PTEN, a unique tumor suppressor gene

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Introduction

For many years, it has been thought that the chromosome region 10q22-24 includes one or more genes that appear to play a role in several human malignancies. PTEN is a new tumor suppressor gene encoding a dual-specificity phosphatase that was cloned simultaneously by three groups (Li & Sun 1997, Li et al. 1997, Steck et al. 1997), two of which used a positional cloning approach to identify genes in chromosome 10 (Li et al. 1997, Steck et al. 1997). While several protein kinases have been implicated as oncogenes, and phosphatases have long been known frequently to antagonize their function, there has been no direct demonstration of the role of phosphatases in tumor development (Myers & Tonks 1997). PTEN characterization as a bona fide tumor suppressor gene has confirmed that a deficient phosphatase activity can lead to cancer, as detailed by studies that are described below.

Since the cloning of PTEN, a considerable number of papers describing its mutational spectrum in a variety of human tumors (Cairns et al. 1997, 1998, Dahia et al. 1997, Liu et al. 1997, Rasheed et al. 1997, Risinger et al. 1997, Tashiro et al. 1997, Teng et al. 1997, Tsou et al. 1997, Wang et al. 1997), as well as its identification as the susceptibility gene to cancer-hamartoma syndromes (Liaw et al. 1997, Marsh et al. 1997a) have been reported in the literature. These studies were followed closely by the initial reports unraveling PTEN’s function in cell signaling. Within a short period of time, it has been demonstrated that PTEN indeed functions as a phosphatase (Myers et al. 1997), as initial structural comparisons indicated. However, lipid residues are PTEN’s major, functionally proven substrates (Maehama & Dixon 1999). Tyrosine- or serine/threonine-proteins that function as targets of PTEN have been more difficult to establish, although PTEN has been shown to bind to focal adhesion kinase (FAK) and to reduce its tyrosine phosphorylation (Tamura et al. 1998). It has also been found that PTEN induces growth suppression via cell cycle arrest and/or induction of apoptosis (Furnari et al. 1998, Myers et al. 1998, Stambolic et al. 1998, Ramaswamy et al. 1999, Sun et al. 1999), and inhibits cell adhesion and migration. Three PTEN mammalian knockout models have been generated (Di Cristofano et al. 1998, Stambolic et al. 1998, Podsypanina et al. 1999) with many common features and some phenotypic disparities which have, for the most part, confirmed the previous in vitro data available. In addition, four knockouts of the PTEN homolog in Caenorhabditis elegans have helped understand, in more detail, the pathways involved in PTEN downstream signaling (Ogg & Ruvkun 1998, Gil et al. 1999, Mihaylova et al. 1999, Rouault et al. 1999), and have set the stage for additional studies that will shed light on yet unknown aspects of its regulation. Thus, genetic, biochemical evidence as well as in vivo disruption have implicated PTEN in the regulation of multiple cellular functions: cell growth, extracellular matrix interactions, cell migration and development interactions. More recently, the crystal structure of PTEN has been solved, providing further clues to PTEN substrate specificity, and also helping in the understanding of some of the biochemical peculiarities of this tumor suppressor phosphatase.

All this wealth of data considered, PTEN has had a remarkable appeal to a broad range of research areas and can certainly be adopted as an excellent paradigm for the fast pace of cancer research that we are presently witnessing. An update on the current knowledge of the genetic and functional aspects of PTEN highlighted above are the scope of this review and will be discussed in more detail in the next sections.

PTEN cloning, structure and mutation analysis

Somatic mutations of PTEN

The chromosome 10q23 region has long been found to represent an important target area in several human tumors, such as glioblastomas, prostate and breast cancer, endometrial neoplasms and hematological malignancies (Pfeifer et al. 1995, Rasheed et al. 1995, Albarosa et al. 1996, Trybus et al. 1996, Kobayashi et al. 1997, Bose et al. 1998). It has also been suspected to be the site for loci associated with certain inherited disorders such as hamartomatous syndromes (Nelen et al. 1996, Jacoby et al. 1997, Zigman et al. 1997). This group of diseases includes Cowden syndrome (CS), an autosomal dominant disorder which comprises multiple...
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hamartomas in tissues derived from the three major embryological layers, and is associated with a higher risk of breast and follicular or papillary thyroid cancer (25–50% of affected females, and 10% of affected individuals respectively), and in a subset of cases, a dysplastic ganglioneuroma of the cerebellum, Lhermitte-Duclos disease, might also develop. Another hamartoma syndrome linked to the same chromosomal region is Bannayan-Riley-Ruvalcaba syndrome (BRR). BRR also has an autosomal dominant trait and is clinically characterized by macrocephaly, lipomatosis and pigmented macules of the glans penis, Hashimoto’s thyroiditis, and mental and developmental delay (Eng 1997). In contrast to CS, an increased risk of malignancy has not previously been documented in a formal manner in BRR. Besides these two highly related syndromes, other inherited disorders such as juvenile polyposis coli (JPC) have been suspected to be linked, in at least a subset of cases, to the 10q23 region (Jacoby et al. 1997). JPC is an autosomal dominant condition with incomplete penetrance characterized by hamartomatous polyps of the gastrointestinal tract and predisposition to gastrointestinal cancer.

Initial linkage analysis studies have localized CS and JPC to 10q23 (Jacoby et al. 1997, Nelen et al. 1997). In particular, by studying families with CS with recombination of this area, a critical CS region was delineated which spanned a 1.5 cm area at 10q23.3 (Liaw et al. 1997).

PTEN was identified as a tumor-suppressor gene located within the CS critical region 10q23.3 and was found to be deleted in a wide variety of human cancers and cell lines, including glioblastomas, prostate, breast and kidney cancer cell lines (Li et al. 1997). This gene has also been designated as MMAC1 (for mutated in multiple advanced cancer) due to its initial apparent association with later-stage tumors (Steck et al. 1997) and TEP1 (transforming growth factor-β)

Figure 1 Frequency of somatic PTEN abnormalities in various tumor types: the mean (± s.d.) results from different reports have been calculated and are depicted as columns (original studies are cited throughout the text). When available, PTEN abnormalities other than mutations, such as transcriptional silencing or low/absent protein expression, are also shown (*; available in only two tumor types, hematological malignancies and breast cancer). Hem. malign, hematological malignancies.
(TGFβ)-regulated and epithelial-cell-enriched phosphatase) for its putative signaling effectors (Li & Sun 1997). The PTEN gene contains nine exons and encodes a 403 amino acid protein that displays high homology in its N-terminal region to dual specificity protein phosphatases and also to tensin, a cytoskeleton protein, and to auxilin, a protein involved in synaptic vesicle transport (Li & Sun 1997, Li et al. 1997). The initial suggestion that PTEN functions as a tumor suppressor gene came from studies in which truncating or inactivating mutations of PTEN accompanied by deletions of the wild-type allele were described with high frequency in malignant gliomas and endometrial cancer (Cairns et al. 1997, Liu et al. 1997, Rasheed et al. 1997, Risinger et al. 1997, Wang et al. 1997), with moderate frequency in prostate cancer (Cairns et al. 1997, Liu et al. 1997, Rasheed et al. 1997, Risinger et al. 1997, Wang et al. 1997) and with a lower rate in other malignancies such as breast, thyroid, bladder, ovary, small-cell lung cancer, and hematological malignancies (Dahia et al. 1997, Cairns et al. 1998, Gronbaek et al. 1998, Kohno et al. 1998, Ueda et al. 1998, Yokomizo et al. 1998). Confirmation of the tumor suppressing function of PTEN was provided by studies showing that ectopic expression of wild-type, but not mutant PTEN, induces growth suppression of cell lines carrying a mutant copy of PTEN (Furnari et al. 1997, Li et al. 1998, Myers et al. 1998) (see more on PTEN function below). A large body of studies is now available reporting PTEN mutation analysis in a variety of different human tumors and results from many of such studies are summarized in Fig. 1. Further, a comprehensive review of PTEN mutation spectra in several human tumors has recently been published (Ali et al. 1999). The initial suggestion that PTEN is mutated in later stages or more advanced tumor types, such as that seen in glioblastomas, did not stand extensive analysis that included other tumor types. It is now known that PTEN abnormalities represent, in fact, an early event in endometrial cancer, in which PTEN mutations can be detected as early as in pre-neoplastic lesions (Maxwell et al. 1998, Obata et al. 1998). In addition, studies attempting to associate PTEN mutations with prognostic indicators in prostate cancer have not revealed a clear correlation (McMenamin et al. 1999). Surprisingly, two of the most common malignancies associated with CS (see below), breast and non-medullary thyroid cancer, have been found to have a low frequency of PTEN mutations (Dahia et al. 1997, Chen et al. 1998, Halachmi et al. 1998, Ueda et al. 1998, Feilotter et al. 1999). In particular, non-CS and non-breast cancer associated gene (BRCA)1 or -BRCA2 associated familial breast cancer syndromes do not have PTEN mutations (FitzGerald et al. 1998, Marsh et al. 1998). Of interest, we found that thyroid adenomas carry higher rates of loss of heterozygosity in the area spanning the PTEN locus than malignant thyroid tumors (Dahia et al. 1997). This finding suggests that benign and malignant thyroid neoplasms arise through two independent pathways, each of which requires the acquisition of distinct molecular defects, and not in a sequential manner, as previously believed.

Occasionally, highly divergent mutation frequencies have been reported for a given tissue type: this may be explained in some cases by the existence of a PTEN pseudogene, PTENP1, also known as psPTEN, PTEN2, PTEN2 (Teng et al. 1997, Dahia et al. 1998, Whang et al. 1998a). This pseudogene carries over 98% homology with PTEN cDNA, and can confound interpretation of the mutation data, especially in studies based on analysis of cDNA-based templates.

**Germline PTEN mutations**

PTEN’s role, however, is not limited to somatic disorders: germline mutations of PTEN are the cause of CS and BRR cases (Liaw et al. 1997, Marsh et al. 1997a). PTEN mutations have been identified in 13–81% and 57–60% of cases of CS and BRR respectively (Lynch et al. 1997, Nelen et al. 1997, Longy et al. 1998, Marsh et al. 1998, 1999, Tsou et al. 1998). PTEN mutations in CS tend to cluster at, but are not limited to, exon 5, which encodes the phosphatase signature motif (see Fig. 2). Not only do the two syndromes share many clinical features, but the mutations in some families are identical, suggesting that these two disorders are allelic. Recent studies have attempted to establish genotype–phenotype correlations in CS and BRR (Marsh et al. 1998a, 1999). Genotype-phenotype analyses in CS alone suggest that the presence of a PTEN mutation may be associated with the development of breast carcinoma (Marsh et al. 1998a). Within the BRR group, an association has been noted between the presence of lipomas and the detection of PTEN mutation (Marsh et al. 1999). It is possible that the presence of a PTEN mutation, whether it be in CS, BRR or BR/CS families, predisposes to tumor development. However, it appears from these initial observations that mutation-positive status in BRR predisposes to benign tumors, compared with malignant tumors in CS or BR/CS overlap families. Among CS, BRR and in BRR/CS overlap families that are PTEN mutation-positive, the mutation spectrum appears similar. Thus, PTEN mutation-positive CS and BRR are possibly different presentations of a single syndrome and, hence, both should receive equal attention with respect to cancer surveillance (Marsh et al. 1999). It is possible that other modulating factors might be responsible for the observed clinical variation between CS and BRR. However, approximately 20% of CS and 40% of BRR families still remain without an identifiable germline PTEN mutation. Individuals from these PTEN mutation-negative families cannot at present rely on a diagnostic test for the disease.

Informative PTEN mutation-negative families have been shown to be linked to the 10q25 region, where PTEN lies (Nelen et al. 1996, Liaw et al. 1997). While mutations in the
promoter region of PTEN may account for some of these cases, it is possible that another gene located in this area might be responsible for these PTEN mutation-negative cases. A recently identified gene mapping centromeric of PTEN in 10q23 (Caffrey et al. 1999, Chi et al. 1999), which encodes a histidine phosphatase with the ability to remove the 3-phosphate from inositol moieties, was examined for mutations in PTEN mutation-negative CS and BRR families (Dahia et al. 2000). We found no mutations in any of the individuals examined. Therefore, the major genetic defect responsible for CS and BRR in cases without detectable PTEN mutation remains to be established. There has been a report describing genetic heterogeneity in CS (Tsou et al. 1997b). No candidate gene has emerged to account for these cases to date.

Besides CS and BRR, a definite role for PTEN in other inherited hamartomatous syndromes has not been clearly confirmed. While an initial report described the detection of germline PTEN mutations in three cases of juvenile polyposis syndrome (JPS) (Olschwang et al. 1998), the study of a larger series of affected families selected by more stringent diagnostic criteria for JPS excluded linkage to an area spanning 29cM surrounding the PTEN locus (Marsh et al. 1997b). Therefore, a definite role for PTEN in JPS pathogenesis still needs to be established.

Other mechanisms of PTEN inactivation

In addition to structural abnormalities, an increasing amount of evidence has now suggested that PTEN may be inactivated by mechanisms other than mutations and/or deletions (Whang et al. 1998b, Dahia et al. 1999). Epigenetic mechanisms might be responsible for a number of cases in which PTEN expression is downregulated or even totally ablated in the absence of a detectable mutation, as seen in prostate cancer (Whang et al. 1998b) as well as in our observation of a series of leukemia and lymphoma cell lines (Dahia et al. 1999). In agreement with these findings and in favor of the existence of other mechanisms of PTEN inactivation is the recent report that lack of PTEN expression in breast cancer sections is not associated with deletions in a subgroup of samples (Perren et al. 1999). The precise mechanisms involved in silencing PTEN expression in such malignancies are not known. It has been suggested that PTEN methylation might occur in a subset of prostate cancers (Whang et al. 1998b) but not in another series of prostate tumors (Cairns et al. 1997) nor in hematological malignancies (Dahia et al. 1999).

Our preliminary in vitro studies have suggested that ubiquitin-mediated degradation is not enhanced in cell lines with low or absent PTEN expression in co-existence with
normal transcript levels and structural defects (P L M Dahia and C Eng, unpublished observations). It remains to be established whether PTEN degradation is in fact mediated by the ubiquitin-proteasome pathway in vivo and whether any component of this cascade is altered under particular circumstances in cells with low or absent PTEN protein without structural or transcriptional defects.

Other mechanisms involving reduction of PTEN protein expression are still elusive. Analysis of structural motifs of PTEN might help unravel such mechanisms. It has recently been suggested that PTEN’s half-life may be reduced in certain mutated cell lines (Georgescu et al. 1999). PTEN has two PEST homology domains and a PSD-95/Dig/20–1 (PDZ)-binding motif on its C-terminal region. While the PEST homology motifs are supposed to assist in protein folding, it is expected that their absence would interfere with protein targeting for degradation. The PDZ-binding motif is responsible for protein–protein interactions. Studies aimed at mapping the functional activity of PTEN constructs lacking these motifs revealed that, not unexpectedly, absence of PEST domains results in faster rates of PTEN degradation (Georgescu et al. 1999). However, no changes in PTEN degradation rates were seen in PDZ-lacking PTEN constructs as compared with the wild-type protein. The tumor suppressor function of the mutants lacking either PEST or PDZ homology motifs was not altered in the study referred to.

More recent work has shown that PTEN associates with a member of a family of membrane-associated scaffold proteins known as MAGUKs (membrane-associated guanylate kinases), MAGI-2 (also known as AIP), through PTEN’s PDZ domain, and that this interaction enhances PTEN efficiency in reducing AKT activity (Wu et al. 2000). It is proposed that MAGUKs function as scaffold proteins to assemble multiprotein signaling complexes and enhance their stability, thereby increasing the efficiency of signal transduction.

While many aspects of PTEN structure–function regulation have been unraveled, it still remains to be established how the stability of PTEN protein is regulated in certain tumors without PTEN mutations but with low or absent protein levels.

Initial observations that hamartomatous tissues from Cowden patients had high frequencies of loss of heterozygosity (LOH) suggested that homozygous PTEN inactivation was required for a tumor suppressor phenotype (Marsh et al. 1998b). This was in agreement with findings that Pten−/− knockout mice developed lymphomas at a high rate where LOH was found at the PTEN locus (Suzuki et al. 1998). A recent study has now suggested that PTEN has a dosage effect and haploinsufficiency may, in fact, be responsible for impaired PTEN activity (Di Cristofano et al. 1999). In this study, heterozygote mutant Pten−/+ mice showed an abnormal apoptotic response and an autoimmune phenotype (discussed below). These results are more in keeping with the findings that heterozygous animals already display hyperplastic and dysplastic phenotypes and with the observations that a number of CS tumors do not have detectable LOH (Marsh et al. 1998). This may reflect the fact that a much broader range of tumors lacking a single copy of the gene may suffer from abnormal PTEN function than was previously anticipated. Table 1 depicts results from current research that favor or against a PTEN dosage effect. To reconcile these apparent conflicting findings, it may eventually be proven that PTEN haploinsufficiency may involve a tissue-dependent or developmentally regulated phenomenon.

| Table 1 Features in favor or against a PTEN haploinsufficiency phenotype based on current data. |
|---------------------------------|---------------------------------|
| In favor                        | Against                         |
| CS hamartomas without LOH      | LOH of hamartomas in CS         |
| Heterozygous mice with          | LOH seen in somatic tumors      |
| lymphoid tissue proliferation   | with PTEN mutations             |
| (an auto-immune like            | (glioblastoma, endometrial      |
| syndrome)                       | carcinoma, prostate             |
| Heterozygous knockout mice      | LOH in the majority of tested    |
| develop tumors                  | lymphomas of Pten−/− knockout   |
| mice                            | mice                            |

PTEN function

In general terms, PTEN has been shown to modulate, mostly as an inhibitor factor, two seemingly distinct cellular functions: cell growth/survival and cell migration/adhesion through its lipid and protein phosphatase activities respectively. These aspects of PTEN function will be discussed in detail below.

PTEN signaling: substrates and pathways

A recent study demonstrated that PTEN acts as a phospholipid phosphatase dephosphorylating the phosphatidylinositol 3,4,5 triphosphate (PtdIns(3,4,5)P3) and phosphatidylinositol 3,4 biphosphate (PtdIns(3,4)P2) specifically at the D3 position of the inositol ring, resulting in PtdIns(4,5)P and PtdIns(4)P respectively. These D3 phospholipids are the direct products of the phosphatidylinositol 3-OH kinase (PI-3 kinase, PI3K), which counteracts PTEN function (Fig. 3). PI3-kinase triggers signaling through multiple pathways, many of which are thought to associate with cell growth and survival (Liscovitch & Cantley 1994, Vanhaesebroeck et al. 1997). PTEN, working in opposition to PI3-kinase, is therefore associated with cell death or arrest signals. Phospholipid residues such as PtdIns(3,4,5)P3 are present in cells upon stimulation by several growth factors,
such as platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), and epidermal growth factor (EGF). Upon activation by growth factor, proteins containing a pleckstrin-homology (PH) domain are recruited to the membrane where they associate with phospholipids. One of the PH domain-containing proteins relevant in this pathway is the serine-threonine kinase, AKT, also known as PKB or RAC1 (reviewed in Coffer et al. 1998). AKT, in turn, and as a consequence of lipid binding, alters its conformation to allow two of its residues, threonine 308 and serine 473, to be phosphorylated and therefore become active. The kinase responsible for phosphorylation of threonine 308 is phosphonositide-dependent kinase 1 (PDK1), an enzyme which also contains a PH domain and is therefore dependent on lipid binding for its full activity (Alessi et al. 1997). There is some preliminary evidence, predominantly from in vitro studies, that a second lipid-dependent, PH domain-containing enzyme, ILK (integrin-linked kinase), is responsible for phosphorylation of the serine 473 (Delcommenne et al. 1998). Further, a recent paper has proposed that the kinase responsible for Ser 473 phosphorylation might in fact be PDK1, when it associates with certain specific proteins, such as PDK1 interacting fragment (PIF), as seen by in vitro studies (Balendran et al. 1999). By dephosphorylating D3 residues on PtdIns(3,4,5)P3 and PtdIns(3,4)P2, PTEN works in opposition to the PI3K/AKT pathway and therefore counters cell survival mechanisms elicited by this signaling.

The mechanisms of cell survival associated with AKT appear to involve multiple pathways, including growth factors, cytokines, c-myc overexpression, UV irradiation, and matrix detachment (Coffer et al. 1998). One of the known signals activated by AKT is its phosphorylation of the Bcl-2 family member, BAD: phosphorylation of BAD results in suppression of apoptosis (Datta et al. 1997). AKT has also been reported to counteract the apoptotic response of several cellular factors. Recently, the transcription factor NF-kappaB has been implicated in the apoptotic response antagonized by the PI3K/AKT pathway (Romashkova & Makarov 1999). In addition, AKT suppresses CD-95/Fas-induced apoptosis (Rohn et al. 1998). Another possible mechanism by which AKT exerts this function is through the regulation of the activity of FKHRL1, a member of the Forkhead family of transcription factors (Brunet et al. 1999). In the presence of survival factors, AKT phosphorylates FKHRL1, leading to FKHRL1’s retention in the cytoplasm. Survival factor withdrawal leads to FKHRL1 dephosphorylation, nuclear translocation, and target gene activation. Within the nucleus, FKHRL1 triggers apoptotic signals, possibly by inducing the expression of genes that are critical for cell death, such as the Fas ligand gene. A role for PTEN in the regulation of the Fas-induced response has also
been suggested by studies with the Pten\textsuperscript{\textminus} mice mentioned above (Di Cristofano \textit{et al.} 1999). In agreement with the involvement of Fas in this pathway, the defect in Fas-mediated apoptosis observed in these Pten\textsuperscript{\textminus} mice is mediated by activation of the PI3K/AKT pathway.

\textit{PTEN}’s involvement in antagonizing the PI3K/AKT pathway was confirmed by subsequent studies which showed that cells lacking wild-type \textit{PTEN} from gliomas, from patients with CS or from Pten-deficient mice have elevated levels of PtdIns(3,4,5)P\textsubscript{3}. As a result, the activity of AKT was also increased in these cells, indicating that \textit{PTEN} exerts its tumor-suppressor function by negatively regulating the antiapoptotic PI-3 kinase/AKT signaling pathway (Fig. 3). Biological evidence that \textit{PTEN} acts as a tumor suppressor came from studies indicating that wild-type \textit{PTEN} suppresses the proliferation and the tumor growth of \textit{PTEN}-deficient glioblastoma cells. In contrast, mutant phosphatase-inactive \textit{PTEN} failed to suppress cell growth. The analysis of mutations from tumor specimens or cell lines derived from tumors highlighted the importance of the phosphatase domain for the tumor-suppressor function of \textit{PTEN}. Indeed, a large proportion of these mutations maps to the region encoding the phosphatase domain (Fig. 2 shows the spectrum of \textit{PTEN} mutations in Cowden syndrome). However, several mutations and deletions are located distal to the phosphatase core motif, in the C-terminal region of \textit{PTEN}. From an \textit{in vitro} functional analysis of mutations located downstream of the phosphatase catalytic domain, in particular those located in exons 7 and 8, most of which resulted in premature stop codons, it was shown that these C-terminal mutants inactivated the tumor-suppressor function of \textit{PTEN} (Georgescu \textit{et al.} 1999). The stability and phosphatase activity of these mutants were also affected. Similar results have been identified by independent groups, in which a functional ‘map’ of \textit{PTEN} defines an area spanned by codons 10 through 353 as a minimum requirement for the lipid phosphatase and cell-cycle arrest properties (S Ramaswamy & W Sellers, personal communication). These findings suggest that \textit{PTEN} lipid phosphatase function is dependent on the integrity of a larger portion of the molecule, which extends far beyond the catalytic motif. The implications of these data are far-reaching, as it is expected that mutations scattered throughout the gene are likely to impair \textit{PTEN} function. Nevertheless, mutations have been reported in glioblastomas (Rasheed \textit{et al.} 1997) which are located at the area coding for the final amino acids of the \textit{PTEN} protein, outside the minimal functional region. The relevance of these mutations for \textit{PTEN} function still remains to be elucidated. In light of the new studies showing the relevance of \textit{PTEN}’s PDZ domain for \textit{PTEN}’s function, it may be found that such glioma mutations alter \textit{PTEN} stability (Wu \textit{et al.} 2000).

\textbf{\textit{PTEN} as an inducer of cell-cycle arrest}

\textit{PTEN}’s ability to induce growth suppression appears to be mediated by at least two mechanisms: it promotes cell-cycle arrest at the G1 phase and also increases apoptosis induced by multiple distinct stimuli. The cell-cycle arrest property of \textit{PTEN} has been detected in some cell types. In the presence of ectopic expression of wild-type \textit{PTEN}, glioblastoma and renal carcinoma cells arrest at G1 (Furnari \textit{et al.} 1998, Ramaswamy \textit{et al.} 1999). This effect is enhanced by nocodazole, a G2 blocker, and by low serum conditions (Furnari \textit{et al.} 1998). It has been demonstrated that this function of \textit{PTEN} is AKT-dependent, and can be rescued by expression of an activated form of AKT (Li \textit{et al.} 1998, Ramaswamy \textit{et al.} 1999). One of the targets of \textit{PTEN} in its ability to induce cell-cycle arrest has been suggested to be the p27\textsuperscript{kip1} cyclin-dependent kinase inhibitor (Li \& Sun 1998, Cheney \textit{et al.} 1999). It has been shown that \textit{PTEN} induces p27 expression and allows the formation of complexes with cyclin E. This, in turn, reduces specifically cyclin-dependent kinase 2 (CDK2) activity and, consequently, results in low phosphorylation of retinoblastoma (Rb) protein. Low Rb phosphorylation leads to an arrest in cell-cycle progression. It remains to be established whether the increase in the formation of p27/cyclinE/CDK2 complexes are truly a direct effect of \textit{PTEN}’s action and which intermediate steps are involved in producing this cell-cycle inhibition signal.

Another potential mechanism of cell-cycle control by \textit{PTEN} may be through inhibition of cyclin D1 accumulation. AKT phosphorylates GSK3 (glycogen synthase kinase 3), leading to its inactivation (Cross \textit{et al.} 1995). Active GSK3 phosphorylates cyclin D1, targeting it for degradation (Diehl \textit{et al.} 1998). AKT, therefore, appears to promote cyclin D1 accumulation (Coff er \textit{et al.} 1998, Cantley & Neel 1999).

\textbf{\textit{PTEN} as an apoptotic factor}

Despite the observed cell arrest properties of \textit{PTEN}, it has been suggested that its tumor suppression function also results from \textit{PTEN}’s ability to increase cellular apoptotic rates (Li \textit{et al.} 1998, Stambolic \textit{et al.} 1998). However, while AKT is a known promotor of cell survival, an increase in \textit{PTEN} has not been accompanied by high indexes of apoptosis in all cell types studied, in particular glioblastoma cells, in which the G1 arrest induced by \textit{PTEN} was extensively documented (Furnari \textit{et al.} 1998, Ramaswamy \textit{et al.} 1999). On the other hand, an increased sensitivity to apoptosis induced by UV, heat shock and radiation was observed upon \textit{PTEN} overexpression in mice null for \textit{PTEN} (Stambolic \textit{et al.} 1998). In addition, breast cancer cell lines showed an increased number of apoptotic cells after \textit{PTEN} expression (Li \textit{et al.} 1998). Further, it has been suggested that an apoptotic response in breast cancer cell lines may ensue after initial G1 arrest, in a serial manner (Weng \textit{et al.} 1999). It is also possible that increased apoptosis is not seen upon \textit{PTEN} expression in certain cell lines, due to additional molecular defects associated with apoptotic functions in these cells.
The mechanism of PTEN induction of apoptosis is still unclear. It has recently been suggested that Pten heterozygous mice have impaired Fas-mediated cell death. This function of PTEN appears to be mediated by PI-3 kinase-AKT signaling, as mentioned above. From these recent studies, a dosage effect of PTEN has been suggested and Pten mice develop an autoimmune-type syndrome similar to that seen in Fas-deficient mice. These animals develop lymph node hyperplasia, splenomegaly, and inflammatory infiltrates in multiple organs, in particular the kidneys in which immune complexes were observed in the glomeruli, in some cases similar to the segmental glomerulosclerosis of human or mice autoimmune syndromes (Di Cristofano et al. 1999). However, the interrelation between the PI3K/PTEN/AKT and the Fas signaling appears to involve additional factors, as highlighted by another study (Wick et al. 1999), in which ectopic expression of PTEN in glioblastoma cells lacking functional PTEN sensitizes these cells to irradiation and Fas-FasL-induced apoptosis via increased caspase 3 activity. Sensitization of a PTEN-null glioblastoma cell, U87MG, to CD95L apoptosis by wild-type PTEN is blocked by IGF-I in a PI3K-dependent manner. Although AKT is a downstream target of PI3K, the protection by IGF-I was not associated with the reconstitution of AKT phosphorylation in this model.

Further insights into the mechanisms of PTEN-induced apoptosis are still required to allow for a complete understanding of its functional role. In particular, the factor(s) responsible for a cell’s decision to induce cell-cycle arrest or apoptosis as a result of PTEN activity remain to be determined. A diagram summarizing currently known and presumed players in PTEN/AKT signaling is shown in Fig. 3.

Are phospholipids the only PTEN substrates?

In the search for PTEN protein substrates, it has been shown that PTEN expression is associated with reduced cell migration, spreading and focal adhesion formation, and these phenomena were linked to the integrin pathway (Tamura et al. 1998) (Fig. 4). It has been noted that wild-type PTEN binds to the same complex as the focal-adhesion kinase (FAK) and reduces its phosphorylation. Phosphatase-active PTEN mutants, including C124S and the substrate-trapping mutant, D92A, do not show the same properties (Tamura et al. 1998). Interestingly, a PTEN mutation found in patients with CS, in which glycine is replaced by glutamine in a residue within the catalytic core motif (G129E), has been shown to behave in a similar manner to wild-type PTEN with regard to migration, spreading and focal adhesion inhibition (Tamura et al. 1998). However, this mutant has been shown to lack phospholipid activity (Furnari et al. 1998, Myers et al. 1998). This finding gives further hints on PTEN functions and couples the Cowden syndrome-related and possibly tumor-associated abnormalities to the phospholipid, but not necessarily protein-related, PTEN activity. There is currently no known PTEN mutant that maintains activity towards lipid substrates and loses protein-substrate function. It will be interesting to dissect these multiple functions of PTEN further. Other substrates tested, such as paxillin, e-src, and tensin, all associated with adhesion, did not show reduced phosphorylation, suggesting that PTEN acts specifically towards FAK (Tamura et al. 1998, 1999).

Additional insight into PTEN signaling came from work from the same group (Gu et al. 1998) which has shown that PTEN inhibits the mitogen-activated protein kinase (MAPK) pathway, via inhibition of S6 phosphorylation. S6 is a SH2-phosphotyrosine-binding adapter protein that associates tyrosine kinases to Ras signaling by recruiting the growth factor receptor binding protein 2 (Grb2)-Sos complex to the plasma membrane in a tyrosine phosphorylation-dependent manner (Rozakis-Adcock et al. 1992). This effect was not observed when a phosphatase-inactive mutant of PTEN was expressed instead. Inhibition of this pathway was rescued, in part, by expression of an upstream molecule in the MAPK signaling pathway, MAP or ERK kinase (MEK1). More recent studies from this group revealed that while both FAK and Shc-mediated PTEN inhibition affect cell migration, the FAK pathway is associated with a directional migration, while Shc is related to random migration (Gu et al. 1999). The differences in the migration pattern are related to the extent of actin cytoskeleton organization, which is more abundant in the FAK pathway. These PTEN migration and adhesion properties have yet to be reproduced by other groups.

Further studies aiming at characterizing PTEN's signaling through the FAK pathway revealed that p130Cas, a downstream target of FAK-mediated cell migration, does not associate directly with PTEN, although p130Cas phosphorylation levels are reduced in the presence of PTEN possibly via inhibition of FAK activity (Tamura et al. 1999). In addition, while it has been shown that overexpression of FAK and p130Cas is able to rescue PTEN-inhibited cell migration and spreading, only FAK expression reversed PTEN effects on cell growth. This suggests that certain functions of PTEN are mediated by pathways that diverge at the level of FAK. In agreement with this notion, a recent report shows that the lipid phosphatase domain of PTEN is not required for PTEN inhibition of migration in glioma cells, suggesting that PTEN exerts functions that are independent of AKT inhibition (Maier et al. 1999).

Crystal structure solved – new clues into PTEN substrate specificity

Recently, the PTEN crystal structure has been solved (Lee et al. 1999b). This information provides insights into putative functions of this tumor suppressor, as it helps in the identification of domains other than the catalytic core for...
Figure 4 PTEN function: an integrated model. This diagram attempts to integrate, in a concise form, the intracellular signals regulated by PTEN, according to recent studies. One of the signaling pathways, triggered by growth factors, involves predominantly PTEN's antagonism to PI-3 kinase activity, which is associated with PTEN dephosphorylation of lipid substrates and relates to control of cell growth and survival. These latter properties appear to be mediated by multiple putative factors whose detailed characterization is still unknown (some of the factors regulated by AKT and presumed to be involved in mediating such functions are shown in the figure). The other, less well studied, pathway of PTEN activity involves (but is not limited to) protein substrates, including FAK and Shc, which mediate cell migration and adhesion functions (see text for details).

PTEN activity, which can be used as the basis for further biochemical studies. The PTEN structure reveals a phosphatase domain that is similar to protein phosphatases but whose active site is extended to accommodate the phosphoinositide substrate (PtdIns(3,4,5)P3). In addition, a C2 domain was identified in the structure. C2 domains have been characterized in a growing number of eukaryotic signaling proteins that interact with cellular membranes and mediate a broad array of critical intracellular processes, including membrane trafficking, the generation of lipid-second messengers, activation of GTPases, and the control of protein phosphorylation (Nalefski & Falke 1996). These domains bind a variety of different ligands and substrates, including Ca2+, phospholipids, inositol polyphosphates, and intracellular proteins. The PTEN C2 domain extends towards the C-terminus of PTEN and binds phospholipid membranes in vitro. In consequence, mutation of critical basic residues at this site results in reduced membrane affinity of PTEN and also impairs its ability to suppress cell growth. The phosphatase and C2 domains associate extensively, suggesting that the C2 domain may serve to assist in positioning the catalytic domain on the membrane. This finding is in agreement with the PTEN null phenotype caused by certain mutations that locate away from the catalytic core (see above). In addition, it has repeatedly been shown that detection of significant catalytic activity of PTEN is only obtained when highly acidic substrates are used (Li & Sun 1997, Myers et al. 1997). The characterization of the structure of PTEN has provided insights into why that might be. Two basic residues located in the catalytic motif, K125 and K128, as well as H93 outside the catalytic motif, give the pocket a highly basic character which is absent from other phosphatases, such as vaccinia H1-related phosphatase (VHR) and protein tyrosine phosphatase 1B (PTP1B) (Lee et
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This is in keeping with PTEN’s preference for highly acidic substrates.

In summary, the crystal structure of PTEN has helped in the understanding of some of the functions attributed to this phosphatase by biochemical studies and has additionally provided several clues with regard to PTEN’s unique features. The C2 domain is involved in PTEN’s binding to the membrane, and the lipid phosphatase activity, as suggested by in vitro and in vivo studies, appears to be critical for PTEN tumor suppressor function. In particular, PtdIns(3,4,5)P3, the only PTEN substrate to carry a phosphate at position D5, is more important to PTEN tumor suppressor activity than the other D3 substrates without a phosphate at this position, PtdIns(3,4)P2 and PtdIns(3)P. This conclusion has been made based on the finding that mutations affecting the histidine at position 93 affect the phosphatase activity almost exclusively towards the triphosphate compound, in comparison with the other two lipid substrates (Lee et al. 1999b). From the data provided by PTEN structure, it does not appear that PTEN carries an additional phosphatase domain that accounts for a protein-directed activity distinct from the lipid activity, as some of the functional studies with mutants, in particular G129E, might have suggested. The phosphatase domain appears to be contained in a single portion of the structure.

More on PTEN knockout mice

As mentioned above, three different PTEN knockout mice were generated. While many features of these animals were common to all three mutants, including embryo lethality, some of the observed phenotypes varied substantially. One of the models describes deficient development of the three germinal layers (Di Cristofano et al. 1998), while a second one reports defects on patterning of the cephalic and caudal regions (Suzuki et al. 1998). A third mutant (Podsypanina et al. 1999) displays aberrant lymph node architecture and hyperplasia, compatible with a B-lymphocyte defect. Lymphomas and non-hamartomatous colonic polyps were also observed in this model. The major features of each one of the described mutants are presented in Table 2. Differences in the mouse background have been raised as a possible explanation for the differences observed, as well as the type of targeting construct used and the time of

Table 2 Comparison between the three PTEN knockout mice models generated to date.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Mouse background</td>
<td>CD1 and intercrosses with C57BL/6J</td>
<td>129/Sv and intercrosses with C57BL/6</td>
<td>129svJ and intercrosses with C57BL/J and B6/129</td>
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<tr>
<td>PTEN exons deleted</td>
<td>3–5</td>
<td>4–5</td>
<td>5</td>
</tr>
<tr>
<td>Embryonic day of death</td>
<td>E9.5</td>
<td>E7.5</td>
<td>E6.5</td>
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<tr>
<td>Developmental features of mutants</td>
<td>Abnormal cephalo-caudal patterning</td>
<td>Abnormal differentiation of the three germ layers in vitro</td>
<td>Abnormal lymph node architecture</td>
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<tr>
<td>Defects in generation of mesodermal lineages</td>
<td>Abnormal placentations</td>
<td>Lymphoid aggregates</td>
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<tr>
<td>Tumorigenic features of heterozygous mutants</td>
<td>T-cell leukemia/lymphomas</td>
<td>Dysplastic and inflammatory colonic polyps</td>
<td>Follicular and papillary thyroid tumors</td>
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<tr>
<td>Adenomatous liver</td>
<td>Prostate hyperplasia</td>
<td>Prostate hyperplasia and neoplasia</td>
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<tr>
<td>Teratocarcinoma</td>
<td>Gonadotrophin tumors</td>
<td>Pre-neoplastic multifocal complex atypical hyperplasia of endometrium</td>
<td></td>
</tr>
<tr>
<td>Prostate carcinoma</td>
<td>Teratoma</td>
<td>Lymphoblastic lymphomas</td>
<td></td>
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<tr>
<td>Hamartomatous colonic polyps</td>
<td>Papillary-like thyroid carcinoma</td>
<td>Poorly differentiated leukemia</td>
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<td>Prostate carcinomas</td>
<td>Myeloid leukemia</td>
<td>Liver adenoma</td>
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<td></td>
<td>Skin hyperkeratosis</td>
<td>Hyperplastic polyps of colon</td>
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<td></td>
<td></td>
<td>with lymphoid infiltrates</td>
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<td></td>
<td></td>
<td>Lymphoid hyperplasia</td>
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<td>(females &gt; males)</td>
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<td></td>
<td></td>
<td>Lymphomas</td>
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<td></td>
<td></td>
<td>Increased apoptotic rates</td>
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<td></td>
<td></td>
<td>(measured by annexin V) in heterozygous mice</td>
<td></td>
</tr>
<tr>
<td>Tumor LOH</td>
<td>Lymphomas</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Other features</td>
<td>Increased resistance to apoptosis</td>
<td>Enhanced anchorage-independent growth</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tumorigenesis accelerated by γ-irradiation</td>
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</table>

Histological abnormalities in bold are commonly found in Cowden syndrome (CS). Other common features of CS that have not been observed in any of the mutants are: breast cancer, multiple hamartomas of skin, breast and thyroid, and macrocephaly. Lymphomas have been described in CS patients; however, it has not been formally established whether they are a true component of the syndrome. ND: not described.
observation of the heterozygote animals. Of interest, few features typical of CS and/or BRR have been described in these animals.

It appears from the knockout data that unknown additional factors possibly help modulate the phenotypical features on a tissue-dependent and time of development basis. Longer observation times of the heterozygotes might be required for additional features to be identified. It is possible that in the months to come, reports will be available on aging mutant animals that will provide such important information. Inter-crosses between these Pten−/− knockout animals with other mutant strains associated with tumors and/or developmental abnormalities are also likely to shed light onto interactive pathways. The role of PTEN in certain tissues only may be further studied using Cre-Lox systems, whereby tissue-specific PTEN inactivation can be attained.

**PTEN homologs – Caenorhabditis elegans**

In the nematode, *C. elegans*, an insulin-related pathway regulates metabolism, development and longevity. Wild-type animals enter the developmentally arrested dauer stage in response to high levels of a secreted pheromone, accumulating large amounts of fat. In this state, metabolic functions are suppressed and it is associated with a prolonged life-span of the worms. Several mutants have been identified in *C. elegans* which associate with defective dauer formation (DAF mutants). DAF-2 mutants, which encode a homolog of the mammalian insulin receptor or insulin receptor-like (IRL), and AGE-1 mutants, homologs of the catalytic subunit of mammalian PI-3 kinase, arrest development at the dauer stage, and this effect can be rescued by expression of DAF-16, a downstream element in this pathway (Ogg et al. 1997). DAF-16 forkhead transcription factor is inactivated by DAF-18, a gene required for dauer formation, antagonizes the function of PI-3 kinase (Ogg & Ruvkun 1999). Detailed study of specific DAF-18 mutants has suggested that certain phenotypes, such as fertility, are less sensitive to PtdIns(3,4,5)P₃ levels than dauer formation. The existence of distinct phenotypes that arise from different DAF-18 mutants raises the interesting possibility that similar diversity may be seen with the mammalian homolog. It is interesting to speculate whether PTEN is involved in regulating glucose metabolism and pathways relating to aging control.

**Future directions of PTEN studies**

Despite the large amount of data presently available on *PTEN*, there are still many open questions with regard to its regulation and cellular role. The recently solved crystal structure of *PTEN* has already provided several insights into the catalytic function of this phosphatase, and has helped us to understand in further detail certain aspects of *PTEN’s* substrate specificity, in comparison with other tyrosine and dual-specificity phosphatases (Lee et al. 1999b). Figure 4 attempts to summarize the current signaling pathways associated with *PTEN* function. However, several issues still remain unresolved: there are no known upstream regulators of *PTEN* levels, although there have been some conflicting reports on the potential role of TGFβ on *PTEN* regulation (Li & Sun 1997, Lee et al. 1999a). Also, there are no current data on the mechanisms of *PTEN* activation; it is not known, for example, whether *PTEN* activity is modulated by post-translational modification such as phosphorylation, and how *PTEN* is degraded or inactivated. The putative role of naturally occurring *PTEN* alternative splice forms is not presently known. Studies determining the actual relevance of a *PTEN* dosage effect and whether haploinsufficiency indeed plays a role in tumorigenesis in humans are expected to be available soon. The nature of the factor or factors involved in *PTEN’s* decision towards inducing cell-cycle arrest or apoptosis in certain cell types is still elusive. Due to its important function as a tumor suppressor, *PTEN* is a natural candidate for gene therapy and it is likely that the next few years will provide preliminary analyses attempting to provide answers to its therapeutic potential in several tumor types.

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