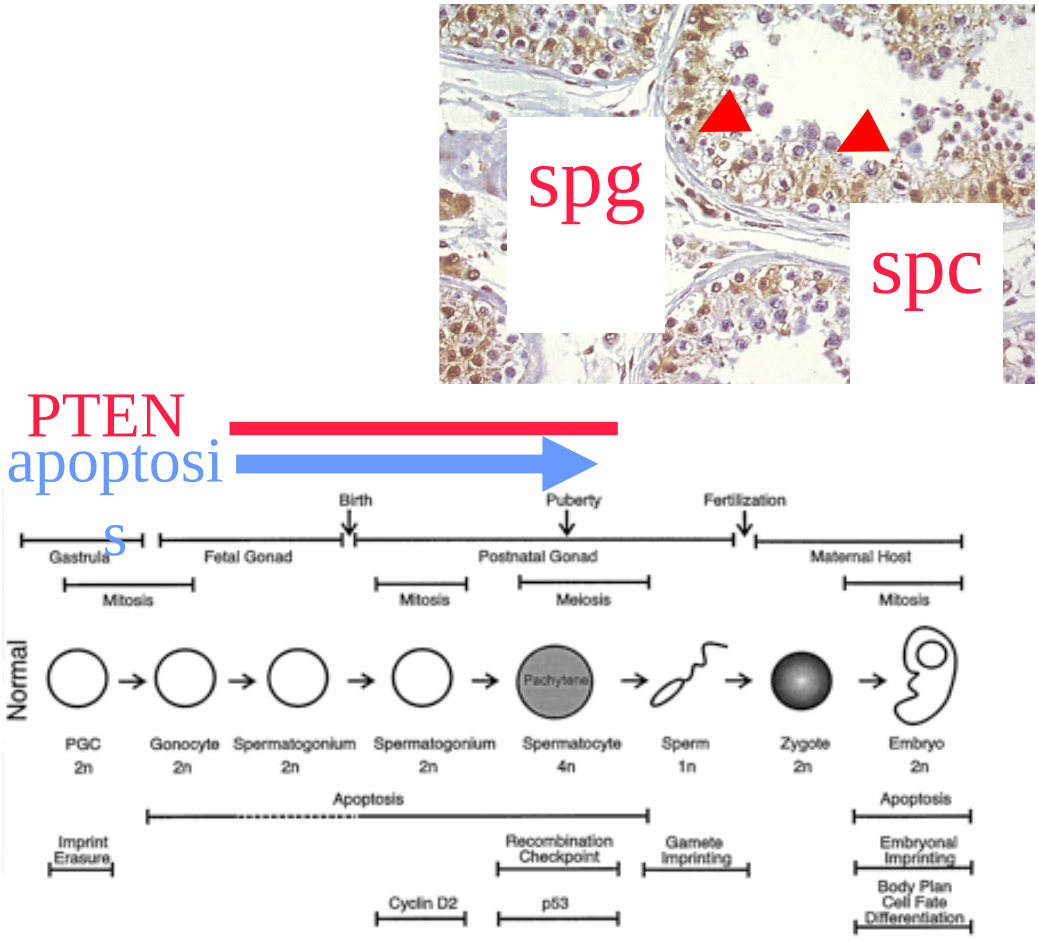


Figure 12. Here is re-proposed the pathway of differentiation that lead from PGC to mature sperm (also seen in figure 9). Added with immunoistochemical staining to underline PTEN expression in spermatogonium (SPG) and spermatocyte (SPC) stage (panel on the right). As red line and blue arrows stress, the expression of PTEN drives apoptotis necessary for correct development and maturation of spermatozoa.

It has been confirmed the differential expression of PTEN in the different cell types in the mouse testis, by Western blot analysis of cell extracts from adult mouse testis fractionated in interstitial, Sertoli, spermatogonia, spermatocytes, spermatids, and spermatozoa. Immunoblot analysis performed on cell types enriched in the different types of germ cells, showed a single product migrating as a 55 kDa protein (Fig. 13B). Among germ cells, PTEN was abundant in spermatogonia, present in spermatocytes and spermatids, absent in spermatozoa in agreement with immunohistochemical results. PTEN protein was also present in the interstitial and Sertoli extract cells.

C. *PTEN* EXPRESSION IN GERM CELL TUMORS

Subsequently we have analysed the expression of PTEN in GCTs. ITGCN was present in 22 tumor samples. In all cases, the neoplastic cells present in ITGCN showed strong PTEN staining. As with normal germ cells, PTEN occurred both in the nuclear and in the cytoplasmic compartment of precancerous cells (Figure 14B). Interestingly, in cases in which tubules with ITGCN were entrapped inside a fully malignant tumor, strong PTEN expression was observed in the cells from ITGCN but not in the adjacent area (Figure 14B): in fact, PTEN staining was weak in the nuclei and cytoplasms of cancer cells. Conversely, endothelial cells showed moderate to strong PTEN expression, and thus served as internal positive controls.

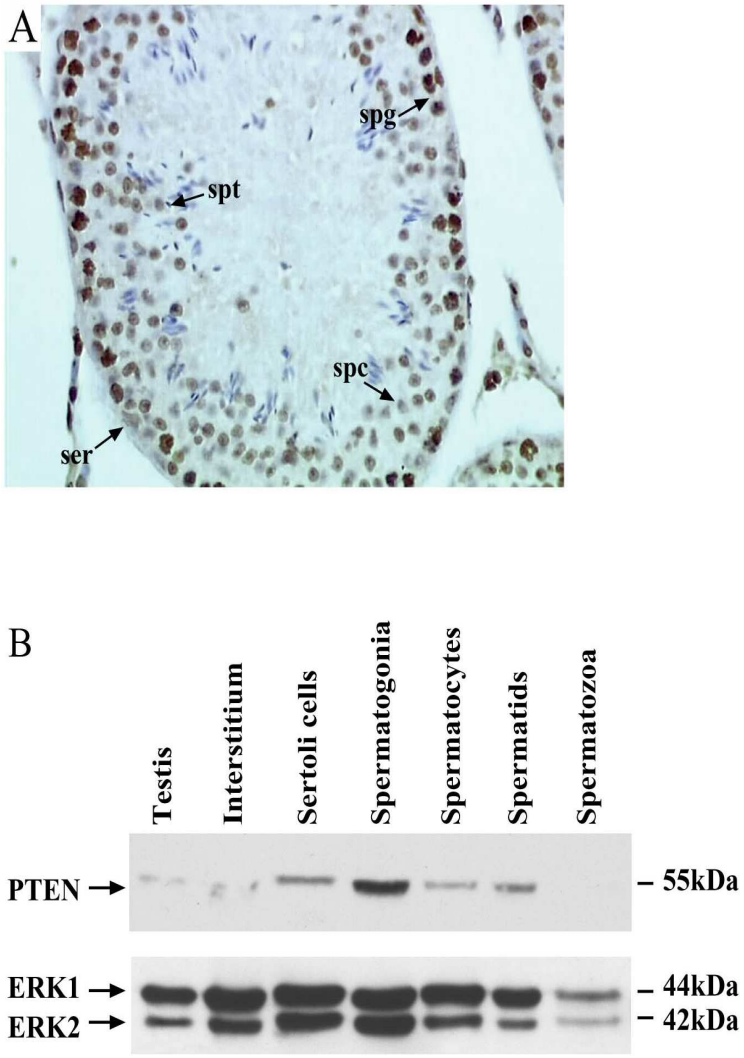


Figure 13. PTEN expression in the mouse testicular cells.
A. Localization of the PTEN protein in sections of adult mouse testis by immunocytochemistry. A representative seminiferous tubule showing staining in spermatogonia (spg), spermatocytes (spc), spermatids (spt), and Sertoli cells (ser) Magnification 400X. **B.** Western blot analysis of PTEN protein in mouse adult testis (lane 1), interstitium (lane 2), Sertoli cells (lane 3), and in normal mouse testis germ cells (lane 4-7) (50g/lane). Whole lysates were detected by anti-PTEN monoclonal serum or with anti-ERK antibodies used as internal standard. ERK antibodies recognize both ERK1 and ERK2, which are expressed at similar levels in all cell types with the exception of spermatozoa (Sette *et al.*, 1999).

PTEN protein expression was reduced in tumors as witnessed by the low signal obtained per single cell and by the decreased number of cells/field stained with the anti-PTEN antibody (Table II). In particular, most embryonal carcinomas (19/22), approximately 60% of seminomas (18/32) and virtually all teratomas (6/6) showed no staining with anti-PTEN antibody. Moreover, the remaining tumors showed weak and focal PTEN staining. A representative immunodetection experiment of PTEN expression is reported in Figure 14, where PTEN-negative seminoma, embryonal carcinoma and teratoma are shown (Figure 14C, D, and E, respectively).

To verify that the monoclonal anti-PTEN antibody (clone A2B1) was suitable for immunostaining experiments (Figure 14 F), serial 5-□ the signal induced by the anti-PTEN antibody, demonstrating the specificity of the reaction. Similar results were obtained with another monoclonal anti-PTEN antibody (#26H9) from Cell Signaling (not shown). Recently, a testis-specific PTEN homologue, denoted PTEN2, has been described (Wu *et al*., 2001). However, since the C-terminal peptides used for the generation of antibodies used in this study, are present in PTEN but absent in PTEN2, it is highly unlikely that the antibodies recognized PTEN2 in immunostaining.

It has been next compared the immunoistochemical PTEN expression with immunoblot data. First the specificity of the monoclonal PTEN antibody to be used in immunoblot experiments was tested. As positive control, it has been used the breast cancer cell line MCF7, which is known to express PTEN, and as negative control it has been used the breast cancer cell line MDA-MB-468 that bears a hemizygous deletion

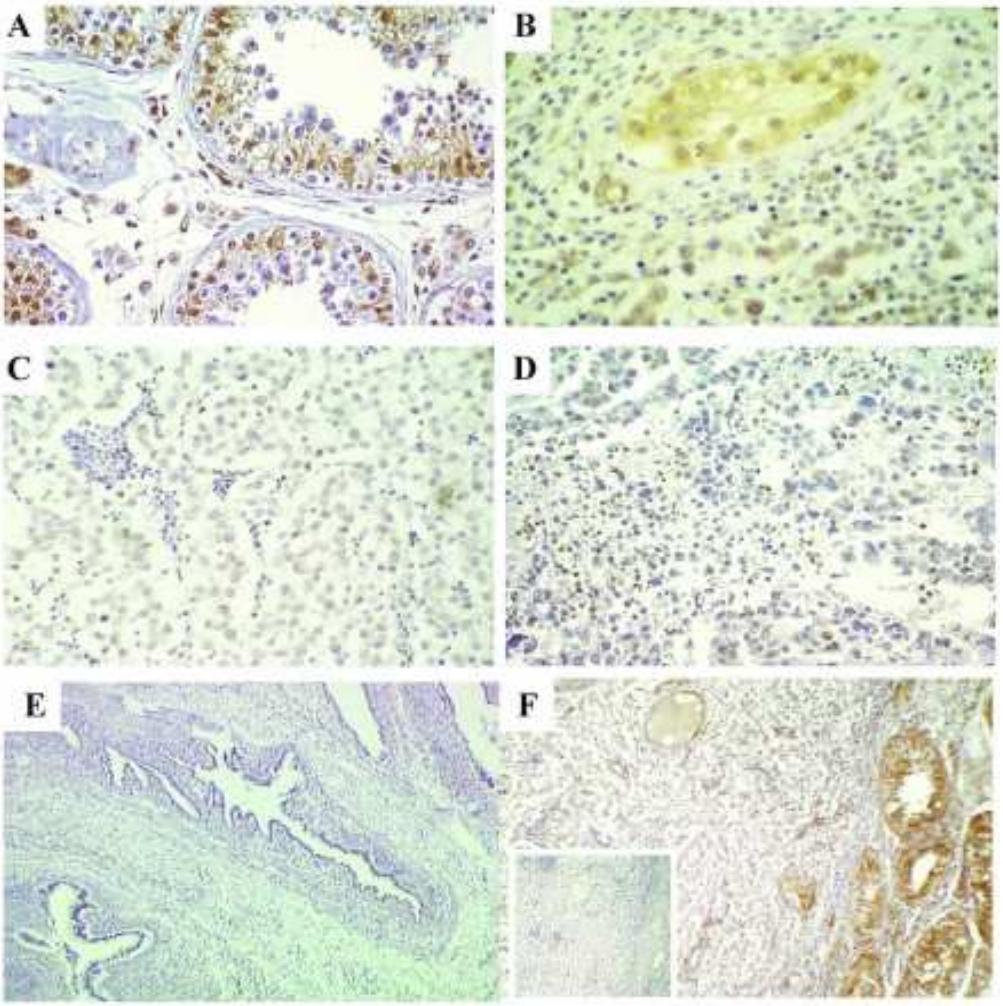


Figure 14. Immunoistochemical analysis of PTEN expression in normal testis, *in situ* neoplasia and germ cell tumors. A. PTEN expression in human normal testis. Magnification 400X . spg, spermatogonia; spc, spermatocytes; spt, spermatids; ser, Sertoli cells. **B.** PTEN expression in intratubular neoplasia. Magnification 400X. **C.** Seminoma with rare focal and faint positivity for PTEN. Magnification 400X **D.** Embryonal carcinoma negative for PTEN staining. Magnification 400X. **E.** Teratoma negative for PTEN staining. Magnification 400X. **F.** Peptide neutralization assay. Serial sections derived from the same biopsy were incubated with monoclonal anti-PTEN antibody with and without (inset) a molar excess peptide antigen. Magnification 150X. sections of the same samples were incubated with anti-PTEN antibody with and without a 10-fold excess of a competing peptide. As shown in the inset of Figure 1F, peptide competition almost completely abolished

No. samples	Diagnosis	PTEN immunohistochemistry
10	Normal testis	+++ (10/10)
22	ITGCN	+++ (22/22)
32	Seminoma	+/- (14/32) - (18/32)
22	Embrional carcinoma	+/- (3/22) - (19/22)
6	Teratoma	- (6/6)

Table II. PTEN expression in germ cell tumors

of PTEN and a truncating mutation in exon 2 of the remaining allele, which results in the loss of PTEN expression (Li *et al*., 1999).

The anti-PTEN antibody recognized a single band of 55-60 kDa only in the MCF-7 cells but not in the MDA-MB-468 cells (Figure 15A).

Then it has been determined PTEN expression in 16 primary germ cell tumors (8 seminomas and 8 embryonal carcinomas) using immunoblotting, selecting them on the basis of PTEN expression, among the samples undergone immunohistochemical analysis. Proteins from 4 non-neoplastic testes served as controls (NT, normal testis). The amount of PTEN protein was high in normal testis (Figure 15B, lane 1) and low in several tumors (6/8 seminomas and 6/8 embryonal carcinomas presented low PTEN expression, respectively). See for an example figure 15 B. A good correlation between the immunostaining and immunoblot data was observed.

Because PTEN activity prevents AKT activation in a variety of human tumors and cell lines (Haas-Kogan *et al*., 1998; Bruni *et al*., 2000), it has been investigated whether the down-regulation of PTEN observed in testicular tumors resulted in AKT activation, measured as increased phosphorylation at specific serine (ser473) and threonine (thr308) residues. To this end, it has been determined the expression and the phosphorylation status of AKT in the same representative set of tumors (Figure 15B). As expected, AKT phosphorylation on Ser473 was higher in some tumors (Figure 15B, lanes 1, 3, 4, 6, 7, 8, 10, 11, 14, 15, and 16) than in normal testis. Ten out of eleven tumors with low PTEN expression had high levels of phosphorylated Akt (Figure 15B, lanes: 1,

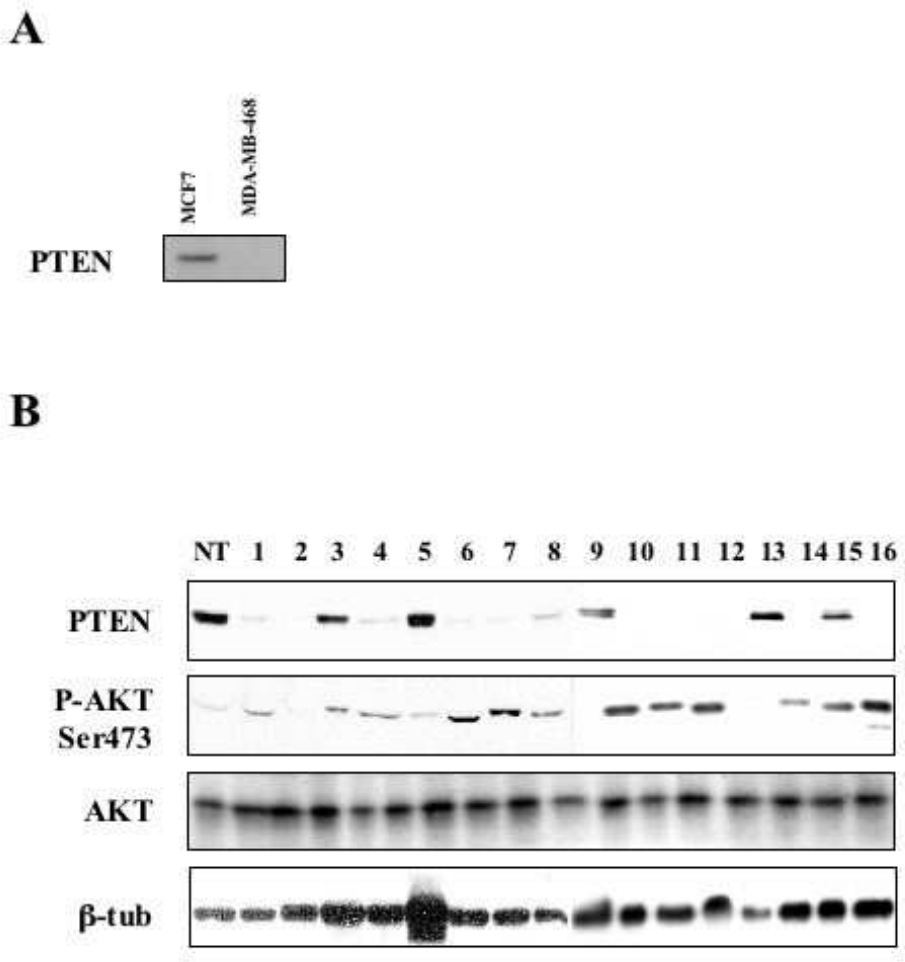


Figure 15. Western blot analysis of PTEN expression in normal and neoplastic testis A. The monoclonal anti-PTEN antibody recognized PTEN protein in MCF7 cells but not in MDA-MB-468 cells used as positive and negative controls, respectively. **B.** PTEN expression and AKT phosphorylation in germ cell. Forty micrograms of total proteins were resolved on 10% SDS-PAGE, transferred to nitrocellulose filters and western blotted with anti-PTEN monoclonal antibody, anti-phospho-Ser473 AKT and anti-total AKT. Antibodies to □-tubulin served as loading control. Lane NT: normal testis; seminomas: lanes 1-4, 9-12 ; embryonal carcinomas: lanes 5-8, 13-16. Films were scanned and the intensity of bands was quantified by the NIH Image 1.57 program.

4, 6, 7, 8, 10, 11, 12, 14, 15 and 16). Anti-β-tubulin antibody was used as a loading control. In general, PTEN expression inversely correlated with the level of phosphorylated Akt. Exceptions were tumor SE2 in lane 2, which showed low Akt phosphorylation in the presence of low PTEN expression and tumor SE3 in lane 3, which showed high Akt phosphorylation in the presence of high levels of PTEN protein. While we do not have any reasonable explanation for tumor SE2, Akt hyperexpression or activating mutations in the PI3K catalytic subunit may account for increased Akt activity in the case of tumor SE3 as recently reported (Samuels *et al*., 2004).

D. ANALYSIS OF PTEN'S mRNA LEVEL IN GERM CELL TUMORS

In order to assess whether loss of PTEN protein, demonstrated by immunohistochemistry and confirmed by immunoblot, was a consequence of reduced mRNA expression, it has been performed mRNA In Situ Hybridization (ISH) on a subgroup of germ cell tumors selected for being negative for the expression of PTEN protein (12 seminomas, 6 embryonal carcinomas, 4 teratomas). Results are reported in Table II. It has been observed a direct correlation between the amount of PTEN protein and PTEN-specific mRNA in 75% of cases. The majority of seminomas (9/12, 75%), embryonal carcinomas (5/6, 83%) and teratomas (3/4, 100%) analysed showed reduced or no mRNA in tumor cells (Fig. 16B and C). An example of seminoma positive for PTEN's mRNA expression is shown in figure 16 D. Staining for PTEN mRNA was observed instead in the samples

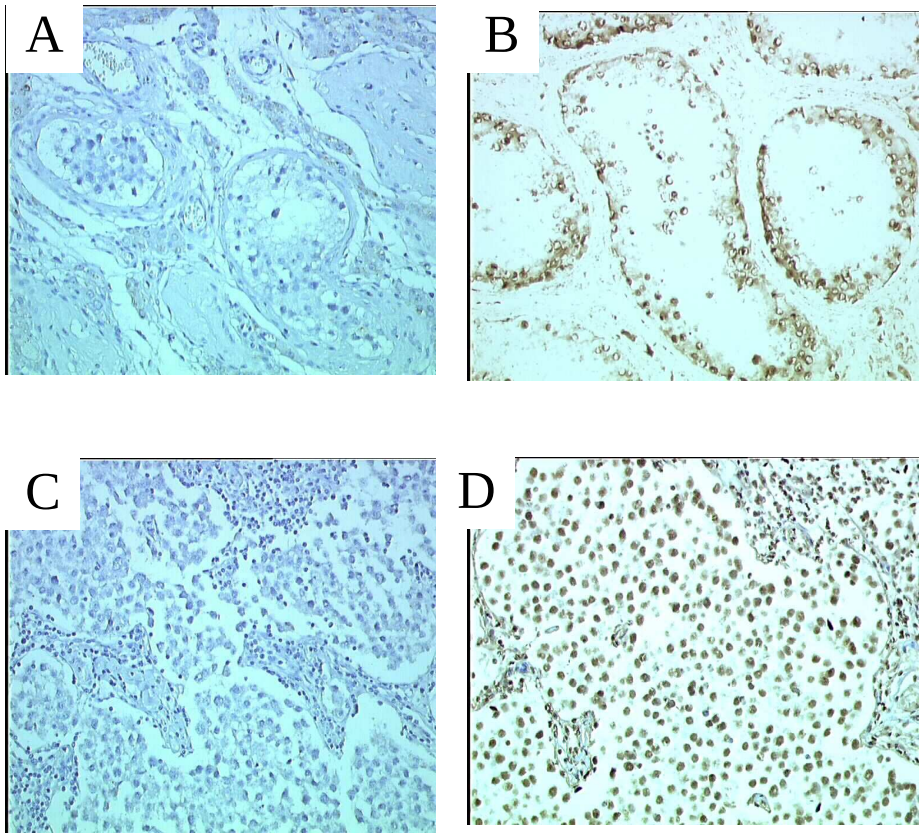


Figure 16. <u>In situ</u> hybridization analysis of PTEN expression in normal testis and germ cell tumors. A. Expression of PTEN mRNA in human normal testis. Magnification 400X . **B.** Expression of PTEN mRNA in a PTEN deficient seminoma. Magnification 400X **C.** Expression of PTEN mRNA in an embryonal carcinoma negative for PTEN staining. Magnification 400X. **D.** Expression of PTEN mRNA in seminoma positive for PTEN staining. Magnification 400X.

positive for PTEN protein (not shown). In all cases, non-neoplastic atrophic tubules, adjacent to the tumor, showed nuclear and cytoplasmic staining of germinal cells, thus functioning as internal control (Fig. 16A). Conversely, 5 out of 22 cases (23%) analysed retained PTEN mRNA despite absence of protein expression (Table III).

E. GENETIC ANALYSIS OF *PTEN* IN GERM CELL TUMORS

PCR-based analysis to determine LOH of markers spanning the PTEN locus was performed on a series of 22 germ cell tumor samples (12 seminomas, 6 embryonal carcinomas and 4 teratomas) and the corresponding adjacent normal tissues. Results are reported in Table III. All samples were analysed for microsatellite markers surrounding the PTEN locus at 10q23 (D10S551, D10S1765 and D10S541). In particular, these markers present a centromere-to-telomere orientation, covering 5 MB of chromosome 10 that includes the PTEN locus. The 5' end of the PTEN gene is approximately 20 Kb downstream D10S1765 and the 3' end 270 Kb upstream of D10S541 (see figure 17). Three samples were non informative (NI) for D10S551 (see Table III), three were non informative for D10S1765 and four for D10S541. Overall, the LOH frequency in germ cell tumors was 41% (9 of 22). Four of 19 informative samples were homozygous for D10S551, 7 samples exhibited apparent LOH for D10S1765 and 3 for D10S541.

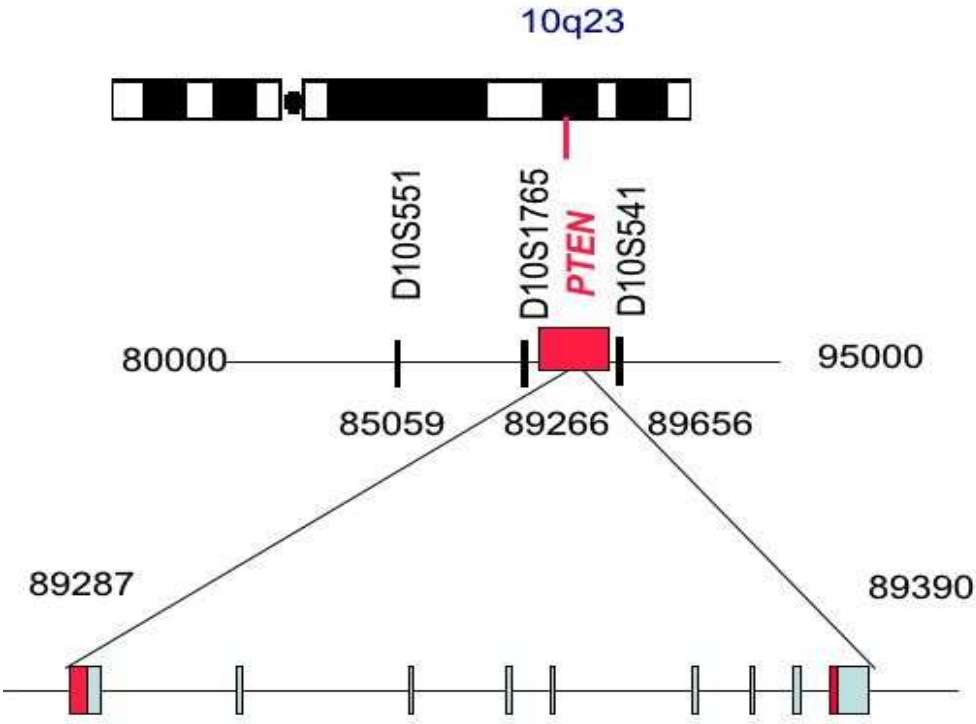


Figure 17. Here it is shown the position of microsatellites analyzed for LOH of PTEN, on chromosome 10. The 5'end of PTEN is about 20kb downstream of DS1765 while the 3'end about 270 kb upstream D10S541

Tumor	D10S551	D10S176	D10S541	PTEN
1		NI	NI	
2				
3	▲	▲	▲	
4		NI		
5	NI			
6	▲			
7	▲			
8		NI		
9				
10	▲	▲		V119G
11		▲	NI	
12	▲			
13	▲			V138Term
14	▲	▲	▲	
15	NI			
16				
17		▲	▲	
18		▲		
19	NI		NI	R233H
20				
21				
22		▲		

Table III. Here they are summarized results concerning the investigation on LOH and point mutations. It has been used the following formula to calculate LOH: (peak height of normal allele 2)/ (peak height of normal allele 1) divided by (peak height of tumor allele 2)/ (peak height of tumor allele 1). LOH at a single locus was considered present when the difference between the two alleles was 50%. NI, not informative

Five samples showed LOH at two loci while the majority of samples showed LOH for just one marker. LOH was slightly more frequent in embryonal carcinomas and teratomas (3 of 6 and 2/4, respectively) than in seminomas (3/12).

Subsequently, we analyzed the same 22 germ cell tumors for the presence of mutations in the coding region of the *PTEN* gene by PCR amplifying all the nine exons of the PTEN gene and subsequent direct automated DNA sequencing of the PCR products. Genomic DNA extracted from paraffin-embedded samples was amplified using intron-specific primers that flanked exons 1-9 as previously described (Bruni *et al.*, 2000). Samples from the corresponding adjacent normal tissues were included as controls. Results are reported in Table III. DNA sequencing of exons 1-9 of *PTEN* gene demonstrated the existence of a pathogenetic mutation in three samples (2 embryonal carcinomas and 1 seminomas): a TAT->TAG transversion at the codon 138 in exon 5 that caused the formation of a premature termination codon (Y_{138}->term) in a patient affected by a seminoma (# 13); a transversion in the codon 119 GTT->GGT that causes V119->G mutation in a patient affected by embrional carcinoma (# 10) (see figure 18); a transition CGC->CAC at the codon 233 in exon 7 that caused a R_{233}->H missense mutation in another patient affected by an embryonal carcinoma (# 19). These mutations likely impair PTEN function: the missense mutation (R_{233}->H) hits a residue that has been reported to be germline mutated in a family affected by Cowden Disease (Liaw *et al*. 1997). On the other

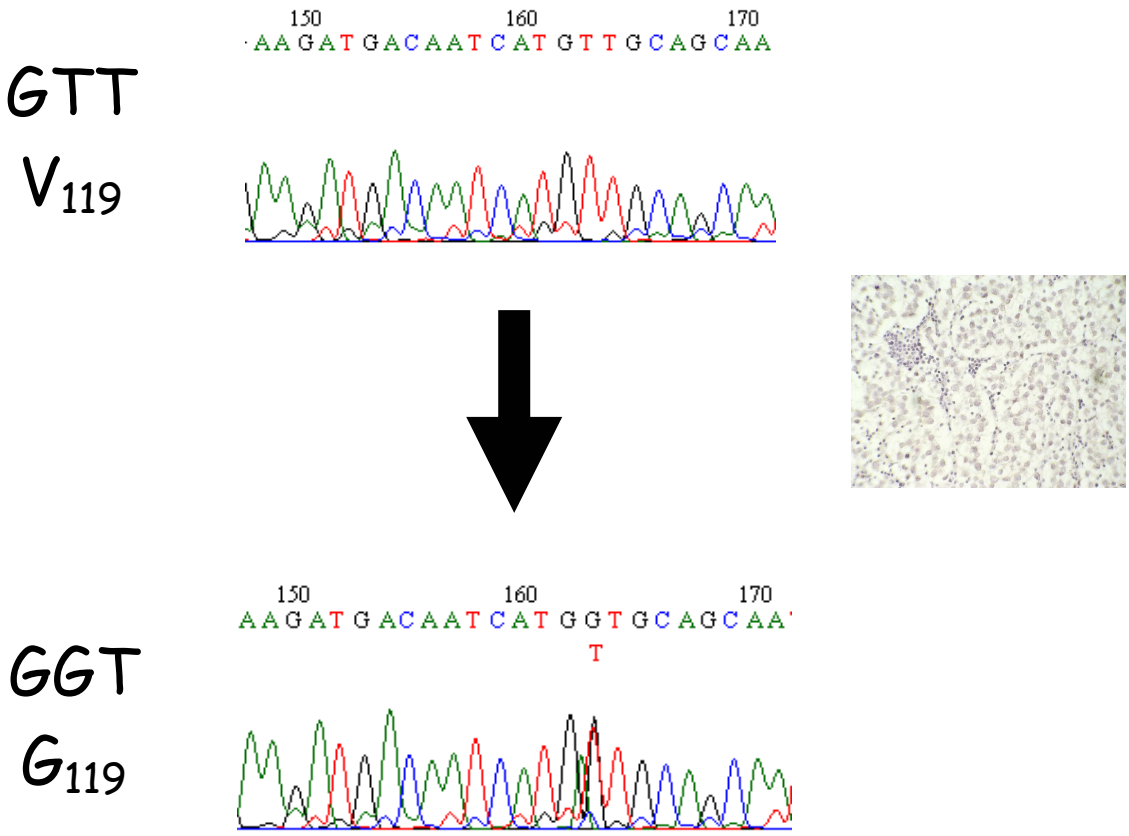


Figure 18. Chromatogram inherent to mutation GTT->GGT which determines the mutation V119G in the 5 exon. In this example of seminoma, PTEN expression is manteined (see panel on the right) but its function is lost.

hand, the mutation Y138term generates a truncated protein whose function is impaired. Accordingly, a mutation that hits the residue 139 has been found in a patient affected by Cowden Disease.

Finally, the other mutation in the 5 exon , probably impair right phosphatase activity of PTEN, since it is located in the catalytic domain. Thus, our analysis demonstrated the presence of somatic mutations (at a frequency of about 9%) in sporadic germ cell tumors.

F. REGULATION OF PTEN EXPRESSION IN EMBRYONAL CARCINOMA CELLS

The finding that post-transcriptional mechanisms are involved in the loss of PTEN expression in at least a quarter of GCTs, made us investigate whether protein degradation was involved in the loss of PTEN expression by using the embryonal carcinoma NT2/D1 cell line as a model system.

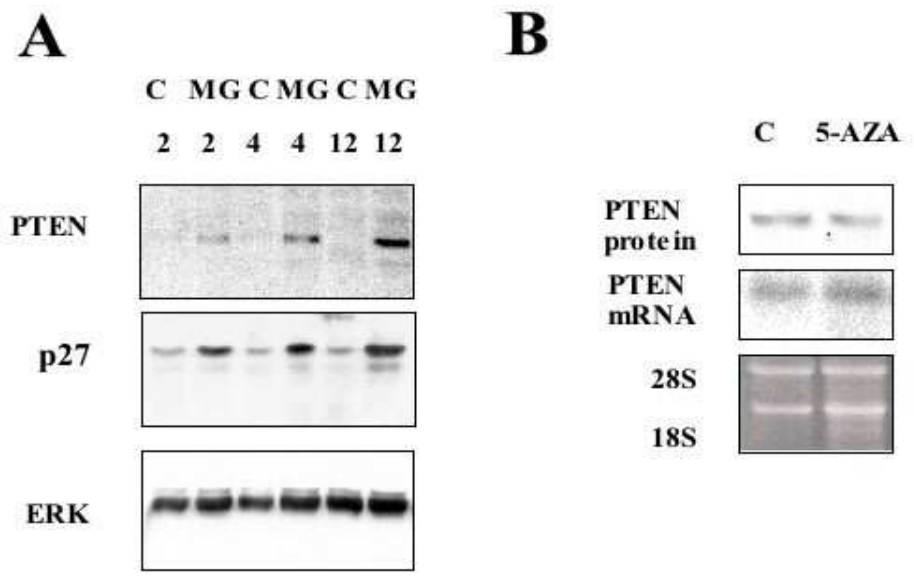
To determine the molecular mechanisms whereby PTEN expression is lost in neoplastic germ cells, we used a well-known model of human embryonal carcinoma cells: the NTERA-2 cell line (NT2/D1) (Andrews, 1984). Though this cell line derives from a late stage lesion (embryonal carcinoma cell line), and does not allow to reproduce the transition from early-lesion (ITGCN) to late lesion (full blown cancer), it still represents a good model because is amenable to manipulation in vitro, allows to study the mechanisms whereby PTEN expression is regulated in EC cells and finally allows to pinpoint the relevant

pathways downstream PTEN.

Treatment of NT2/D1 cells with two highly specific proteasome inhibitors (the peptide aldheyde *N*-acetyl-leucyl-leucine norleucinal or LLnL and the inhibitor MG132) increased the level of PTEN. Treatment of NT2/D1 cells for 2, 8 or 12 hours with 20 □M of MG132 (Figure 19A, lanes MG), or 50 □M of LLnL (not shown) resulted in 2-4.5 fold increase in the level of PTEN expression compared to DMSO-treated cells (Figure 19A, lanes C), suggesting that in embryonal level of PTEN RNA and protein (Figure 19B), suggesting that *PTEN* promoter methylation is not implicated in the down-regulation of *PTEN* expression in NT2/D1 cells.

G. ADOPTIVE EXPRESSION OF PTEN INTO EMBRYONAL CARCINOMA CELLS INDUCES GROWTH ARREST

Subsequently, we used the NT2/D1 cells also as a model system to determine the effects exerted by PTEN in neoplastic germ cells. NT2/D1 cells were plated in 10-mm dishes and transfected with wild type or mutant (C124S, G129E) FLAG-tagged PTEN constructs (FLAG-PTEN, FLAG-PTEN/C124S or FLAG-PTEN/G129E) or with the control empty vector. Forty-eight hours after transfection, cells were collected and analysed by FACS. Enforced PTEN expression in NT2/D1 cells resulted in G1 arrest but not apoptosis at 24-48 hours (Figure 20A). In fact, 50.3% of NT2/D1 cells transfected with wild type FLAG-PTEN were in G_1 phase versus 29.4% of vector-transfected cells. Neither C124S nor G129E PTEN mutants suppressed growth (30% and 30.5% of cells were in the G_1 compartment, respectively).



A.

Figure 19. Regulation of PTEN expression in embryonal carcinoma cells
A. Treatment of NT2/D1 cells with with 20 □M DMSO (C) or MG132 (MG) for the indicated times (2, 8, 12 hours). **B.** Northern blot analysis of PTEN expression in NT2/D1 cells treated with solvent alone or 5 □M 5-azacytidine for 2 days.

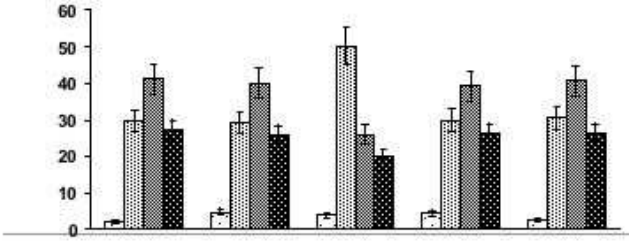
Because the G129E PTEN mutant has lost the lipid phosphatase activity but not the protein phosphatase activity, these findings demonstrate that the growth suppression induced by PTEN in NT2/D1 cells requires the ability to dephosphorylate lipid but not protein substrates. Statistical analysis was performed using the one-way ANOVA with post-hoc multiple comparisons assessed with the 2-tailed Dunnett's t test, and the differences resulted significative ($p<0.05$).

Treatment with pharmacological PI3K inhibitors LY294002 or wortmannin for 24 h decreased the proliferation of breast cancer cell lines (Lu *et al*., 1999). To determine the relevance of the PI3K/PTEN/AKT pathway in embryonal carcinoma cells, we investigated the effects exerted by PI3K inhibitors (LY294002 and wortmannin) on NT2/D1 cells. As with PTEN, treatment of NT2/D1 cells with 20 □M LY294002 (or 25 □M wortmannin, not shown) greatly reduced S phase entry as determined by flow cytometry (Figure 20B) and BrdU incorporation (Figure 12A). Therefore, inhibition of the PI3K pathway induces G1 arrest in NT2/D1 cells.

H. PTEN-DEPENDENT GROWTH ARREST IN EMBRYONAL CARCINOMA CELLS REQUIRES P27^{KIP1}

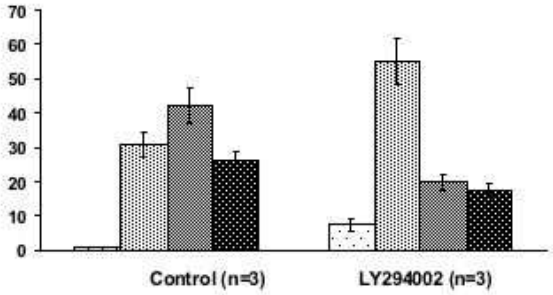
Previously, it has been have demonstrated that p27^{kip1} is a key regulator of the growth and differentiation of NT2/D1 cells (Baldassarre *et al.*,

A



	Control (n=3)	FLAG (n=3)	FLAG-PTEN (n=3)	C124S (n=3)	G129E (n=3)	ANOVA p
<G0/G1	2.0±0.4	4.8±0.9*	3.8±0.7*	4.5±0.8*	2.6±0.5	0.002
G0/G1	29.9±3.0	29.4±2.9	50.3±5.6*	30.0±3.5	30.5±2.9	<0.001
S	41.0±3.9	39.9±4.1	26.1±2.7*	39.1±3.7	40.7±3.9	0.003
G2/M	27.2±3.0	25.9±2.8	19.8±1.7*	26.4±2.8	26.2±3.2	0.047

B



Apoptosis	0.9±0.2	7.4±1.8‡
G0/G1	30.8±3.7	55.3±6.6†
S	42.2±5.1	19.9±2.4‡
G2/M	26.1±2.9	17.4±1.9*

Figure 20. PTEN-induced growth suppression in NT2/D1 cells. A. Flow cytometry of NT2/D1 cells transfected with wild type and mutant PTEN constructs. Values are means ± SD, of 3 experiments. Statistical analysis was performed using the one-way ANOVA with post-hoc multiple comparisons assessed with the 2-tailed Dunnett's t test. * $p<0.05$ vs Control. **B.** Flow cytometry analysis of NT2/D1 cells treated with DMSO alone or with the PI3K inhibitor LY294002. Values are means ± SD, of 3 experiments. Data are mean value SD. Data are mean value SD. Statistical analysis was performed using the unpaired 2-tailed Student's *t* test. *$p<0.05$, †$p<0.01$, ‡$p<0.005$ vs Control.

1999; Baldassarre *et al.*, 2000). The function of $p27^{kip1}$ is regulated by the activity of the PTEN/PI3K/Akt pathway through different strategies. In different cell lines the PTEN/PI3K/Akt pathway regulates both expression and localization of $p27^{kip1}$ (Da-Ming & Hong, 1998; Bruni *et al.*, 2000; Viglietto *et al.*, 2002). Therefore, it has been investigated: (1) whether inhibition of PI3K signalling either by PTEN or by change of localization and (2) whether $p27^{kip1}$ up-regulation was required for the growth-inhibitory effects exerted by blocking the PI3K pathway.

FLAG-PTEN expression in NT2/D1 cells or treatment with the PI3K inhibitor LY294002 reduced AKT phosphorylation (Figure 21A, lanes 2 and 3, and Figure 21B, lanes 2-4, respectively), and induced a two-fold increase in the levels of $p27^{kip1}$ (Figure 21A, lane 2, and Figure 21B, lanes 2 and 3, respectively).

Furthermore, adoptive expression of PTEN and/or treatment of NT2/D1 cells with LY294002 induced cytoplamic re-localization of $p27^{kip1}$ (Figure 22A). The effects exerted by PTEN or by LY294002 on $p27^{kip1}$ were similar, in agreement with the concept that wild type PTEN as well as LY294002 block PI3K-dependent activation of Akt; conversely, the mutant PTEN allele G129E has no effect on the localization and the phosphorylation of $p27^{kip1}$ (see figure 22B).

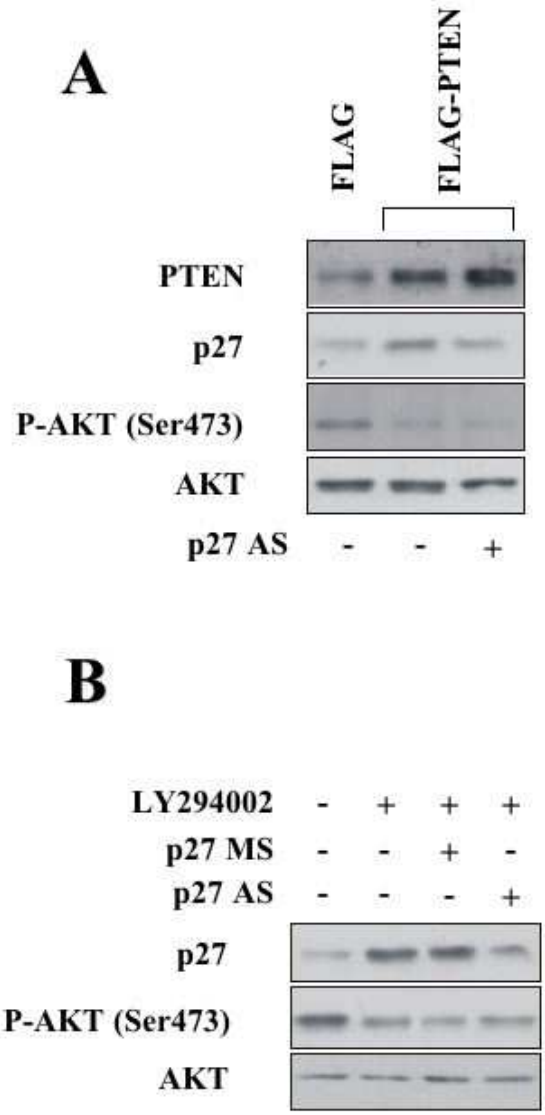


Figure 21. Up-regulation of $p27^{kip1}$ in NT2/D1 cells by blockage of the PI3K pathway. A. Immunoblot analysis of PTEN, AKT and $p27^{kip1}$ expression in NT2/D1 cells transfected with PTEN constructs. Lane 1, FLAG-transfected cells; lane 2, FLAG-PTEN-transfected cells; lane 3, FLAG-PTEN-transfected in the presence of $p27^{kip1}$ antisense oligonucleotides. **B.** Immunoblot analysis of analysis of PTEN, AKT and $p27^{kip1}$ expression in LY290042-treated NT2/D1 cells, in the presence or in the absence of anti-$p27^{kip1}$ antisense oligonucleotides. Lane 1, proliferating NT2/D1 cells; lane 2, LY290042-treated NT2/D1 cells; lane 3, LY290042-treated NT2/D1 cells in the presence of control oligonucleotides; lane 4, LY290042-treated NT2/D1 cells in the presence of $p27^{kip1}$ antisense oligonucleotides.

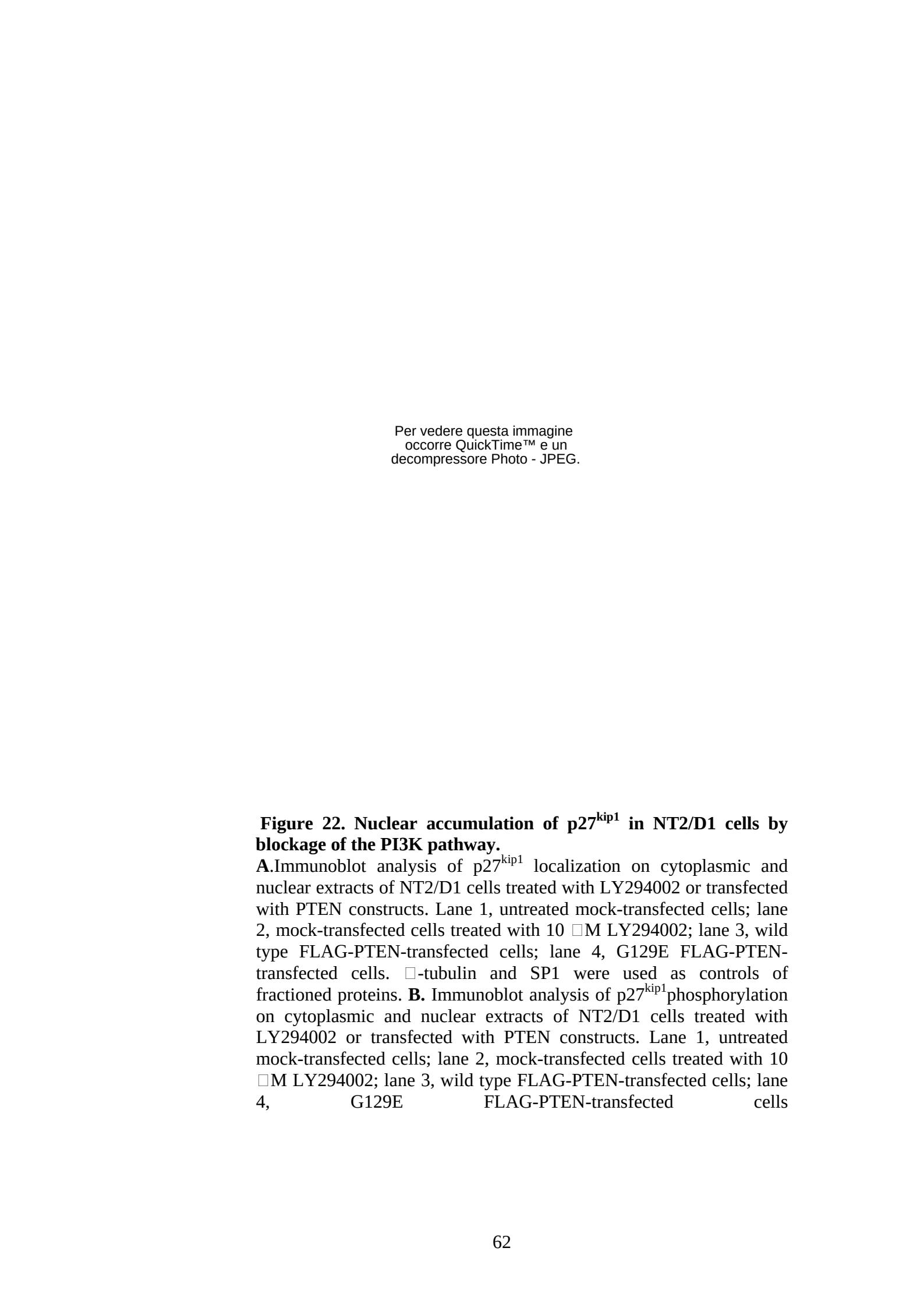


Figure 22. Nuclear accumulation of $p27^{kip1}$ in NT2/D1 cells by blockage of the PI3K pathway.

A.Immunoblot analysis of $p27^{kip1}$ localization on cytoplasmic and nuclear extracts of NT2/D1 cells treated with LY294002 or transfected with PTEN constructs. Lane 1, untreated mock-transfected cells; lane 2, mock-transfected cells treated with 10 □M LY294002; lane 3, wild type FLAG-PTEN-transfected cells; lane 4, G129E FLAG-PTEN-transfected cells. □-tubulin and SP1 were used as controls of fractioned proteins. **B.** Immunoblot analysis of $p27^{kip1}$phosphorylation on cytoplasmic and nuclear extracts of NT2/D1 cells treated with LY294002 or transfected with PTEN constructs. Lane 1, untreated mock-transfected cells; lane 2, mock-transfected cells treated with 10 □M LY294002; lane 3, wild type FLAG-PTEN-transfected cells; lane 4, G129E FLAG-PTEN-transfected cells

To determine whether $p27^{kip1}$ was necessary for the growth arrest induced by wild type PTEN and LY294002 in NT2/D1 cells, we suppressed $p27^{kip1}$ expression by using antisense oligonucleotides, and measured S phase entry by determining the rate of BrdU incorporation. Antisense oligonucleotides (1 □M) spanning the ATG initiation codon of $p27^{kip1}$ efficiently blocked the increase in $p27^{kip1}$ expression induced by PTEN or LY294002 in NT2/D1 cells (Figure 23A, lane 3, and Figure 23B, lane 4) and almost completely rescued the growth arrest induced by PTEN or by PI3K inhibitors (Figure 23A and B, respectively).

Transfected cells were identified by cotransfection of relevant plasmids with pEGFP, a plasmid encoding the eukaryotic green autofluorescent protein (EGFP). pFLAG-transfected cells incorporated BrdU (yellow arrows); whereas FLAG-PTEN-transfected cells did not (white arrows).However, when NT2/D1 cells were transfected with FLAG-PTEN inthe presence of 1 □M of $p27^{kip1}$ antisense oligonucleotides, PTEN-transfected cells incorporated BrdU (yellow arrow). Analogous results were obtained when cells were treated with LY294002. Taken together, these results indicate that the cyclin-dependent inhibitor $p27^{kip1}$ is required for PTEN growth-suppressing activity in embryonal carcinoma cells and that this effect is mediated by AKT.

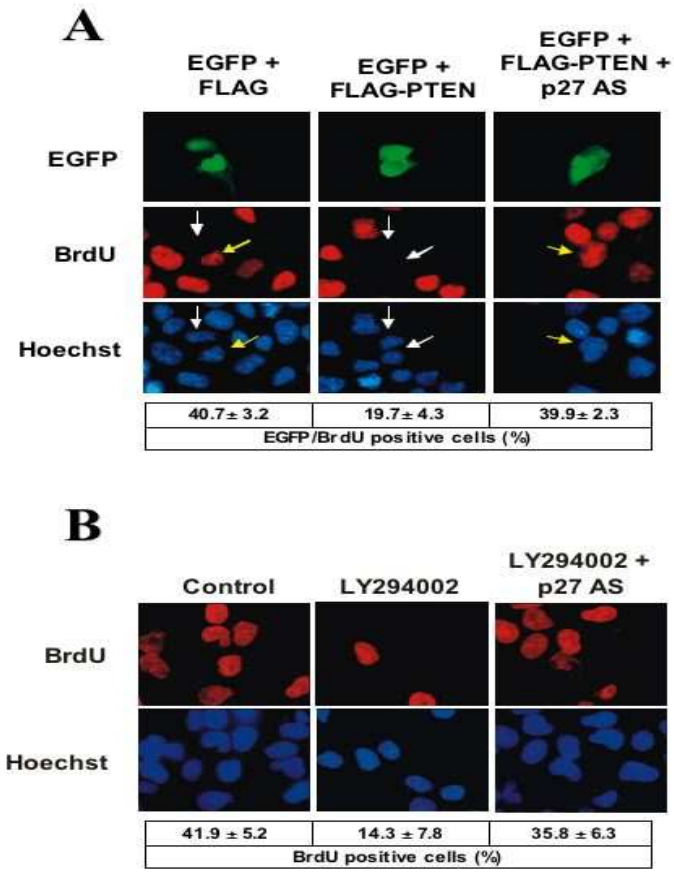


Figure 23. PTEN exerts its growth suppression activity through $p27^{kip1}$ A. BrdU incorporation assay of NT2/D1 cells transfected with PTEN constructs in presence or absence of $p27^{kip1}$ antisense oligonucleotides. First column: green FLAG-transfected cells incorporate BrdU (yellow arrow). Second column: green FLAG-PTEN-expressing cells do not incorporate BrdU (white arrows). Third column: green FLAG-PTEN-transfected NT2/D1 cells incorporate BrdU in the presence of $p27^{kip1}$ antisense oligonucleotides (yellow arrow). A 100X Neo-Achromat Zeiss lens was used. Data are mean ± SD, of 3 experiments; $p<0.01$. **B.** BrdU incorporation assay of NT2/D1 cells treated with DMSO alone (column 1), LY294002 (column 2) or with LY294002 in the presence of excess of $p27^{kip1}$ antisense oligonucleotides (column 3). First row: transfected cells are identified by green fluorescence of EGFP; second row: cells that incorporate BrdU are stained with Texas Red –conjugated secondary antibodies (red); third row: cell nuclei stained with Hoechst (blue). A 100X Neo-Achromat Zeiss lens was used. Data are mean ± SD, of 3 experiments; $p<0.01$.

DISCUSSION

Inactivation of the tumor suppressor gene *PTEN* leads to the development of testicular germ cell cancer in heterozygous mice (Suzuki *et al*., 1998; Di Cristofano *et al*., 1998; Podsypanina *et al*., 1999; Kimura *et al*., 2003). In this study we address whether PTEN is also implicated in the development of human GCTs (seminomas, embryonal carcinomas and teratomas). Our results clearly demonstrate that the loss of PTEN expression marks the transition from non-invasive ITGCN to invasive cancer, being PTEN expression retained in ITGCN, the presumed precursor lesion of germ cell tumors, and lost in tumors. Since ITGCN frequently progresses to invasive cancer, the findings reported in this study, suggest that PTEN loss is required at later stages of cancer development to facilitate the emergence of a more aggressive phenotype.

This conclusion is in agreement with the concept that PTEN may be inactivated at different stages of tumor development (initiation and/or progression) in different tissues, and thus serves for different purposes depending on cell type (Iqbal, 2000): In endometrial cancer PTEN expression/activity is already absent in early, precancerous lesions (complex atypical hyperplasia) (Mutter *et al*., 2000); conversely, PTEN loss is associated with a high Gleason score in prostate cancer (McMenamin *et al*., 1999); with advanced pathological stage in high-

grade glioblastomas (Wang *et al*., 1997; Rasheed *et al*., 1997), and late stage disease (metastatic) in melanomas (Zhou *et al*., 1998).

In situ hybridization analysis of GCT samples which had lost PTEN protein supported the hypothesis that loss of PTEN protein reflect the reduction of PTEN mRNA levels. Moreover, the genetic analysis of GCTs performed in this study with microsatellites spanning about 5 Mb around the *PTEN* locus at 10q23 (D10S551, D10S1765 and D10S541), clearly implicated PTEN loss in the development of a subset of GCTs (approximatively 35%). Consistent with the idea that PTEN is the major target of deletion at 10q23 in GCTs, LOH was most frequent for D10S1765, which is closest to *PTEN* (Table III). In GCTs, loss of genetic material associated with chromosome 10q23 is observed in seminomas, embryonal carcinomas and teratomas. Moreover, DNA sequence analysis of exons 1-9 of the *PTEN* gene uncovered the presence of mutations in the *PTEN* gene in three cases (13%); in all cases, the inactivating mutation was found in a sample that retained a certain degree of PTEN expression (#10, #13, #19) and was not accompanied by LOH. Overall, our results demonstrate that one copy of PTEN is lost in 50% of GCTs. These results are consistent with previous studies on the cytogenetic profile of human tumors, that have shown a range of 10-15% loss of chromosome 10q in GCTs (Mertens *et al*., 1997), and with the report of 60% LOH and 33% mutations in cultured testicular cancer cell lines (Teng *et al*., 1997). The observation that about 25% of GCTs retain PTEN mRNA expression despite decreased PTEN protein levels, along with the finding that, in NT2/D1 cells, PTEN expression is up-regulated by pharmacological inhibition

of the proteasome, indicate that increased turnover pf PTEN protein may account for the loss of PTEN expression in an additional 25% of GCTs.

These results are in agreement with the recent finding that the regulation of PTEN expression may occur through the control of the stability of the protein (Torres J, & Pulido R, 2001; Wu *et al*., 2003; Okahara *et al*., 2004). However, we can not rule out the existence of other mechanisms, such as promoter methylation, that contribute to inactivate PTEN gene in GCTs, especially in those cases that did not apparently show LOH, mutations or retainment of *PTEN* mRNA.

Previous works have failed to detect the presence of PTEN protein in the seminiferous tubule of the 17-day embryo in the human (Gimm *et al*., 2000) and PTEN mRNA in the mouse embryo (Lukko K *et al*., 1999). However, PTEN mRNA is easily detected by Northern blot in the whole testis (Suzuki *et al*., 1998) and by immunoblot and immunostaining in maturating germ cells in adult testis (this work). Furthermore, targeted inactivation of *Pten* in mouse predisposes for development of teratocarcinomas and teratomas (Suzuki *et al*., 1998; Di Cristofano *et al*., 1998; Podsypanina *et al*., 1999; Kimura *et al*., 2001).

In the testis, germ cells undergo a complex program of proliferation and differentiation to form mature sperms (Chaganti & Houldsworth, 2000). Correct proliferation and apoptosis is required to regulate the size of cell lineages and the timing of differentiation (Matsui, 1998; Chaganti & Houldsworth, 2000). Therefore, the loss of PTEN expression observed in GCTs may serve multiple purposes in germ cell

transformation. The window in which PTEN is expressed in mouse and human testis, as observed in this work (spermatogonia -> spermatocytes -> spermatids), overlaps the timing of massive apoptosis that occurs during maturation of germ cells in the seminiferous tubule after birth (Chaganti & Houldsworth, 2000). By preventing apoptosis-driven germ cell selection and maturation, PTEN loss would allow clones of germ cells to elude programmed cell death and undergo malignant transformation. In agreement with this thought, the targeted disruption of Akt1 in the mouse, as well as the "knockin" mice of Stem Cell Factor/Kit receptor mutated in the docking site for the regulatory subunit of the PI3K, attenuates spermatogenesis and induces testicular atrophy, due to increased apoptosis restricted to the germ cell compartment (Chen *et al*., 2001).

PTEN loss may also result in unrestrained cell cycle progression and prevention of terminal differentiation. Accordingly, a recent paper has suggested that estrogen-mediated PTEN down-regulation markedly increases the growth of primordial germ cells in culture and that PTEN-deficient germ cells are much more sensitive to tumorigenic transformation induced by proliferative stimuli (Moe-Behrens *et al*., 2003). Indeed, primordial germ cells from *pten*-/- mice exhibit an increased proliferative capacity (Kimura *et al*., 2003).

The serine/threonine kinase PKB/Akt is an important cellular target downstream PTEN that transmits proliferative and anti-apoptotic signals (Datta *et al*., 1999). Accordingly, the loss of PTEN in GCTs inversely correlated with Akt activation. Moreover, the adoptive expression of PTEN in embryonal carcinoma NT2/D1 cells and

pharmacological inhibition of the PI3K pathway induced a reduction in the level of Akt activation.

Blockage of the PI3K/PTEN/Akt pathway arrests the growth of embryonal carcinoma cells. PTEN-transfected or LY294002-treated NT2/D1 cells accumulate in G1 phase but show no sign of apoptosis, at least after 24-48 h. Defective regulation of cell cycle progression in PTEN-deficient germ cells may depend either on increased expression of cyclins or on decreased expression of CDK inhibitors. In fact, Akt increases the stability of cyclin D1 by suppressing glycogen synthase kinase-3 (GSK-3) activity, which targets cyclin D1 to phosphorylation-mediated degradation (Diehl *et al*., 1998). As the threonine residue phosphorylated by GSK-3 is highly conserved in all D-type cyclins, it is likely that Akt regulates also the levels of cyclin D2 and D3. Thus, the loss of PTEN function may contribute to the overexpression of cyclin D2 frequently observed in germ cell tumors (Chaganti & Houldsworth, 2000).

On the other hand, the cyclin-dependent kinase inhibitor $p27^{kip1}$ is a key target downstream the PI3K/Akt signalling pathway (Bruni *et al*., 2000). Also in embryonal carcinoma cells the effects exerted by inhibition of the PI3K/Akt pathway on cell cycle progression are dependent on $p27^{kip1}$. In fact, our results demonstrate that the adoptive expression of PTEN and the pharmacological inhibition of PI3K activity with LY294002 moderately up-regulates $p27^{kip1}$ in NT2/D1 cells and that suppression of $p27^{kip1}$ synthesis by antisense oligonucleotides prevents growth arrest induced either by PTEN or by LY294002. It is noteworthy that the PTEN mutant, which lacks lipid

phosphatase activity but retains protein phosphatase activity (i.e. G129E) neither induces $p27^{kip1}$ expression nor blocks S phase entry.

The PI3K pathway also regulates sub-cellular localization of $p27^{kip1}$ through Akt-dependent phosphorylation of $p27^{kip1}$ (Viglietto *et al*., 2002). Accordingly, PTEN-dependent inactivation of Akt in NT2/D1 cells results in the accumulation of $p27^{kip1}$ in the nuclear compartment. This suggests that regulation of $p27^{kip1}$ localization may contribute, along with regulation of $p27^{kip1}$ expression, to the proliferative arrest induced either by PTEN or by LY294002 in NT2/D1 cells.

In conclusion, inactivation of PTEN is a critical step in the progression of germ cell cancer, and the cyclin-dependent kinase inhibitor $p27^{kip1}$ is a key target of PTEN signalling pathway. Further studies are necessary to identify the molecular targets that act downstream PTEN in the transformation of the germ cell.

MATERIALS AND METHODS

Preparation of mouse testicular cells

Testicular cells were prepared from testes of adult CD1 mice (Charles River Italia). Testes were freed from the albuginea membrane, and digested for 15 min in 0.25% (w/v) collagenase (type IX, Sigma) at room temperature under constant shaking. Seminiferous tubules were cut into pieces, with a sterile blade and further digested in minimum essential medium containing 1 mg/ml trypsin for 30 min at 30°C. Digestion was stopped by adding 10% fetal calf serum; released germ cells were collected after sedimentation (10 min at room temperature) of tissue debris. Germ cells were centrifuged for 13 min at 1,500 rpm at

4°C and the pellet resuspended in 20 ml of elutriation medium (120.1 mM NaCl, 4.8 mM KCl, 25.2 mM $NaHCO_3$, 1.2 mM MgS_4 ($7H_2O$), 1.3 mM $CaCl_2$, 11 mM glucose, 1X essential amino acid (Life Technologies, Inc.), penicillin, streptomycin, 0.5% bovine serum albumin. Pachytene spermatocyte and spermatid germ cells were obtained by elutriation of the unfractionated single cell suspension as described elsewhere (Meistrich, 1977). Homogeneity of cell populations ranged between 80 and 85% (pachytene spermatocytes) and 95% (spermatids), was routinely monitored morphologically. Mature spermatozoa were obtained from the cauda of the epididymus of mature mice as described previously (Sette *et al*., 1997). Spermatogonia and Sertoli cells were obtained from prepuberal mice as previously described (Rossi *et al*., 1993; Grimaldi *et al*., 1993).

Cell lines and Reagents

The embryonal carcinoma NT2/D1 and the breast MCF7 and MDA-MB-468 tumor cell lines that have been used in this study are described elsewhere (Lu *et al*., 1999; Andrews, 1984). Cells were grown in Dulbecco's modified Eagle's Medium (DMEM) containing 10% foetal calf serum (FCS) (Invitrogen). MG132 and 5-aza-cytidine were from Sigma-Aldrich (St. Louis, MO, USA).

Tissue samples and Immunohistochemistry

Paraffin-embedded specimens were obtained from the di Scienze Biomorfologiche e Funzionali, Università Federico II (Naples, Italy). For PTEN detection, sections were dewaxed and incubated with

primary antibody for 1 h at room temperature. The conventional avidin-biotin complex procedure was used according to manufacturer's protocol (LSAB Plus DAKO, Carpinteria, CA, USA). Monoclonal anti-PTEN antibodies were purchased from Santa Cruz Biotechnology Inc. (clone A2B1) and from Cell Signaling (#26H9). Positive signal was revealed by DAB chromogen, according to the supplier's conditions. Nuclei were counterstained with Mayer hematoxylin. For peptide neutralization control, the reaction with anti-PTEN antibody was preceded by overnight incubation with a ten-fold excess of the corresponding peptide antigen (Santa Cruz Biotechnology, Inc.).

In Situ Hybridization

In situ hybridization was performed using biotin-labelled probes at 5'OH, which were obtained tailing reaction using biotin-dUTP as marker. Hybrid detection was achieved by amplification using biotinylated tyramide (Gen Point K620 Kit, DAKO, Carpinteria, CA, USA). Sections were prepared from each sample and assayed according to the instructions of the Dako Gen Point K620 Kit. Briefly, sections were deparaffinized, re-hydrated, treated with proteinase K (6g/ml) in a buffer of Tris-HCl 0.05M, pH7.6, and then incubated in 0.3% H_2O_2, at RT, for 20 minutes, to quench endogenous peroxidase. Optimal hybridization and stringent wash temperatures were determined and slides were rinsed in the stringent solution provided with the kit. Amplified detection was performed using an anti-digoxigenin antibody coupled to a peroxidase (HRP) which precipitated biotinylated tyramide. The precipitated biotin bound to streptavidin-linked HRP,

which in turn precipitated the dimethylaminobenzidine (DAB) chromogen provided with the kit. Nuclei were counterstained with Mayer's hematoxylin. Negative controls were obtained using an anti-sense probe.

Protein extraction and immunoblotting

Total proteins were prepared as described (Baldassarre *et al*., 1999a). Differential extraction of nuclear or cytoplasmic proteins was obtained by lysing cells in ice-cold Nonidet-P40 (NP-40) lysis buffer (0.2% NP-40, 10 mM Hepes pH 7.9, 1 mM EDTA, 60 mM KCl) supplemented with protease and phosphatase inhibitors (aprotinine, leupeptine, PMSF, and okadaic acid) and incubated on ice for 5 min. The cytosolic fraction was collected by centrifugation. Nuclei were separated through a 30% sucrose cushion and lysed by resuspension in ice-cold hypertonic buffer (250 mM Tris-HCl pH 7.8, 60 mM HCl supplemented with phosphatase and protease inhibitors) followed by repeated cycles of rapid freeze and thaw. Proteins were separated by electrophoresis in SDS-containing polyacrylamide gels, transferred to nitrocellulose membranes (Hybond C, Amersham Pharmacia Biotech, Inc.), blocked in 5% non-fat dry milk, incubated with primary and secondary antibodies for 2 hours and 1 hour, respectively, and revealed by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Inc.). Polyclonal antibodies to phospho-AKT-Ser473 and AKT were purchased from New England Biolabs (Lake Placid, NY); monoclonal anti-$p27^{kip1}$ and anti-ß-Tubulin were acquired, respectively, from Transduction Laboratories and NeoMarkers. Two PTEN antibodies

were used in this study both for immunoblot and immunostaining: a monoclonal antibody elicited to a C-terminal peptide (clone A2B1) from Santa Cruz inc. and a monoclonal antibody elicited to a C-terminal peptide (PTEN 26H9) from Cell Signaling. The anti phospho-Akt motif antibody was from Cell Signaling (#9611).

Vectors and Transfections

The PTEN constructs are described elsewhere (Bruni *et al*., 2000). Transfection experiments were performed as described (Baldassarre *et al*., 1999). NT2/D1 cells were seeded at a density of $2x10^6$ cells per 100-mm dish. The next day, cells were transiently transfected by the lipofectamine 2000 procedure (Invitrogen). Forty-eight hours post-transfection, cells were scraped into ice-cold PBS and lysed in NP-40 lysis buffer. Where needed, the $p27^{kip1}$ antisense oligonucleotides (5'–GTCTCTCGCACGTTTGACAT -3') were used at a concentration of 1 M.

DNA preparation and mutation analysis by direct DNA sequencing

Paraffin-embedded germ cell tumors and the corresponding adjacent normal tissue samples were selected from the pathology files of Dipartimento di Anatomia Patologica, Università Federico II (Naples, Italy). Genomic DNA from 22 testes (normal or cancer tissues) was isolated with a High Pure polymerase chain reaction (PCR) Template Preparation Kit (Roche Molecular Biochemicals, Mannheim, Germany and the *PTEN* mutation status was determined. Briefly, DNA from tumor samples and from the corresponding normal tissues was

extracted from 3-4 8-□ thick serial sections and subjected to PCR amplification for exons 1-9 as previously described (Scala *et al*., 1998; Bruni *et al*., 2000). PCR amplification of each single *PTEN* exon was performed by use of intronic primers designed at the 5' and 3' ends of each exon, followed by reamplification with nested primer pairs. Primer sequences for PCR amplification of each *PTEN* exon were previously reported (Steck *et al*, 97). Amplified DNA was purified using Microspin S300HR Columns (Pharmacia Biotech) and sequenced using the Big Dye Terminator cycle sequencing kit (ABI PRISM, Applied Biosystems, CA) and the ABI 3100 PRISM DNA sequencer (Applied Biosystems).

LOH analysis at the PTEN locus

LOH on chromosome 10 was studied by PCR-based microsatellite analysis as previously described (Mutter *et al*., 2002). Three polymorphic markers spanning the *PTEN* gene (D10S551, D10S1765, D10S541) were selected to cover deletions at the whole *PTEN* locus on chromosome 10q23. DNA from normal testis adjacent to tumors on histological sections from the same patient was used as reference. LOH was calculated according to the following formula: (peak height of normal allele 2)/(peak height of normal allele 1) divided by (peak height of tumor allele 2)/(peak height of tumor allele 1). LOH at a single locus was considered present when the signal corresponding to one allele showed at least a 45% reduction of intensity.

Immunofluorescence analysis

5-Bromo-2'deoxyuridine-5'-monophosphate (BrdU) incorporation assay was performed as described previously (Baldassarre *et al*., 1999). Briefly, 5 X 10^5 cells were transfected with 6 g each of control empty vector or of wild type or mutant PTEN constructs, respectively, together with 3 g of a vector encoding green fluorescent protein (Clontech). Labelling was carried out as recommended by the manufacturer (Roche). Fluorescence was visualized with Zeiss 140 epifluorescent microscope equipped with filters that discriminated between Texas Red and fluorescein. All assays were performed 3 times in duplicate.

Fluorescence-activated cell sorter (FACS) analysis

Cells were washed into ice-cold PBS and fixed by adding drop-wise ice-cold 70% ethanol. Fixed cells were washed with cold PBS, labelled with 10 □g/ml propidium iodide (Sigma) and 5 □g/ml Rnase A (New England Biolabs) and analysed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA) interfaced with a Hewlett Packard computer (Palo Alto, CA). Cell cycle analysis was performed with the CELL-FIT programme (Becton Dickinson). All FACS were performed in triplicate.

Northern blot analysis

Northern blot analysis was performed according to a standard procedure. In brief, equal amounts of total RNA (20 □g/lane) were denatured and resolved electrophoretically through formaldehyde-agarose gels. The RNA was transferred onto a nylon membrane and

cross-linked by UV irradiation, Human PTEN cDNA was labeled with ^{32}P-dCTP using a random primer labeling kit (Amersham Pharmacia Biotech), and hybridization was performed at 42°C in the presence of 50% formamide.

REFERENCES

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. (1987). *Nucleic Acids Res.* **25**(17), 3389-3402.

Ali, I. U., Schriml, L. M. and Dean, M. (1999). *J. Natl. Cancer Inst.* **91**, 1922-1932.

Andrews PW. (1984). *Develop Biol,* **103**, 285-293.

Angers-Loustau, A., Cote, J. F., Charest, A., Dowbenko, D., Spencer, S., Lasky, L. A. and Tremblay, M. L. (1999). *J. Cell Biol.* **144**, 1019-1031.

Baldassarre G, Romano A, Armenante F, Rambaldi M, Paoletti I, Sandomenico C, Pepe S, Staibano S, Salvatore G, De Rosa G, Persico MG and Viglietto G. (1997). *Oncogene*, **15**, 927-936.

Baldassarre G, Barone, MV, Belletti, B, Sandomenico C, Bruni, P, Spiezia,S., Boccia, A., Vento MT, Romano, A Pepe, S, A. Fusco and Viglietto G. (1999). *Oncogene*, **18**, 6241-6251

Baldassarre G, Boccia A, Bruni P, Sandomenico C, Barone MV, Pepe S, Angrisano T, Belletti B, Motti ML, Fusco A and Viglietto G. (2000). *Cell Growth & Differentiation*, **11**, 517-526.

Barker K, Martinez A, Wang R, Bevan S, Murday V, Shipley J, Houlston R, Harper J. (2001). *J Med Genet* **38**, 480-481.

Bartkova J, Thullberg M, Rajpert-De Meyts E, Skakkebaek NE, Bartek J. (2000). *Oncogene* **19**, 4146-4150.

Bergstrom R, Adami HO, Mohner M, Zatonski W, Storm H, Ekbom A, Tretli S, Teppo L, Akre O, Hakulinen T. (1996). *J Natl cancer Inst* **88**, 727-733.

Besson, A., Robbins, S. M. and Yong, V. W. (1999). *Eur. J. Biochem.* **263**, 605-611.

Biesecker LG, Happle R, Mulliken JB, Weksberg R, Graham JM Jr, Viljoen DL, Cohen MM Jr. (1999). *Am J Med Genet* **84**, 389-395.

Bonneau, D. and Longy, M. (2000). *Hum. Mutat.* **16**, 109-122.

Bosl G. J., Motzer R. J. N. (1997). *Engl. J. Med.* **337**, 242-253.

Brunet A, Datta SR, Greenberg ME. (2001) *Curr. Opin. Neurobiol***. 11**, 297.

Bruni P, Boccia A, Baldassarre G, Trapasso F, Santoro M, Chiappetta G, Fusco A, Viglietto G. (2000). *Oncogene*, **19**, 3146-3155.

Chaganti R. S. K., Houldsworth J. (1998). *Acta Pathol. Microbiol. Immunol. Scand.*, **106,** 80-84.

Campbell RB, Liu F, Ross AH. (2003). *J Biol Chem* **278**, 33617–20.

Cantley LC, Neel BG. (1999). *Proc Natl Acad Sci U S A*. **96**(8),4240-5.

Cantley L.C. (2002) *Science*, **296**, 1655-1657.

Celebi JT, Tsou HC, Chen FF, Zhang H, Ping XL, Lebwohl MG, Kezis J, Peacocke M. (1999). *J Med Genet* **36**, 360-364.

Chaganti RSK & Houldsworth J. (2000). *Cancer Res*, **60**,1475-1482.

Chen, J., Lindblom, P. & Lindblom, A. (1998) *Hum. Genet.* **102,** 124–125.

Chen WS, Xu PZ, Gottlob K, Chen ML, Sokol K, Shiyanova T, Roninson I, Weng W, Suzuki R, Tobe K, Kadowaki T, Hay N. (2001). *Genes Dev*, **15**, 2203-2208.

Clement S, Krause U, Desmedt F, Tanti JF, Behrends J, Pesesse X, Sasaki T, Penninger J, Doherty M, Malaisse W, Dumont JE, Le Marchand-Brustel Y, Erneux C, Hue L, Schurmans S. (2001) *Nature* **409**, 92.

Conlon I., Raff M. (1999). *Cell*. **9,** 235-244.

Czene K, Lichtenstein P, Hemminki K. (2002). *Int J Cancer* **99**, 260-266.

Dahia PL, Marsh DJ, Zheng Z, Zedenius J, Komminoth P, Frisk T, Wallin G, Parsons R, Longy M, Larsson C, Eng C. (1997). *Cancer Res* **57**, 4710-4713.

Da-Ming L and Hong S. (1998). *Proc Natl Acad Sci USA*, **95**, 15406-15411.

Das S, Dixon JE, Cho W. (2003). *Proc Natl Acad Sci USA*.**100,** 7491–6.

Datta SR, Brunet A, Greemberg ME. (1999). *Genes Dev*, **13**, 2905-2927.

Davies, M. A., Lu, Y., Sano, T., Fang, X., Tang, P., LaPushin, R., Koul, D., Bookstein, R., Stokoe, D., Yung, W. K. et al. **(**1998). *Cancer Res.* **58**, 5285-5290.

Davies, M. A., Koul, D., Dhesi, H., Berman, R., McDonnell, T. J., McConkey, D., Yung, W. K. and Steck, P. A (1999). *Cancer Res.* **59**, 2551-2556.

de Graaff WE, Oosterhuis JW, de Jong B, Dam A, van Putten WL, Castedo SM, Sleijfer DT, Schraffordt Koops H. (1992). *Lab Invest* **66**, 166-168.

Depowski PL, Rosenthal SI, Ross JS. (2001). *Mod Pathol***14**, 672–6.

Derrey S, Proust F, Debono B, Langlois O, Layet A, Layet V, Longy M, Freger P, Laquerriere A. (2004). *Surg Neurol* **61** (5), 447-454.

Di Cristofano A, Pesce B, Cordon-Cardo C, Pandolfi PP. (1998). *Nat Genet*, **19**, 348-355.

Di Cristofano A, Pandolfi PP. (2000). *Cell* **100**(4), 387-90.

Di Cristofano, A., Kotsi, P., Peng, Y. F., Cordon-Cardo, C., Elkon, K. B. and Pandolfi, P. P. (1999). *Science* **285**, 2122-2125.

Diehl JA, Cheng M, Roussel MF, Sherr CJ. (1998). *Genes Dev,* **12**, 3499-3511

Dong C, Hemminki K. (2001). *Int J Cancer* **92**, 144-150.

Eng C , Parsons (1998) New York, Mac Graw Hill, 519-526.

Eng C. (2000). *J Med Genet*. **37**, 828-830.

Eng C, Brody LC, Wagner TM, Devilee P, Vijg J, Szabo C, Tavtigian SV, Nathanson KL, Ostrander E, Frank TS; Steering Committee of the Breast Cancer Information Core (BIC) Consortium. (2001). *J Med Genet* **38**, 824-833.

Eng C.(2003). *Human Mutation* **22**, 183-198.

Fossa SD, Nesland JM, Pettersen EO, Amellem O, Waehre H, Heimdal K. (1991). *Br J Cancer* **64**, 948-952.

Frisch, S. M., Vuori, K., Ruoslahti, E. and Chan-Hui, P. Y. (1996). *J. Cell Biol.* **134**, 793-799.

Frisch, S. M. and Ruoslahti, E. (1997). Integrins and anoikis. *Curr. Opin. Cell Biol.* **9**, 701-706.

Freeman DJ, Li AG, Wei G, Li HH, Kertesz N, Lesche R. *Cancer Cell*.(2003). **3,** 117–30.

Fruman DA, Meyers RE, Cantley LC. (1998) *Annu. Rev. Biochem*. **67**, 481

Furnari FB, Lin H, Huang HS, Cavenee WK. (1997). *Proc Natl Acad Sci USA*, **94**,12479-12484.

Furnari, F. B., Huang, H. J. and Cavenee, W. K. (1998). *Cancer Res.* **58**, 5002-5008.

Gao, X., Neufeld, T. P. and Pan, D. (2000). *Dev. Biol.* **221**, 404-418.

Gimm, O., Attie-Bitach, T., Lees, J. A., Vekemans, M. and Eng, C. (2000). *Hum. Mol. Genet.* **9**, 1633-1639.

Ginn-Pease ME, Eng C. (2003). *Cancer Res* **63**, 282-286.

Gorlin RJ, Cohen MM Jr, Condon LM, Burke BA. (1992). *Am J Med Genet* **44**, 307-314.

Greenwood, J. A., Theibert, A. B., Prestwich, G. D. and Murphy-Ullrich, J. E. (2000). *J. Cell Biol.* **150**, 627-642.

Grimaldi P, Piscitelli, D, Albanesi C, Blasi, F, Geremia R and Rossi P. (1993). *Mol. Endocrinol*., **7**, 1217-1225.

Groszer M, Erickson R, Scripture-Adams DD, Lesche R, Trumpp A, Zack JA, Kornblum HI, Liu X, Wu H. (2001). Science **294** (5549), 2186-2189

Gu, J., Tamura, M. and Yamada, K. M. (1998). *J. Cell Biol.* **143**, 1375-1383.

Gu, J., Tamura, M., Pankov, R., Danen, E. H., Takino, T., Matsumoto, K. and Yamada, K. M. (1999*J. Cell Biol.* **146**, 389-403.

Haas-Kogan D, Shalev N, Wong M, Mills G, Yount G, Stokoe D. (1998). *Curr Biol*, **8**, 1195-1198.

Hanssen AM and Fryns JP, (1995). *J Med Genet* **32**, 117-119.

Han, D. C., Shen, T. L. and Guan, J. L. (2000) *J. Biol. Chem.* **275**, 28911-28917.

Hlobilkova, A., Guldberg, P., Thullberg, M., Zeuthen, J., Lukas, J. and Bartek, J. (2000). *Exp. Cell Res.* **256**, 571-577.

Huang, H., Potter, C. J., Tao, W., Li, D.-M., Brogiolo, W., Hafen, E., Sun, H. and Xu, T. (1999). *Development* **126**, 5365-5372.

International Testicular cancer Linkage Consortium. (1998). APMIS 10664-70.

Iqbal UA. (2000). *Journal of National Cancer Institute*, **92**, 861-862.

Jang and Nadeu, 2001.

Jiang LI, Nadeau JH. (2001). *Mamm Genome* **12**, 89-94.

Kimura T, Suzuki A, Fujita Y, Yomogida K, Lomeli H, Asada N, Ikeuchi M, Nagy A, Mak TW, Nakano T. (2003). *Development*, **130**,1691-1700.

Klemke, R. L., Cai, S., Giannini, A. L., Gallagher, P. J., de Lanerolle, P. and Cheresh, D. A. (1997). Regulation of cell motility by mitogen-activatedprotein kinase. *J. Cell Biol.* **137**, 481-492.

Kobayashi M, Nagata S, Iwasaki T, Yanagihara K, Saitoh I, Karouji Y, Ihara S, Fukui Y. (1999). *Proc Natl Acad Sci U S A.* **96**(9), 4874-4879

Kurose K, Zhou XP, Araki T, Eng C. (2000). *Gene Chrom. Cancer* **29**, 166-172.

Kurose K, Zhou XP, Araki T, Cannistra SA, Maher ER, Eng C. (2001). *Am J of Pathol*. **158**, 2097-2106.

Kurose K, Gilley K, Matsumoto S, Watson PH, Zhou XP, Eng C. (2002). *Nat Genet* **32**, 355-357.

Labosky PA, Barlow DP, Hogan BL. (1994). Ciba Found Symp. **182**,157-168.

Lawlor MA, Alessi DR. (2001) *J Cell Sci*. **114**, 2903-2910.

Lee JO, Yang H, Georgescu MM, Di Cristofano A, Maehama T, Shi Y. (1999).*Cell* **99,** 323–34.

Leevers, S. J., Vanhaesebroeck, B. and Waterfield, M. D. (1999). *Curr. Opin. Cell Biol.* **11**, 219-225.

Leupin N, Cenni B, Novak U, Hugli B, Graber HU, Tobler A. (2003). *Br J Haematol* **121**, 97–100.

Levine RL, Cargile CB, Blazes MS, van Rees B, Kurman RJ, Ellenson LH. (1998). *Cancer Res* **58**, 3254–8.

Liliental, J., Moon, S. Y., Lesche, R., Mamillapalli, R., Li, D., Zheng, Y., Sun, H. and Wu, H. (2000). *Curr. Biol.* **10**, 401-404.

Li DM, Sun H. (1997). *Cancer Res*, **57**, 2124-2129.

Li, D. M. and Sun, H. (1998). *Proc. Natl. Acad. Sci. USA* **95**, 15406-15411.

Li J, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliaresis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R. (1997). *Science*, **275**,1943-1947.

Li, J., Simpson, L., Takahashi, M., Miliaresis, C., Myers, M. P., Tonks, N. and Parsons, R. (1998). *Cancer Res.* **58**, 5667-5672.

Li, L. and Dixon, J. E. (2000). *Semin. Immunol.* **12**, 75-84.

Liaw D, Marsh DJ, Li J, Dahia PL, Wang SI, Zheng Z, Bose S, Call KM, Tsou HC, Peacocke M, Eng C, Parsons R. (1997). *Nat Genet*, **16**, 64-67.

Ling LE, Druker BJ, Cantley LC, Roberts TM. (1992) *J Virol*66:1702–8.

Liu, W., James, C. D., Frederick, L., Alderete, B. E. & Jenkins,R. B. (1997) *Cancer Res.* **57,** 5254–5257.

Longy M, Lacombe D. (1996). *Ann Genet* **39**, 35-42.

Looijenga LH, Oosterhuis JW. (1999). *Rev Reprod* **4**(2), 90-100.

Lu, Y., Lin, Y. Z., LaPushin, R., Cuevas, B., Fang, X., Yu, S. X., Davies, M. A., Khan, H., Furui, T., Mao, M. et al. **(**1999). *Oncogene* **18**, 7034-7045.

Lu Y, Yu Q, Liu JH, Zhang J, Wang H, Koul D. (2003). *J Biol Chem*

278, 40057–66.

Luukko K, Ylikorkala A, Tiainen M, Makela TP. (1999). *Mech Dev* **83**, 187-190.

Lynch ED, Ostermeyer EA, Lee MK, Arena JF, Ji H, Dann J, Swisshelm K, Suchard D, MacLeod PM, Kvinnsland S, Gjertsen BT, Heimdal K, Lubs H, Moller P, King MC. (1997). *Am J Hum Genet* **61**, 1254-1260.

Maehama T and Dixon JE. (1998). *J Biol Chem*, **273**, 13375-13378.

Maier, D., Jones, G., Li, X., Schonthal, A. H., Gratzl, O., Van Meir, E. G. and Merlo, A. (1999). *Cancer Res.* **59**, 5479-5482.

Mallory SB. (1995). *Dermatol Clin* **13**, 27-31.

Marsh DJ, Dahia PL, Zheng Z, Liaw D, Parsons R, Gorlin RJ, Eng C. (1997). *Nat Genet*, **16**,333-334.

Marsh DJ, Dahia PL, Caron S, Kum JB, Frayling IM, Tomlinson IP, Hughes KS, Eeles RA, Hodgson SV, Murday VA, Houlston R, Eng C. (1998). *J Med Genet* **35**, 881-885.

Marsh DJ, Coulon V, Lunetta KL, Rocca-Serra P, Dahia PL, Zheng Z,

Liaw D, Caron S, Duboue B, Lin AY, Richardson AL, Bonnetblanc JM, Bressieux JM, Cabarrot-Moreau A, Chompret A, Demange L, Eeles RA, Yahanda AM, Fearon ER, Fricker JP, Gorlin RJ, Hodgson SV, Huson S, Lacombe D, Eng C, et al. (1998). *Hum Mol Genet* **7** (3), 507-515.

Marsh DJ, Kum JB, Lunetta KL, Bennett MJ, Gorlin RJ, Ahmed SF, Bodurtha J, Crowe C, Curtis MA, Dasouki M, Dunn T, Feit H, Geraghty MT, Graham JM Jr, Hodgson SV, Hunter A, Korf BR, Manchester D, Miesfeldt S, Murday VA, Nathanson KL, Parisi M, Pober B, Romano C, Eng C. (1999). *Hum Mol Genet* **8**, 1461-1472.

Marte BM and Downward J. (1997).*Trends Biochem Sci*, **22**, 355-358.

Matsui Y, Zsebo K, Hogan BL.(1992). *Cell* **70**(5):841-847.

Matsui Y. (1998). *Acta Pathol. Microbiol. Immunol Scand*, **106**, 142-148

McMenamin ME, Soung P, Perera S, Kaplan I, Loda M, Sellers WR. (1999).*Cancer Res*, **59**, 4291-4296

Meistrich ML. (1977). *Methods Cell Biol*, **15**, 15-54.

Mertens F, Johansson B, Hoglund M, Mitelman F. (1997). *Cancer Res*, **57**, 2765-2780.

Miller RT, Jones DT and Thornton JM. (1996). *FASEB J* **10**, 171-178.

Moe-Behrens GH, Klinger FG, Eskild W, Grotmol T, Haugen TB, De Felici M. (2003). *Mol Endocrinol Dec*, **17**, 2630-2638.

Moscatello DK, Holgado-Madruga M, Emlet DR, Montgomery RB, Wong AJ. (1998). *J BiolChem* **273**, 200–6.

Motzer R. J., Amsterdam A., Prieto V., Murty V. V. V. S., Mazumdar M., Sheinfeld J., Bosl G. J., Bajorin D. F., Chaganti R. S. K., Reuter V. E. (1998) J Urol., **159,** 133-138.

Mutter GL, Lin MC, Fitzgerald JT, Kum JB, Baak JP, Lees JA, Weng LP, Eng C. (2000). *J Natl Cancer Inst*, **92**, 924-930.

Mutter GL, Ince TA, Baak JP, Kust GA, Zhou XP, Eng C. (2001).*Cancer Res* **61**, 4311-4314.

Myers MP, Pass I, Batty IH, Van der Kaay J, Stolarov JP, Hemmings BA, Wigler MH, Downes CP, and Tonks NK. (1997). *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). *Proc. Natl. Acad. Sci. USA* **95**, 13513-13518.

Nelen MR, van Staveren WC, Peeters EA, Hassel MB, Gorlin RJ, Hamm H, Lindboe CF, Fryns JP, Sijmons RH, Woods DG, Mariman EC, Padberg GW, Kremer H. Hum (1997). *Mol Genet*, **6**,1383-1387.

Nelen MR, Kremer H, Konings IB, Schoute F, van Essen AJ, Koch R, Woods CG, Fryns JP, Hamel B, Hoefsloot LH, Peeters EA, Padberg GW. (1999). *Eur J Human Genet* **7**, 267-273.

Nicholson PW, Harland SJ. (1995). *Br J Cancer* **71**, 421-426.

Okahara F, Ikawa H, Kanaho Y, Maehama T. (2004). *J Biol Chem.* **279**, 45300-45303.

Okami, K., Wu, L., Riggins, G., Cairns, P., Goggins, M., Evron, E., Halachmi, N., Ahrendt, S. A., Reed, A. L., Hilgers, W., *et al.* (1998) *Cancer Res.* **58,** 509–511.

Owens, L. V., Xu, L., Craven, R. J., Dent, G. A., Weiner, T. M., Kornberg, L., Liu, E. T. and Cance, W. G. (1995). *Cancer Res.* **55**, 2752-2755.

Padberg GW, Schot JD, Vielvoye GJ, Bots GT, de Beer FC. (1991). *Ann Neurol*. **29**(5), 517-23.

Perren A, Weng LP, Boag AH, Ziebold U, Thakore K, Dahia PL, Komminoth P, Lees JA, Mulligan LM, Mutter GL, Eng C. (1999). *Am J of pathol* **155**,1253-1260.

.

Perren A, Komminoth P, Saremasiani P, Matter C, Feurer S, Lees JA, Heitz PU, Eng C. (2000). *Am J Pathol*, **157**, 1097-1103

Penninger JM, WoodgetJ, (2001). *Science*, **294**(5549), 2116-8.

Persad, S., Attwell, S., Gray, V., Delcommenne, M., Troussard, A., Sanghera, J. and Dedhar, S. (2000). *Proc. Natl. Acad. Sci. USA* **97**, 3207-3212.

Podsypanina K, Ellenson LH, Nemes A, Gu J, Tamura M, Yamada KM, Cordon-Cardo C, Catoretti G, Fisher PE, Parsons R. (1999). *Proc Natl Acad Sci U S A*, **96**, 1563-1568.

Ramaswamy, S., Nakamura, N., Vazquez, F., Batt, D. B., Perera, S., Roberts, T. M. and Sellers, W. R. (1999). *Proc. Natl. Acad. Sci. USA* **96**, 2110-2115.

Rameh, L. E. and Cantley, L. C. (1999). *J. Biol. Chem.* **274**, 8347-8350.

Rasheed BK, Stenzel TT, McLendon RE, Parsons R, Friedman AH, Friedman HS, Bigner DD, Bigner SH. (1997). *Cancer Res*, **57**, 4187-4190.

Reiske, H. R., Kao, S. C., Cary, L. A., Guan, J. L., Lai, J. F. and Chen, H. C. (1999). *J. Biol. Chem.* **274**, 12361-12366.

Resnick JL, Bixler LS, Cheng L, Donovan PJ. (1992). *Nature*, **359**(6395), 550-551

Risinger JI, Barrett JC, Watson P, Lynch HT, Boyd J. (1996). *Cancer* **77**, 1836-1843.

Risinger JI, Hayes AK, Berchuck A, Barrett JC. (1997). *Cancer Res*, **57**, 4736-4738.

Rodriguez E, Mathew S, Reuter V, Ilson DH, Bosl GJ, Chaganti RS. (1992). *Cancer Res* **52**, 2285-2291.

Robinson S and Cohen AR, (2000). *Neurosurgery* **46** (2), 371-383.

Rossi P, Dolci, S, Albanesi C, Grimaldi P, Ricca R and Geremia R. (1993). *Dev Biol,* **155,** 68-74.

Samuels Y, Wang Z, Bardelli A, Silliman N, Ptak J, Szabo S, Yan H, Gazdar A, Powell SM, Riggins GJ, Willson JK, Markowitz S, Kinzler KW, Vogelstein B, Velculescu VE. (2004). *Science*, **304**, 554-8.

Schottenfeld D., Warshuer M. E. Testis Schottenfeld D. Fraumeni J. F. (1982). *Cancer Epidemiology and Prevention*, 947-957.

Schwartz D., Goldfinger N., Kam Z., Rotter V. (1999). *Cell Growth Differ*. **10,** 665-675.

Sette C, Bevilacqua A, Bianchini A, Mangia F, Geremia R and Rossi P. (1997). *Development*, **124**, 2267-2274.

Singh B, Reddy PG, Goberdhan A, Walsh C, Dao S, Ngai I, Chou TC, O-Charoenrat P, Levine AJ, Rao PH, Stoffel A. (2002). *Genes Dev*. **16**(8), 984-93.

Simpson L, Parsons R. (2001). *Exp Cell. Res*, **264**, 29-41.

Skakkebaek N. E., Berthelsen J. G., Giwercman A., Müller J. Int. J. Androl.(1987). **10,** 19-28.

Skakkebaek, N. E., Rajpert-de Meyts, E., Jorgensen, N., Carlsen, E., Meidahl Petersen, P., Giwercman, A., Andersen, A-G., Kold Jensen, T., Andersson, A-M., Müller. (1998). *J. Acta Pathol. Microbiol.*

Immunol. Scand., **106**, 3-12.

Skotheim RI, Lothe RA. (2003). *APMIS*, **111**, 136-150.

Smith JM, Kirk EP, Theodosopoulos G, Marshall GM, Walker J, Rogers M, Field M, Brereton JJ, Marsh DJ. (2002). *J Med Genet* **39**, 937-940.

Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW. (1998). *Cell*, **95**, 29-39.

Starink TM, van der Veen JP, Arwert F, de Waal LP, de Lange GG, Gille JJ, Eriksson A. (1986). *Clin Genet* **29**, 222-233.

Steck PA, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DH, Tavtigian SV. (1997). *Nat Genet*, **15**, 356-362.

Stevens LC, (1967). *J Natl Cancer Inst*. **38**(4), 549-52.

Stevens LC, (1984). *Transplant Proc.* **16**(2), 502-504.

Sulis ML, Parsons R (2003).*Trends in Cell Biology* **13**(9), 478-483.

Sun, H., Lesche, R., Li, D. M., Liliental, J., Zhang, H., Gao, J., Gavrilova, N., Mueller, B., Liu, X. and Wu, H. (1999). *Proc. Natl.*

Acad. Sci. USA **96**, 6199-6204.

Suzuki A, de la Pompa JL, Stambolic V, Elia AJ, Sasaki T, del Barco Barrantes I, Ho A, Wakeham A, Itie A, Khoo W, Fukumoto M, Mak TW. (1998). *Curr Biol*, **8**, 1169-1178.

Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R. and Yamada, K. M. (1998). *Science* **280**, 1614-1617.

Tamura, M., Gu, J., Danen, E. H., Takino, T., Miyamoto, S. and Yamada,
K. M. (1999a). *J. Biol. Chem.* **274**, 20693-20703.

Tamura, M., Gu, J., Takino, T. and Yamada, K. M. (1999b). *Cancer Res.* **59**, 442-449.

Tamura, M., Gu, J., Tran, H. and Yamada, K. M. (1999c). *J. Natl. Cancer Inst.* **91**, 1820-1828.

Tashiro H, Blazes MS, Wu R, Cho KR, Bose S, Wang SI, Li J, Parsons R, Ellenson LH. (1997). *Cancer Res*, **57**, 3935-3940.

Taylor, V., Wong, M., Brandts, C., Reilly, L., Dean, N. M., Cowsert, L. M., Moodie, S. and Stokoe, D. (2000). *Mol. Cell. Biol.* **20**, 6860-6871.

Teng DH, Hu R, Lin H, Davis T, Iliev D, Frye C, Swedlund B, Hansen KL, Vinson VL, Gumpper KL, Ellis L, El-Naggar A, Frazier M, Jasser S, Langford LA, Lee J, Mills GB, Pershouse MA, Pollack RE, Tornos C, Troncoso P, Yung WK, Fujii G, Berson A, Steck PA, et al. (1997). *Cancer Res*, **57,** 5221-5225.

Tonks, N. K. and Neel, B. G. (1996). *Cell* **87**, 365-368.

Torres J, Pulido R. (2001). *J Biol Chem.*, **276**, 993-998.

Tsou HC, Teng DH, Ping XL, Brancolini V, Davis T, Hu R, Xie XX, Gruener AC, Schrager CA, Christiano AM, Eng C, Steck P, Ott J, Tavtigian SV, Peacocke M. (1997). *Am J Hum Genet*, **61**(5), 1036-43.

Tsuchiya KD, Wiesner G, Cassidy SB, Limwongse C, Boyle JT, Schwartz S. (1998). *Gene Chrom Cancer* **21**, 113-118.

Ulbright TM. (1993). *American Journal of Surgical Pathology*, **17**,

1075-1091.

Vazquez, F. and Sellers, W. R. (2000). *Biochim. Biophys. Acta* **1470**, M21-35.

Vazquez F, Ramaswamy S, Nakamura N, Sellers WR. (2000). *Mol Cell Biol* . **20,** 5010–8.

Viglietto G, Romano A, Maglione D,Rambaldi M, Paoletti I, Lago C, Califano D, Monaco C, Mineo A, Santelli G, Manzo G, Botti G, Chiappetta G and Persico MG. (1996). *Oncogene*, **13**, 577-587.

Viglietto G, Motti ML, Bruni P, Melillo RM, D'Alessio A, Califano D, Vinci F, Chiappetta G, Tsichlis P, Bellacosa A, Fusco A, Santoro M. (2002). *Nature Medicine*. **8,** 1136-1144

Vivanco I, Sawyers CL. (2002). Nat Rev Cancer. **2**, 489–501

Waite KA and Eng C .(2002). *Am J Hum Genet*. **70**, 829-844.

Wang SI, Puc J, Li J, Bruce JN, Cairns P, Sidransky D, Parsons R. (1997). *Cancer Res*, **57**, 4183-4186.

Wanner M, Celebi JT, Peacocke M. (2001). *J Am Acad Dermatol*. **44**, 183-187.

Webster MA, Hutchinson JN, Rauh MJ, Muthuswamy SK, Anton M, Tortorice CG, *Mol Cell Biol* 1998;18:2344–59.

Weng, L. P., Smith, W. M., Brown, J. L. and Eng, C. (2001). *Hum. Mol. Genet.* **10**, 605-616.

Wymann, M. P., Sozzani, S., Altruda, F., Mantovani, A. and Hirsch, E. (2000). *Immunol. Today* **21**, 260-264.

Wu Y, Dowbenko D, Pisabarro MT, Dillard-Telm L, Koeppen H, Lasky LA. (2001) *J Biol Chem.* **276**: 21745-21753.

Wu W, Wang X, Zhang W, Reed W, Samet JM, Whang YE, Ghio AJ. (2003). *J Biol Chem*., **278**, 28258-28263.

Wylie C .(1999). *Cell*, **96**(2):165-74.

Yu, D. H., Qu, C. K., Henegariu, O., Lu, X. and Feng, G. S. (1998). *J. Biol. Chem.* **273**, 21125-21131.

Zhou XP, Gimm O, Hampel H, Niemann T, Walker MJ, and Eng V. (2000). *Am J Pathol,* **157**, 1123-1128

Zhou XP, Woodford-Richens K, Lehtonen R, Kurose K, Aldred M, Hampel H, Launonen V, Virta S, Pilarski R, Salovaara R, Bodmer WF, Conrad BA, Dunlop M, Hodgson SV, Iwama T, Jarvinen H, Kellokumpu I, Kim JC, Leggett B, Markie D, Mecklin JP, Neale K, Phillips R, Piris J, Rozen P, Houlston RS, Aaltonen LA, Tomlinson IP, Eng C. (2001). *Am J Hum Genet* **69**, 704-711.

Zhou XP, Hampel H, Thiele H, Gorlin RJ, Hennekam RCM, Parisi M, Winter RM, Eng C.(2001). *Lancet* **358**, 210-211.

Zhou XP, Kuismanen S, Nystrom-Lahti M, Peltomaki P, Eng C. (2002). *Hum Mol Genet* **11** (4), 445-450.

Zhou XP, Waite KA, Pilarski R, Hampel H, Fernandez MJ, Bos C, Dasouki M, Feldman GL, Greenberg LA, Ivanovich J, Matloff E, Patterson A, Pierpont ME, Russo D, Nassif NT, Eng C.. (2003). *Am J Hum Genet*. **73(**2), 404-411.

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