Genetic and cell biological aspects of PTEN in prostate cancer

Printed by Printpartners Ipskamp

The research described in this thesis was performed at the Department of Pathology of the Josephine Nefkens Institute, ErasmusMC, Rotterdam.

The printing of this thesis was financially supported by the Department of Pathology of the ErasmusMC and the Erasmus University Rotterdam.

Genetic and Cell Biological Aspects of PTEN in Prostate Cancer

Genetische en celbiologische aspecten van PTEN in prostaatkanker

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. S.W.J. Lamberts

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
woensdag 19 november 2008 om 9.45 uur

door

Petra Wilhemina van Duijn

geboren te Utrecht

Promotiecommissie

Promotor:

Prof.dr.ir. J. Trapman

Overige leden:

Prof.dr. L.H.J. Looijenga

Prof.dr. J.A. Schalken

Dr.ir. G.W. Jenster

Contents

Chapter 1

General Introduction

Introduction

The dual specific phosphatase PTEN (Phosphatase and TENsin homolog deleted on chromosome 10) is one of the most extensively studied proteins of the last decade. It was the first phosphatase identified as a tumor suppressor and in sporadic cancers *PTEN* is one of the most frequently altered genes. Its deregulation is also implicated in several other diseases. In addition, PTEN is critically important during embryonic development and is implicated as a key player in maintaining normal stem cell function. Unraveling of the physiological regulation and function of PTEN will augment our understanding of tumorigenesis and ultimately lead to novel therapeutic options.

1.1 PTEN structure and function

1.1.1 PTEN protein function

In 1997 three groups independently identified a novel tumor suppressor gene at 10q23.3, denoted PTEN, MMAC1 (Mutated in Multiple Advanced Cancers), or TEP1 (TGFβ regulated and Epithelial cell-enriched Phosphatase) [1-3]. The *PTEN* gene encodes a protein of 403 amino acids that shares sequence homology with the family of protein tyrosine phosphatases (PTP), as well as with the cytoskeletal protein tensin [1,3]. The protein contains the HCXXGXXR catalytic signature motif present in all PTPs and in dual specificity phosphatases, which catalyze the hydrolysis of phospho-seryl, -threonyl, and -tyrosyl residues. In addition, this phosphatase signature motif is very well conserved across evolution indicating its functional significance [4]. Recombinant PTEN has been shown to dephosphorylate protein substrates *in vitro* on serine, threonine, and tyrosine residues indicating that PTEN can indeed function as a dual specificity protein phosphatase [5]. The best known protein substrate of PTEN is Focal Adhesion Kinase (FAK) [6]. In addition PTEN might possess protein phosphatase activity towards itself and the Plateled Derived Growth Factor Receptor (PDGFR) [7,8]. Recombinant PTEN, however, exhibited a high activity towards a highly negatively charged, and multiple phosphorylated substrate like the $(Glu\text{-}Tyr)_n$ polymer, suggesting a preference for highly acidic substrates [5]. Maehama and Dixon (1998) were the first to show that PTEN can also dephosphorylate phosphatidylinositol lipids both *in vitro* and *in vivo*, with a preference for the phosphate group at the D3 position of the inositol ring [9] (Figure 1A).

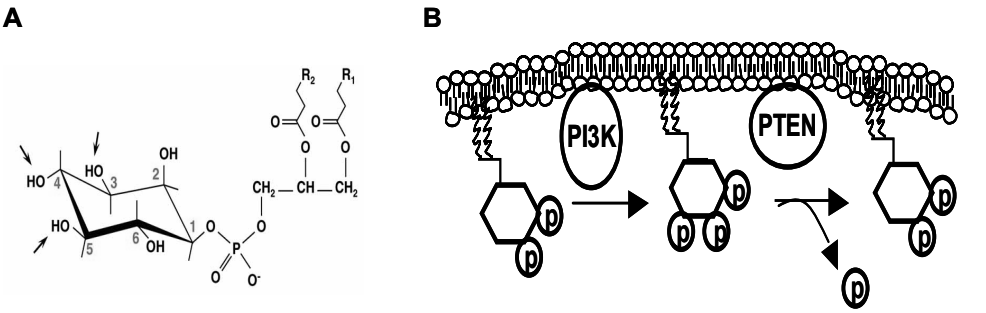


Figure 1. (A) Structure of phosphatidylinositol. Candidate phosphorylation sites are indicated by arrows. PTEN preferentially dephosphorylates PtdIns at the D3 position. (B) PTEN antagonizes the function of PI3K, thereby negatively regulating PI3K dependent signal transduction pathways.

Although PTEN can dephosphorylate the phospholipids PI(3)P, PI(3,4)P2, and PI(3,4,5)P3 *in vitro*, PIP3 is its most important substrate *in vivo*. PIP3 is a lipid second messenger produced by PI3K (phosphatidylinositol-3 kinase) and plays an important role in signal transduction pathways that regulate a variety of cell biological processes. PTEN and PI3K have opposing effects on the PIP3 level (Figure 1B) and as a consequence they counteract each other's actions in the regulation of important cell biological processes like proliferation and survival. The lipid phosphatase activity of PTEN is critical for its tumor suppressive function [10].

1.1.2 PTEN protein structure

Analysis of the crystal structure of PTEN identified two major domains, the phosphatase domain and a C2 domain [11]. Although the structure of the N-terminal phosphatase domain is similar to other protein phosphatases, the active site of PTEN is larger and more basically charged, thereby allowing binding of the lipid substrate PIP3 [11]. Many proteins involved in signal transduction and vesicle trafficking contain C2 domains, which mediate binding to phospholipid membranes [12]. Whereas the majority of C2 domains bind Ca^{2+} via their acidic residues that bridge them to the membrane, the presence of more basic residues in PTEN predicts a direct contact with the membrane [11]. The C2 domain of PTEN is thought to have a function both in protein stability and in the regulation of catalytic activity by productive orientation of the catalytic site [11,13]. Besides the C2 domain, the carboxy-terminal tail of PTEN contains two PEST (Proline-Glutamic acid-

Serine-Threonine) sequences, which are involved in the regulation of protein stability, and a PDZ binding motif (also known as Glycine-Leucine-Glycine-Phenylalanine (GLGF) repeat) that might play a role in protein-protein interactions (reviewed in ref. 14) (Figure 2).

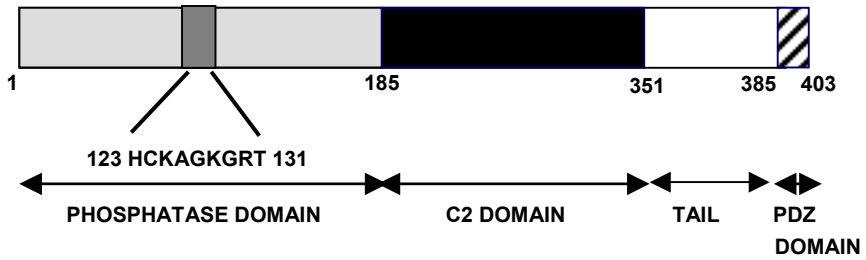


Figure 2. Schematic representation of the domain structure of PTEN. In the N-terminal phosphatase domain the catalytic core sequence is depicted. The C-terminal domain contains the lipid-binding C2 domain, PEST sequences in the tail region and a PDZ domain.

1.1.3 Regulation of PTEN expression and activity

PTEN expression and activity is regulated by complex control mechanisms at both the mRNA and protein level. PTEN was originally identified as a gene transcriptionally regulated by TGFβ, and to date several factors have been demonstrated to regulate PTEN transcription both positively (p53, PPARγ and EGR-1) and negatively (c-Jun and NFκB) (reviewed in ref. 15). However, the physiological significance of most of these transcription factor binding sites remains to be elucidated.

Several groups have reported *PTEN* promoter hypermethylation as a mechanism to suppress PTEN expression in various types of cancer [16-23]. The genomic sequence of *PTEN* is 98% identical to a highly conserved processed *PTEN* pseudogene (*ψPTEN*) at 9p13 and this sequence identity extends a few hundred base pairs into the promoter region [24]. A study by Zysman et al., (2002) showed that the pseudogene and not *PTEN* is predominantly methylated in cell lines and tumors, indicating that data on PTEN promoter methylation must be interpreted with caution [18].

Recently, several miRNAs were reported to be involved in the regulation of PTEN expression [25-27]. MiRNAs are short, endogenous, single-stranded RNAs that could modulate gene expression by inducing degradation, or inhibiting translation

caused by base-pairing to sequences located in the 3'UTR of their target mRNA [28]. The first miRNA that was found to influence PTEN expression was miR-21 [25]. Inhibition of miR-21 in human hepatocellular cells significantly increased the expression of PTEN. In ovarian cancers over-expression miR-214 was found to target the 3'UTR of PTEN, leading to down-regulation of PTEN and activation of the Akt pathway [26]. Finally, the miR-17-92 cluster was found to suppress the expression of PTEN in lymphocytes of miR-17-92 transgenic mice [27]. Although a growing number of miRNAs have been implicated in promoting or suppressing tumorigenesis in a variety of tissues, their precise role in the regulation of PTEN expression and activity remains to be further elucidated.

At the protein level phosphorylation of the C-terminal tail of PTEN contributes to the regulation of its activity and stability. Several phosphorylation sites are involved in modulation of PTEN stability, activity, and subcellular distribution [29,30]. The phosphorylation of Ser380, Thr382, and Thr383 by casein kinase-II decreases the affinity of PTEN for the membrane thereby affecting its phosphatase activity. Furthermore, the casein kinase-II mediated phosphorylation of PTEN increases its stability by preventing its proteasomal degradation [29,30]. In contrast, phosphorylation of Thr366 plays a role in destabilizing PTEN [31].

It is thought that phosphorylated PTEN exists in a more closed conformation that masks the PDZ-binding domain. The more open conformation of unphosphorylated PTEN allows interaction with other PDZ domain containing proteins like MAGI-1/2/3, hDLG, and hMAST [30,32,33]. Binding of PTEN to specific PDZ-domain containing proteins affects its stability and phosphorylation [30,33].

A common mechanism to regulate protein levels posttranslationally is ubiquitin mediated proteasomal degradation. PTEN contains two PEST motifs (see above), which are frequently found in proteins targeted for degradation by the ubiquitin pathway. In addition, treatment of cells with proteasome inhibitors increased the PTEN protein level, indicating a role for ubiquitin mediated proteasomal degradation in the regulation of PTEN [34,35]. Recently, the E3 ubiquitin ligase for PTEN, NEDD4-1, was identified. NEDD4-1 negatively regulates PTEN stability by catalyzing PTEN polyubiquitination [36].

PTEN activity can further be affected by reversible oxidation of Cys124 at the phosphatase active site, which has also been described for other PTP family members [37,38]. Oxidation reduces the catalytic activity of the protein, and several reports describe the regulation of PTEN activity by redox processes [39-42]. Finally,

the catalytic activity of PTEN can be negatively affected by acetylation at Lys125 and Lys128. This acetylation is promoted by the interaction of PTEN with the nuclear histone acetyl-transferase-associated PCAF [43].

1.1.4 Nuclear PTEN

The structural characteristics of PTEN led to the general understanding that PTEN is localized in the cytoplasm. However, several studies described nuclear PTEN staining in a number of different cell types [44-46]. Interestingly, PTEN was found to be predominantly localized to the nucleus in primary, differentiated, and resting cells suggesting that loss of nuclear PTEN might correlate with increased tumorigenicity [45,47]. Despite the presence of many components of the PI3K signaling pathway in the nuclear compartment, Lindsay et al., (2006) reported that PTEN does not dephosphorylate the nuclear pool of PIP3 [48]. Moreover, conflicting data are reported about the Akt mediated effects of nuclear PTEN [49,50]. Nuclear PTEN has been proposed to act as a pro-apoptotic factor, a regulator of chromosomal integrity, and as a cell cycle regulator through suppression of cyclin D1 activity [51-53]. The mechanism underlying the migration of PTEN to the nucleus is not completely understood since PTEN lacks obvious nuclear import/export signals, although PTEN might contain two non-canonical Nuclear Localization Signals (NLS) [54]. Recently, it was suggested that nuclear import of PTEN is regulated by monoubiquitination, since increased nuclear PTEN expression was associated with increased monoubiquitination of the protein [50,55].

1.2 Somatic and germline *PTEN* mutations

Since the discovery of *PTEN* in 1997, many papers have described its frequent inactivation in a wide array of sporadic human cancers, as well as its identification as the susceptibility gene in cancer predisposition syndromes [56-58]. Germline *PTEN* mutations have been implicated Proteus syndrome and in the autosomal dominant disorders Cowden disease (CD), Bannayan-Zonana Syndrome (BZS, also called Bannayan-Riley-Ruvalcaba Syndrome), and Lhermitte-Duclos disease (LDD) [56,59-62].

CD is characterized by multiple hamartomas (benign growths composed of tissue elements normally found at the affected site, but which are growing in a disorganized mass) and an increased risk of breast, thyroid, and endometrial cancer. LDD, or dysplastic gangliocytoma of the cerebellum, is the neurological

manifestation of CD [63,64]. *PTEN* mutations have been identified in up to 80% of CD and LDD cases, and approximately two-thirds of these mutations were found in exons 5, 7 and 8 [14]. The characteristics of BZS include macrocephaly, lipomatosis, and speckled penis, and ~ 60% of cases have been found to harbor germline *PTEN* mutations [14,65]. Additionally, up to 20% patients with Proteus syndrome and approximately 50% of Proteus-like syndrome cases contain germline *PTEN* mutations [66]. Since the *PTEN* mutation spectra of these disorders appeared to be similar it has been suggested that syndromes characterized by the presence of *PTEN* germline mutations can be grouped together for clinical purposes and classified as PTEN Hamartoma Tumor Syndrome (PHTS) [67].

Somatic deletions and mutations of *PTEN* have been identified in a large fraction of tumors placing *PTEN* among the most commonly affected genes in human cancer [57]. Inactivating *PTEN* mutations are described with high frequency in glioblastomas, endometrial cancers, and also in prostate cancer [1,68-70]. In other tumor types such as breast, thyroid, bladder, ovary, small cell lung cancer, and hematological malignancies lower inactivation rates were found [57]. *PTEN* point mutations are scattered over the entire gene although a clustering of missense mutations in exon 5, encoding the phosphatase domain, is observed [58]. Two important mutants are recognized in the phosphatase core motif of PTEN, the C124S mutant in which both its protein and lipid phosphatase activity is completely blocked, and the naturally occurring G129E mutant that retained only the PTEN protein phosphatase activity [5,9,10].

1.3 Molecular targets of PTEN

1.3.1 PTEN signaling

As previously mentioned PTEN counteracts the action of PI3K and thereby functionally antagonizes the many signaling pathways that rely on the activity of PI3K [71] (Figure 3). The PI3K product PIP3 is localized at the inner surface of the cell membrane and acts as docking site for proteins that contain a pleckstrin homology (PH) domain. An important PH domain containing protein that interacts with PIP3 is PDK1 (3-phosphoinositide-dependent protein kinase-1) [72]. PDK1, member of the AGC (cAMP-dependent, cGMP-dependent, and protein kinase C) protein kinase family, has been found to activate other members of this family such as Akt, PKC, SGK, $p70^{s6k}$, and PKN3 by phosphorylating their activation loop [73]. Because of its function as a master upstream kinase controlling the activation of

various AGC kinase family members, PDK1 is involved in the regulation of diverse cellular processes [73].

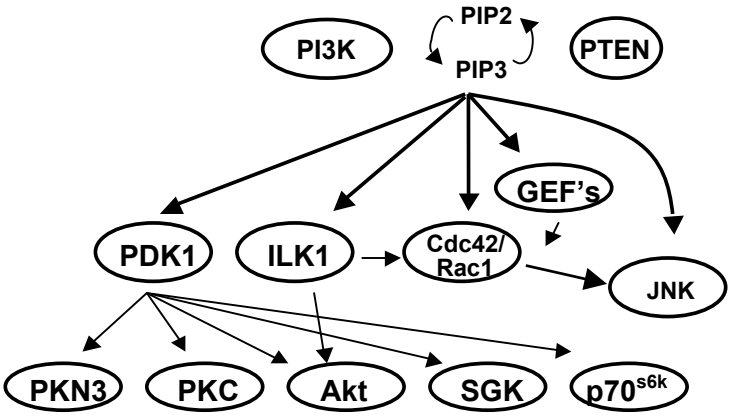


Figure 3. Schematic representation of PTEN signaling pathways. PDK1 activates other members of the AGC protein kinase family thereby affecting many cellular responses such as proliferation and apoptosis. The PDK1 targets Akt and $p70^{s6k}$ will be discussed in futher detail in section 1.3.2.

The activation of ILK (Integrin-Linked Kinase) is also dependent on PI3K signaling. ILK contains a PH-like domain and is implicated in the regulation of growth, inhibition of apoptosis, and migration [74]. It was found that PTEN can inhibit ILK activation, and inhibition of ILK was shown to abolish Akt activation and induce apoptosis [75-77]. Recently, ILK was reported to be involved in the activation of the Rho GTPases Rac1 and Cdc42 [78-80].

In addition to the ILK dependent activation of Rac1 and Cdc42, it was demonstrated that also GEFs (Guanine nucleotide Exchange Factors), which can activate Rho GTPases like Rac1 and Cdc42 by conversion of GDP to GTP, can be activated in a PIP3 dependent manner [79,81]. Interestingly, all members of the Dbl protein family of GEFs contain a PH domain that might be involved in their PI3K dependent activation and indeed it was shown that in PTEN negative fibroblasts a marked increase in the active GTP-bound forms of Rac1 and Cdc42 could be detected [82].

Recently, the JNK signaling pathway was identified as a functional target of PTEN by gene set enrichment analysis [83]. Further research showed that PTEN null cells displayed an increased transcriptional activity of c-Jun, a dependence on

JNK1 and JunD for *in vitro* proliferation and tumor growth, and a synergy of JNK and Akt, which was further strengthened by the association of phospho-jun and phospho-Akt staining in human prostate cancers [83]. In addition, growth factor induced JNK activation in PTEN null cells was found to be dependent on PI3K and mediated by Rac1.

1.3.2 The Akt pathway

The best studied signaling pathway that relies on the activity of PI3K and PTEN, is the Akt signal transduction route, which is involved in the regulation of many cellular processes [84]. Akt is a serine/threonine kinase that is recruited to the membrane upon the formation of PIP3. Once located at the membrane Akt becomes phosphorylated by PDK1 at Thr308. Although this phosphorylation step is sufficient for the activation of Akt, maximal activation is achieved after phosphorylation of Ser473 by the so-called hydrophobic motif kinase PDK2 [85]. Although several kinases have been proposed to function as the Akt hydrophobic motif kinase, biochemical studies have identified the TORC2 complex as the elusive PDK2 [86,87]. In mammals three highly homologous Akt isoforms have been identified, Akt1, Akt2, and Akt3. These isoforms are broadly expressed but next to their overlapping functions, isoform specific knockout mice revealed distinct phenotypes indicating the presence of isoform specific functions [88]. The minimal substrate consensus sequence of Akt is RXRXXS/T and to date, numerous Akt substrates have been identified [89] (Table 1). A few prominent downstream effectors of Akt such as TSC1/2, mTOR, FOXO transcription factors, GSK3 (Glycogen Synthase Kinase-3), and MDM2 (Mouse Double-Minute 2) will be discussed in further detail.

Important targets in the Akt pathway are the FOXO transcription factors. The FOXO protein family consists of FOXO1, FOXO3a, FOXO4, and FOXO6. These transcription factors regulate the expression of many genes involved in proliferation and apoptosis, like *CCNG2* (Cyclin G2), *BIM*, and *FASL* [90]. The FOXO family was initially identified in humans because three family members, FOXO1, FOXO3a, and FOXO4, were found to be located at chromosomal translocation points in human tumors [91,92]. Biochemical studies showed that Akt directly phosphorylates FOXO1, FOXO3a, and FOXO4 at three key residues (Thr32, Ser253 and Ser315 in the FOXO3a sequence) resulting in inactivation of these transcription factors due to nuclear export [93,94]. FOXO6, however, remains

mostly nuclear because its shuttling ability is dramatically impaired due to lack of the conserved third Akt regulatory site [95,96]. Although FOXO6 can be considered constitutively nuclear, its transcriptional activity is still regulated in an Akt dependent manner [96]. In addition to the nuclear export and following sequestration into the cytoplasma, Akt specific phosphorylation has also been reported to play a role in the proteasomal degradation of FOXO1 and FOXO3a [97,98].

Table 1. Overview of Akt target proteins

Target protein	Phosphorylation site	Ref. no.
Androgen Receptor	Ser210, Ser790	99
	Ser588, Thr642	100
AS160		
Ask1	Ser83	101, 102
Bad	Ser136	103
Caspase 9	Ser196	104, 105
Creb	Ser133	106
eNos	Ser1179	107
FOXO	Thr32, Ser253, Ser315	93
GSK3 beta	Ser9	108
Ikk alpha	Thr23	109
IRS	Ser629	110
Merlin	Ser230, Thr315	111
MDM2	Ser166, Ser186	112
mTOR	Thr2446, Ser2448	113
$P21^{cip1/waf}$	Thr145	114
$P27^{kip1}$	Ser10, Thr187, Thr198	115
Pras40	Thr246	116
Raf1	Ser259	117
Synip	Ser99	118
TSC2	Ser939, Ser1086/1088, Thr1422	119
Wnk	Thr60	120
Xiap	Ser87	121
Y box binding protein	Ser102	122

An other key player of the Akt pathway is mTOR (mammalian Target Of Rapamycin) that functions as a serine/threonine kinase regulating protein synthesis and cell size [123-126]. The Akt mediated regulation of mTOR activity is complex as indicated in Figure 4.

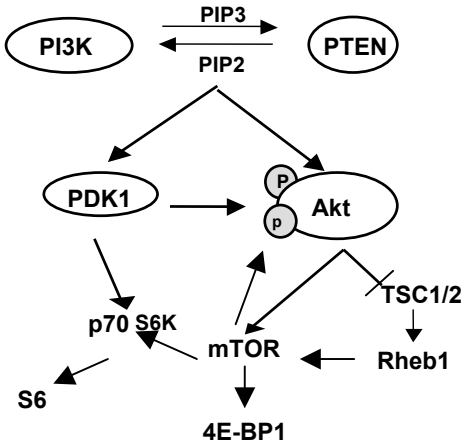


Figure 4. Schematic representation of PI3K/Akt dependent mTOR activation.

Akt might directly phosphorylate mTOR since the protein contains two putative Akt phosphorylation sites; Thr2446 and Ser2448. It was shown both *in vitro* and *in vivo* that Akt indeed phosphorylates Ser2448, although the role of this phosphorylation remained unclear, because it was not required for signaling downstream of mTOR [113,127-129]. Akt more indirectly affects mTOR activity through regulation of the TSC1 (Hamartin)/TSC2 (Tuberin) complex [119,130,131]. This complex inhibits the activity of the small GTP-ase Rheb, which in turn leads to the inactivation of mTOR [119,130,132,133]. Akt was found to phosphorylate and thereby inactivate TSC2, thus upon phosphorylation of TSC2 by Akt, the TSC1/TCS2 complex is no longer capable of inactivating Rheb resulting in an increased activity of mTOR [119,130,131].

The important role of mTOR in translation initiation is mainly mediated through the effector proteins $p70^{s6k}$ and 4E-BP1 [134]. mTOR phosphorylates $p70^{s6k}$ at Thr389 and thereby activates the protein which in turn phosphorylates the ribosomal protein S6, leading to an increase in translation of a subset of mRNAs [135]. Next to phosphorylation by mTOR, several reports suggested that $p70^{s6k}$ can also be directly phosphorylated at Thr229 by PDK1, the kinase that is also required for the activation of Akt [73,136]. Both phosphorylation events may however be

complementary to each other, since phosphorylation of $p70^{s6k}$ by mTOR would enable PDK1 to bind to and phosphorylate $p70^{s6k}$ thereby fully activating $p70^{s6k}$ kinase activity [137]. The other effector of mTOR, 4E-BP1 normally binds eIF4E, the mRNA cap-binding protein, thereby preventing cap-dependent translation by competitively blocking the binding of eIF4G to eIF4E. Phosphorylation of 4E-BP1 by mTOR causes its dissociation from the eukaryotic initiation factor eIF4E, which can in turn bind to the 5'cap of mRNAs and recruit other members of translation initiation complex that finally results in increased translation initiation [138].

Another interesting target protein whose function is impaired upon phosphorylation by Akt is GSK3. Initially GSK3 was identified as an enzyme that regulates glycogen synthesis in response to insulin. Nowadays it is clear that GSK3 phosphorylates a broad range of substrates and is therefore a key component in a large number of cellular processes and diseases [139-141]. GSK3α and GSK3β are two closely related isoforms that are ubiquitously expressed in mammalian tissues. Two sites of phosphorylation directly affect its kinase activity. GSK3 activity is facilitated by phosphorylation of Tyr216 (GSK3β) and Tyr279 (GSK3α), although little is known about the regulation of this process. In contrast, phosphorylation of Ser9 (GSK3β) and Ser21 (GSK3α) dramatically impaired the kinase activity of GSK3 [139,140]. Several kinases are reported to phosphorylate these serine residues, including Akt. Interestingly, GSK3 was the first characterized physiological substrate of Akt [108]. Akt dependent inactivation of GSK3 was reported to affect both apoptosis and proliferation [142,143].

The Akt target MDM2 (mouse double-minute 2), which was originally identified on double-minute chromosomes of spontaneous transformed mouse 3T3 fibroblasts[144], is an oncogene that is amplified in several types of cancer [145]. MDM2 functions as an E3 ubiquitin ligase of which the tumor suppressor p53 is one of its main targets [146]. Akt is able to phosphorylate MDM2 at Ser166 and Ser186, and this phosphorylation is required for the translocation of MDM2 to the nucleus where it can interact with p53 [112,147,148]. Akt dependent phosphorylation of MDM2 is further suggested to function in the regulation of MDM2 protein stability [149].

1.4 Cell biological effects of PTEN

PTEN has a crucial function in the regulation of multiple cellular processes. Thus far, the current understanding of PTEN function in tumorigenesis is primarily based on its lipid phosphatase activity. However, emerging evidence is suggestive

of other, phosphatase independent, functions of PTEN both in the cytoplasm as well as in the nuclear compartment that might contribute to the regulation of tumorigenesis [150]. In this chapter the various cellular processes that are influenced by PTEN will be discussed whereby the role of PTEN in cell cycle regulation, and in particular the G_1-S transition, will be specifically emphasized.

1.4.1 PTEN dependent cell cycle regulation

Movement through the cell cycle is orchestrated by the activity of cyclin dependent kinases (CDK). Their activation, in turn, depends on association with cyclin regulatory subunits, whereas they become inactivated after association with cyclin dependent kinase inhibitors (CDKI). In response to mitogenic signals, like growth factor stimulation, cells are induced to progress from the first gap phase (G_1) of the cell cycle to the DNA synthesis phase, the S-phase. This progression requires assembly of D-type cyclins with CDK4/6, and in late G_1 the cyclin E/CDK2 complex formation (Figure 5). These active complexes phosphorylate and thereby inactivate Retinoblastoma (RB) resulting in the release of the E2F transcription factors that in turn activate the transcription of the so-called S-phase genes [151]. The CDK1/Cyclin B1 complexes are in turn pivotal in the regulation of G_2/M transition.

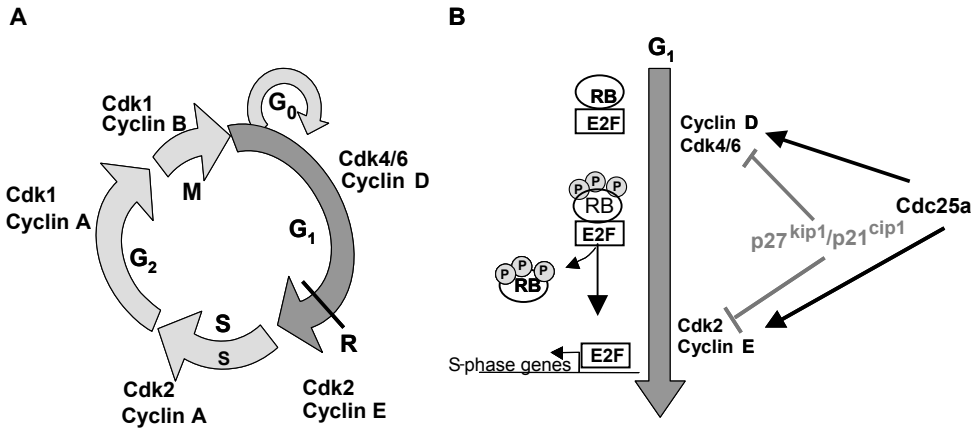


Figure 5. Schematic overview of the mammalian cell cycle

(A) Overview of the different phases of the mammalian cell cycle and the corresponding cdk/cyclin complexes. (B) Schematic representation of G_1 phase of the cell cycle

PTEN has been described as an important regulator of cell cycle progression. Both its lipid and protein phosphatase activity are thought to be involved in this regulation [152]. A large amount of data exists on the role of the PTEN/Akt pathway influencing the expression of cell cycle regulators at the protein level by altered distribution, or phosphorylation and thereby stability [52,152-157]. Recent evidence appeared that also PTEN dependent transcription regulation can contribute to cell cycle control [158] (see also chapter 3).

In several PTEN-null tumor cell lines it has been reported that forced expression of PTEN leads to the induction of G_1 cell cycle arrest [154,159-162]. An important mediator of this PTEN dependent G_1 cell cycle arrest is the Akt signaling pathway. One mechanism by which Akt could control cell cycle regulation is by direct phosphorylation of the cell cycle inhibitors $p21^{cip1/Waf1}$ and $p27^{kip1}$ resulting in their accumulation in the cytoplasm [153,155-157]. The effect of PTEN/Akt signaling on $p27^{kip1}$ is, however, complex, and might occur at several levels. Next to cytoplasmic sequestration upon phosphorylation, Akt can also function through the ubiquitin ligase Skp2 to regulate proteasomal degradation of $p27^{kip1}$ [163-166] (Chapter 2). Recently, it was described that PTEN together with cyclin D1 modulate the assembly of the SCF^{Skp2} complex and thereby affecting $p27^{kip1}$ proteolysis [166]. At the transcriptional level the FOXO family of transcription factors is implicated in the regulation of $p27^{kip1}$ in several cell lines [167-169]. Although PTEN induced cell cycle arrest was associated with altered $p27^{kip1}$ expression, we and others described a lack of correlation between PTEN and total $p27^{kip1}$ expression [160,163,170] (see also Chapter 2). The observed effects might be cell line specific, but, since these studies did not discriminate between nuclear and cytoplasmic $p27^{kip1}$ expression, it is possible that in these cell lines PTEN signaling altered the cellular distribution of $p27^{kip1}$ without affecting its total expression level.

Cyclin D1 might be another mediator of PTEN/Akt dependent cell cycle arrest. Several groups reported a down regulation of cyclin D1 expression upon inhibition of PTEN/Akt signaling, although the underlying regulatory mechanism is complex [52, 171, 172]. Roles for mTOR/$p70^{s6k}$, FOXO, and GSK3 have been suggested in the regulation of cyclin D1 expression levels [142,171,172]. Moreover, it was described that PTEN not only affected the expression level of cyclin D1 but also prevented its nuclear localization, thereby inhibiting its stimulating effect on cell cycle progression [52]. Besides its Akt mediated effect on cyclin D1 expression, others

have suggested that the PTEN protein phosphatase activity was responsible for the down regulation of cyclin D1 [152].

Another target that is involved in PTEN dependent cell cycle regulation is RB-like protein 2/p130, which has an important role in the restraining of the G_1/S phase transition [173,174]. RBL2 has been reported as a FOXO target in certain cell lines [174]. However, in LNCaP prostate cancer cells the PTEN dependent regulation of RBL2 is independent of the Akt/FOXO pathway (see Chapter 3). Other transcriptional targets of PTEN signaling involved in the regulation of G_1 growth arrest include cyclin G2, E2F2, and Cdc25a (Chapter 3).

Next to the effect PTEN/Akt signaling on the G_1/S transition, several reports also suggest a role for Akt in the regulation of the G_2/M checkpoint [175-179]. However, the mechanism by which Akt regulates progression through the G_2/M phase remains largely unclear.

1.4.2 Apoptosis

Programmed cell death, or apoptosis, is a normal cellular process controlling excessive proliferation. In several cell lines manipulating PTEN/PI3K signaling has led to altered apoptosis rates [162,180-183]. PTEN mediated apoptosis can be rescued by the expression of constitutively active Akt, implicating an important role of PI3K/Akt signaling in the regulation of apoptosis [159,181]. However, Akt dependent survival may be cell line specific, since knockdown of Akt in various cell lines resulted in apoptosis in only half of the lines [181]. Akt inhibits apoptosis through phosphorylation of downstream substrates including, FOXO, BAD, and IκB, that directly or indirectly control the apoptotic machinery [180].

PTEN is also involved in a specialized apoptotic pathway, termed anoikis, which is the induction of apoptosis in cells after loss of contact with the extra cellular matrix [184-186]. Anoikis has been linked to altered expression of FAK, which can be dephosphorylated by the protein phosphatase activity of PTEN [187].

1.4.3 Cell Growth

Cell growth may be defined as the increase of cell size as well as the increase of total cell number (proliferation). The processes of cell size control and proliferation are closely coupled, since cells need to grow to a critical size before progressing through the cell cycle, and PTEN is implicated in the regulation of both [188] .

The first indication that PTEN might function in cell size regulation came from studies in the fruitfly, *Drosophila Melanogaster* [189-191]. It was shown that loss of dPTEN caused increased cell size, organ size and even animal size. Studies utilizing Pten conditional knockout mice demonstrated a role for Pten also in mammalian cell size regulation [192]. However, Pten null ES cells, MEFs, and thymocytes do not show an apperant size control defect suggesting that PTEN may control cell size in a cell type specific manner [193]. The control of cell size by the PTEN signaling pathway is thought to be due to the ability of this pathway to regulate protein synthesis via its downstream target mTOR and its corresponding effector proteins $p70^{s6k}$ and 4E-BP1 [123,124].

1.4.4 Angiogenesis

Angiogenesis is a physiological process involving the growth of new blood vessles from pre-existing vessels and is a fundamental step in the transition of tumors to a malignant state. Already several years ago, PTEN inactivation in clinically localized prostate cancers was found to be associated with increased angiogenesis [194]. Further research revealed that PTEN modulates angiogenesis by Akt mediated regulation of HIF-1 and VEGF expression [195,196].

1.4.5 Cell motility

The movement of a cell towards a chemoattractant signal is a fundamental process involved in a wide range of cellular responses, including morphogenesis during development, wound healing, and metastasis of tumor cells [197]. When cells sense a chemoattractant gradient, they dramatically change their shape, polarizing in the direction of the gradient. Several studies have shown that the generation of a PIP2/PIP3 gradient is a conserved mechanism for chemoattractant directional sensing and cell polarization, thereby implicating a regulatory role for PTEN in this process [198,199]. Important effectors of the PTEN lipid phosphatase dependent control of cell motility are its downstream targets Rac1 and Cdc42 [82,200]. Furthermore, it has been suggested that PTEN negatively regulates cell migration by directly dephosphorylating FAK [6]. Besides these phosphatase dependent mechanims it has also been reported that PTEN can regulate cell migration through its C2 domain [7,198].

1.4.6 Stem Cell regulation

All stem cells have the capacity to self-renew and to differentiate in mature cell types in order to sustain homeostatic control in normal tissues. Pten was found to control the self-renewal capacity of neural stem/progenitor cells [201,202]. More recently it was observed that Pten might also be implicated in the maintaining of normal stem cell function in the hematopoietic system [203,204]. Like in normal tissue homeostasis, cancers are thought to be maintained by a minor sub-population of cells within the tumor called the cancer stem cells, and many pathways that are known to promote tumorigenesis have also been implicated in stem cell self-renewal [205]. However, Yilmaz et al., (2006) showed that in the hematopoietic system loss of Pten resulted in excessive proliferation of leukemic stem cells but also in depletion of normal hematopoietic stem cells, suggesting different self-renewal mechanisms for maintaining pools of leukemic stem cells and normal hematopoietic stem cells [204].

Interestingly, FOXO transcription factors were recently implicated to play a critical role in hematopoietic stem cell homeostasis [206-208]. In mice the conditional knock-out of *FoxO1*, *FoxO3,* and *FoxO4* in the hematopoietic system resulted in a marked reduction of the hematopoietic stem cell number [207]. However, the remaining hematopoietic stem cell pool showed a striking increase in the proportion of cells in the $S/G_2/M$ phases of the cell cycle, suggesting that FoxO transcription factors are important for the preservation of the replicative and self-renewal capacity of hematopoietic stem cells by maintaining their quiescent state [207].

1.4.7 Genomic instability

Chromosomal instability is an important hallmark of many cancers and several studies have recently suggested a role for PTEN in maintaining normal chromosome structure and function [53,209]. Loss of PTEN expression in primary human breast cancers has been associated with an increased prevalence for aneuploidy [209]. It was suggested that PTEN suppresses aneuploidy through Akt dependent pathways. In contrast to this PTEN phosphatase dependent regulation of chromosomal stability, nuclear PTEN was recently shown to be involved in the maintenance of genomic stability through physical interaction with the centromers and by the control of DNA repair in a phosphatase independent manner [53]. This

suggests that cytoplasmic and nuclear PTEN might have different functions in regulating certain cell biological processes.

1.5 PTEN expression and function in other organisms

The phosphatase signature motif $C(X)_5R$ of PTEN, which is essential for catalysis, is highly conserved across evolution suggesting that the phosphatase activity of PTEN could be comparable among the various species (Table 2) [4]. In the fission yeast *Schizosacchharomyces pombe* the *PTEN* homologue, *ptn1*, was also found to suppress PIP3 levels [210]. Genetic analysis of the nematode *Caenorhabditis elegans* genetic identified *daf-18* as the homologue of the mammalian *PTEN* [211,212].

Daf-18 loss of function mutants displayed a reduced life-span and blocked entry into dauer stage, a dormant state in response to unfavorable conditions like starvation, by affecting the daf-2 (insulin receptor) /AGE-1 (PI3K) pathway [211-214]. The Drosophila PTEN homolog, *dPTEN*, was shown to suppress hyperplastic growth in flies by reducing cell size and number [190]. Genetic experiments indicated that these effects were primarily mediated by the antagonistic effect of dPTEN on Chico (Insulin Receptor Substrate) / Dp110 (PI3K) signaling pathway [189,190]. Loss of function mutants displayed distinct phenotypes compared to flies in which PI3K was activated by overexpression of Dp110 or dAkt, suggesting that dPTEN acts through both PI3K dependent and independent mechanisms [215].

Table 2. PTEN phosphatase signature motifs across various species

Species	Gene symbol	Phosphatase signature motif
H. sapiens	*PTEN*	VAAIHCKAGKGRTG
M. musculus	*Pten*	VAAIHCKAGKGRTG
R. norvegicus	*Pten*	VAAIHCKAGKGRTG
C. elegans	*daf-18*	VIAVHCKAGKGRTG
D. melanogaster	*dPTEN*	VVAVHCKAGKGRTG
S. Pombe	*ptn1*	VHCKAGKGRTG

Mouse *Pten* shares 98% homology with its human counterpart. The physiological functions of Pten in mouse tissues have been extensively studied utilizing different (conditional) knock-out models [216-219]. Homozygous knock-out of *Pten* was found to be embryonic lethal within a range of embryonic days 6.5-9.5 indicating that Pten is essential for embryonic development. Heterozygous $Pten^{+/-}$ animals frequently die within a year, mostly due to leukemia. The survivors developed a

broad range of tumors in various other organs like, breast, endometrium, and prostate, indicating that Pten defects can indeed predispose to cancer [216-218]. However, the onset and spectrum of tumor development varied among the *Pten*$^{+/-}$ mice of different research groups. Recently, one study reported that the genetic background of the animals could influence the onset, the spectrum, and the progression of tumorigenesis caused by *Pten* mutation [220]. Although all published mutations were generated around exon 5 of the *Pten* gene, each group has employed slightly different strategies to create their specific mutation, which might contribute to the differences found between the various groups [220].

Because complete knock-out of Pten function was embryonic lethal, many groups generated conditional *Pten* knock-out mice utilizing the Cre-loxP system to analyze the function of Pten in specific tissues, including mammary gland, prostate, brain, and pancreas (reviewed in ref. 221). These various tissue specific Pten knock-out models have shown distinct functions of Pten in different organs [222]. Moreover, using the ubiquitous ROSA26 promoter to drive inducible Cre recombinase, it was shown that also gender might affect tumor latency and spectrum [223]. Female animals quickly developed lymphomas compared to a much lower incidence of lymphoma formation in males, which frequently developed intestinal cancers [223]. An indication that also in mice Pten exerts its action through regulation of the PI3K/Akt pathway came from a study in which *Akt1* was deleted in *Pten*$^{+/-}$ mice. It was shown that deficiency of *Akt1* is sufficient to dramatically inhibit tumor progression in *Pten*$^{+/-}$ mice, but despite Akt is necessary it is not sufficient to drive tumorigenesis in *Pten* deficient animals [150,224]. Furthermore, it was found that in a mouse line, in which *Pten* was conditionally deleted in the urigenital epithelium, *Pten* deficiency in the prostate leads to the activation of FOXO3a and p70^{s6k} pathways, whereas in the bladder, which is less sensitive to tumor formation, *Pten* deficiency leads to decreased levels of p27^{kip1}, increased levels of p21$^{Cip1/Waf}$, and a decrease in proliferation rates [225]. These findings indicated that in different tissues *Pten* deficiency could result in the activation of different downstream signaling pathways and cellular processes [225].

1.6 PTEN and prostate cancer

Inactivation of *PTEN* has been described in a wide variety of tumors but with the highest frequency in glioblastomas, endometrium, and prostate cancer [57]. However, the effects of *PTEN* inactivation might be tissue specific as suggested by data from human tumors and animal models [220,225,226]. Therefore this chapter primarily focuses on the various aspects of PTEN in prostate cancer.

1.6.1 Prostate cancer

The human prostate is the largest of the male accessory sex glands and can be subdivided in three major zones, the central zone, the peripheral zone, and the transition zone [227]. In elderly men the prostate is the site for two common diseases, benign prostatic hyperplasia (BPH) and prostate cancer. Whereas BPH occurs predominantly in the transition zone, prostate tumors mainly arise in the peripheral zone. The development of prostate cancer is a multistep process (Figure 6). The first step is the emergence of the pre-invasive, prostatic intraepithelial neoplasia (PIN) lesions, although proliferative inflammatory atrophy (PIA) has been proposed by some researchers as a precursor of PIN [228,229]. PIN lesions can be classified in low grade and high grade and consist of excessive growth of prostatic epithelial cells within pre-existing glands. These cells often show enlargement of nuclei and especially in case of high grade PIN the lesions often show a discontinuous basal cell layer. High-grade PIN lesions are considered to be the preinvasive stage of prostate cancer carcinomas, and in time metastases might develop from these prostatic adenocarcinomas.

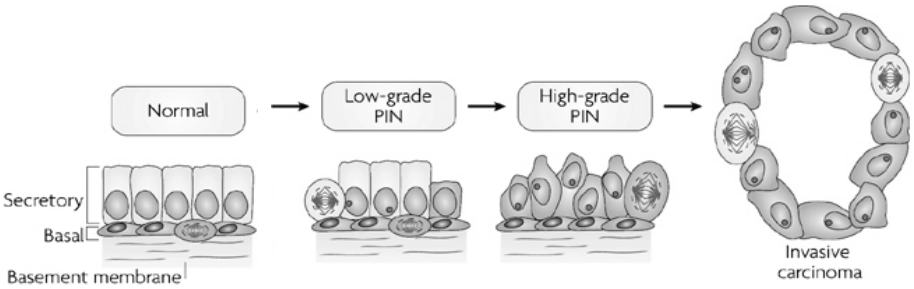


Figure 6. Model representing the different stages of prostate cancer progression (Adapted from Ref. 228).

Prostate cancer is the most frequently diagnosed and the second leading cause of male cancer death in the Western world [230]. The incidence of prostate cancer has significantly increased during the last decades due to increasing proportions of elderly men in the population and earlier disease detection due to PSA screening. Besides age, a positive family history, race, and diet are important risk factors [231].

Treatment options for prostate cancer are limited. Localized prostate cancer is primarily treated with radiotherapy, surgical removal of the prostate, or watchful waiting [232,233]. Because most prostate cancers are dependent on androgens for their growth, metastasized prostate cancers are treated with androgen ablation therapy. Despite the initial rapid response, the disease recurs in most cases within a few years and has then become androgen independent. To date, only palliative treatment is available for androgen independent prostate cancer, although recently a survival benefit for hormone-refractory prostate cancer patients treated with docetaxel-based chemotherapy has been reported [234,235].

1.6.2 Prostate cancer genetics

The majority of prostate cancer cases are sporadic, but epidemiology studies have demonstrated familial clustering of prostate cancer, suggesting that hereditary factors are also important in the development of the disease [236]. Although the association of certain genetic markers to prostate cancer is difficult because of the heterogeneity of the disease, linkage analysis studies have identified at least seven susceptibility loci for hereditary prostate cancer on different chromosomes [237].

In sporadic prostate cancer a number of chromosomal regions have been identified for their frequent deletions or amplifications [238,239]. The most frequent alterations are losses of chromosomes 5q, 6q, 8p, 10q, 13q, 16q, 17p and 18q and gain of chromosomes 1q, 7, 8q, 16p, 17q and Xq. Some of these chromosomal aberrations could already be linked to the locations of either known or candidate prostate cancer tumor suppressor or onco-genes, such as the loss of *PTEN* at 10q, *RB1* (13q), *CDH1* (16q), *APC* (5q), *SMAD4* (18q) and gain of *MYC* at 8q and the *AR* at Xq [238]. Besides gains and losses a specific chromosomal abberation namely the fusion of the androgen regulated gene of *TMPRSS2* to members the *ETS* transcription factor family, has been recently identified as a common rearrangement in prostate cancer [240,241].

Table 3. Overview of PTEN inactivations in prostate cancer

Source material	No of Samples	LOH	HD	Mutation	Type of mutation	Ref. no.
RP and metastases	60 RP and 20 metastases	29% (11/60 + 12/20)	6/80[1]	4/80[1]	Frameshift mutation, exon 7 Nonsense mutation, exon 6 4 bp deletion donor splice site, exon 3 9 bp deletion, exon 8	70
Cell lines and xenografts	15		5/15	4/15	2 frameshift mutations, exons 1, 4 2 nonsense mutations, exon 5	247
RP	60	17% (10/60)	8/60	0/60		248
Metastases	19	55% (10/18)[2]	2/18	4/18	1 missense mutation, exon 5 1 nonsense mutation , exon 5 2 frameshift mutations, exon 7	249
CaP	22	27% (6/22)	0/22	1/22	1 frameshift mutation, exon 7	250
RP	40	0% (0/40)	0/40	1/40	1 missense mutation, exon 9	251
RP	32 RP	ND	ND	5/32 RP	1 nonsense mutation, exon1 4 missense mutations, exons 2, 5 (2x), and 8	252

metastases	6 metastases				2/6 metastases	1 nonsense mutation, exon 5 1 missense mutation, exon 9	252
RP	51	49%	(25/51)	0/51	1/51	1 missense mutation, exon 6	253
TURP	37	70%	(26/37)		5/37		254
TURP	40	55%	(11/19)[3]	9/40	3/40	1 nonsense mutation, exon 7 2 frameshift mutations, exons 7, 8	255
RP	35	74%	(26/35)	2/35	ND		256
RP	107	39%	(42/107)	5/107	ND		257
Xenografts	10	ND		1/10	0/10		261
CaP	40	35%	(14/40)	0/40	5/40	5 no further specified mutations in coding region	265

Abbreviations: LOH, Loss of Heterozygousity; HD, Homozygous Deletion; RP, radical prostatectomy; CaP, Prostate cancer of unknown source type; TURP, Transurethral resection of the prostate; ND, not determined. [1] 7 of the 10 tumors with inactivated *PTEN* were metastases. [2] 1 out of 19 samples was not informative and therefore not included. [3] Only tumor DNAs of which corresponding normal blood DNA was available were investigated (19/40).

1.6.3 PTEN inactivation in prostate cancer

Already before the identification of *PTEN* in 1997, several studies had defined loss of chromosome 10q as a frequent chromosomal alteration in prostate cancer suggesting the presence of a tumor suppressor gene in this region [242-245]. The finding that transfer of chromosome 10cen-q23 into a rat prostate cancer cell line suppressed the metastatic ability of these cells further supported this idea [246]. To date, *PTEN* is considered to be the most frequently inactivated gene in prostate cancer.

In prostate cancer cell lines and xenografts complete *PTEN* inactivation was found in up to 60% of cases [247]. In clinical prostate cancers the frequency of complete *PTEN* inactivation varies widely, probably due to differences in tissue preparation, stage of the disease and the methodology used to detect *PTEN* defects [70,248-257] (Korsten et al., unpublished data) (Table 3). *PTEN* inactivating point mutations have been reported in up to 15% of primary prostate cancers, whereas the reported frequency of point mutations in *PTEN* in metastasis is 20-30% and 25% in prostate cancer cell lines and xenografts, suggesting that mutation of *PTEN* is correlated with a later step in tumor progression [247,249,251-254,258].

Gene deletions are often more difficult to detect in DNA from clinical tumor samples, due to contaminating normal DNA in these samples. In addition, *PTEN* loss might involve only small genomic regions [259] thus the assessment of *PTEN* copy number changes requires sensitive methods, such as the q-WPR (quantitative-Wild-type/Pseudogene Ratio analysis) technique described in chapter 5 of this thesis. It must therefore be noted that the reported percentages of up to 15 percent of homozygous *PTEN* deletion might be an underestimation of the frequency of gene deletions in clinical samples [70,248-251]. A comprehensive study to detect *PTEN* inactivation in 40 locally progressive clinical prostate cancer specimens, utilizing a variety of complementary technical approaches, revealed that bi-allelic deletion of *PTEN* was detected in more than 20% of cases, thereby indicating that bi-allelic loss is the major mechanism of *PTEN* inactivation in these tumors (Chapter 4).

Besides inactivation through homozygous gene loss or point mutation, epigenetic mechanisms like promoter hypermethylation also contribute to *PTEN* inactivation in several tumor types [18-23]. Although *PTEN* inactivation by methylation has been reported in prostate xenograft cells, in clinical prostate cancer samples evidence for promoter hypermethylation is lacking [260,261].

16.4 PTEN and Haploinsufficiency

Whereas classical tumor suppressor genes are thought to require mutation or loss of both alleles to drive tumorigenesis [262,263], it became clear that for some genes loss of a single allele is by itself sufficient to promote tumor progression, a phenomenon called haploinsufficiency [264]. In various malignancies, including prostate cancer, the rate of LOH at 10q23.3 is often higher than the apparent rate of inactivation of the retained *PTEN* allele suggesting that *PTEN* might be haploinsufficient in tumor suppression [70,248-250,253,265]. A recent study, investigating the percentage of *PTEN* deletions in various stages of prostate cancer by interphase FISH, showed *PTEN* loss in 23 percent of high grade PIN and 68 percent of prostate cancer samples [256]. This high frequency of *PTEN* loss observed in prostate cancers versus precursor lesions implicates a role for PTEN haploinsufficiency in the transition from preneoplastic PIN to prostate cancer.

1.7 Cell biological effects of PTEN in prostate cancer

Several studies were undertaken to investigate the biological effects of PTEN in prostate cancer cells [182,196,266-272] (see also Chapter 3). One approach to study the biological effects of the PTEN lipid phosphatase activity is the use of small molecules that inhibit PI3K function, like wortmannin and LY294002. This pharmacological inhibition of PI3K in prostate cancer cells resulted in growth inhibition, apoptosis or in both [266,269,271]. These results are comparable with those obtained upon (inducible)-expression of PTEN in the PTEN negative cell lines PC3 and LNCaP, but besides a reduced proliferation rate, forced PTEN expression in PC3 and LNCaP cells was also reported to inhibit migration [182,196,267,268,270,272]. However, in the chapters 2 and 3 of this thesis it is described that G_1 growth arrest is one of the most predominantly affected processes upon modulation of PTEN signaling in LNCaP and PC3 cells. In PC3 cells blocking of PI3K activity by wortmannin resulted in an accumulation of cells in the G_0/G_1 phase of the cell cycle and a concomitant reduction of the S and G_2/M phase [163] (Chapter 2). The increased number of cells in G_0/G_1 following wortmannin treatment correlated with an increased $p27^{kip1}$ protein expression mediated by the Akt dependent regulation of Skp2 [163]. In LNCaP cells it was shown that induced expression of PTEN lead to cell cycle arrest at the G_1 phase, which was associated with the PTEN dependent transcription regulation of the cell cycle regulators Cdc25a, cyclin G2, RBL2, and E2F2 (Chapter 3).

Since prostate cancer is a heterogenous disease, the identification of the cell of origin of prostate cancer will contribute to a full understanding of the disease. An indication for a role of PTEN/Akt signaling in these so called prostate cancer stem cells came from a recent study demonstrating that the Sca-1 surface antigen could be used to enrich for prostate cells with a regenerative capacity [273]. Introduction of constitutively active Akt in these Sca-1 enriched prostate regenerating cells resulted in the initiation of prostate tumorigenesis [273]. Moreover, prostate specific Pten inactivation leads to an increased prostatic stem/progenitor cell subpopulation and tumor initiation [274] (Korsten et al., unpublished).

1.8 Crosstalk between PTEN/Akt and AR signaling pathways

As for normal cells of the prostate, androgens are essential for the growth and survival of prostate cancer cells, and although initially most prostate cancers respond to androgen ablation therapy eventually the tumors will relapse and become androgen independent [275]. One possibility whereby prostate cancers cells develop androgen independence is the activation of parallel or alternative signaling pathways. However, a role for PTEN signaling in the progression of prostate cancer to an androgen independent state is arguable. To date several lines of evidence have suggested that loss of PTEN function might be associated with the progression to androgen independence [275-286]. Although the underlying mechanism remains to be fully elucidated many reports described a role for Akt in the prostate cancer progression from androgen-dependent to androgen-independent stage [279,280,282,287]. In addition, one study proposed that the combined activation of Akt and Erk pathways stimulates epithelial AR function and counteracts antagonistic effects of the stroma thereby leading to androgen-independent cancer [277].

In contrast, we could not observe any correlation between androgen dependency and PTEN status in our panel of fourteen prostate cancer cell lines and xenografts derived from various stages of clinical prostate cancer (unpublished data). The xenografts PCEW, PC82, PC295, PC310, PC329, PC346, PC374 and the LNCaP cell line were AR dependent, or sensitive in case of PC346 and PC374, but only the xenografts PC310 and PC329 contain a functional PTEN protein. In addition, from the androgen independent cell lines and xenografts PC133, PC135, PC324, PC339, DU145 and PC3 only half had lost PTEN expression [247].

Due to the key role of the AR, as well as the loss of PTEN function and subsequent activation of the Akt signal transduction cascade in prostate cancer

growth, many reports have been focused on the investigation of a functional link between these two signal transduction pathways [288]. Interactions between AR and PTEN/Akt signaling can occur at several levels of regulation. First, it was shown that Akt could bind to and phosphorylate the AR at either S213 and S791 [99,289]. However, these studies used an *in vitro* kinase assay to determine Akt dependent phosphorylation sites of the AR, and *in vivo* analysis failed to confirm phosphorylation of AR by Akt at the indicated sites S213 and S791 [290]. The effect of Akt dependent phosphorylation on AR activity is still a matter of debate. Whereas Lin et al., (2001) found that Akt mediated the suppression of AR transactivation, others reported that the Akt induced AR phosphorylation stimulated the AR transactivation [99,289]. In support of the latter study are the results obtained by Li et al., (2001) showing that PTEN is able to repress the transactivation activity of the AR [291].

Secondly, it has been described that PTEN can affect the activity of the AR through regulation of its expression level [292,293]. Inhibition of Akt activity significantly reduced the AR protein expression in both normal and tumoral epithelial cells [292].

In recent years, steroids have been found to control the activity of multiple signaling pathways via non-genomic mechanisms [294]. This transcription independent action of the AR is a third mechanism of crosstalk between PI3K/Akt and AR pathways. It was shown by several groups that androgens could activate PI3K in a transcription independent manner [295,296].

1.9 Prostate cancer mouse models

Animal models can be of great value to study the potential relationship between molecular mechanisms and clinical progression of a disease, but since conventional *Pten* null mice were not viable several groups generated conditional prostate specific *Pten* knock-out mice using the Cre-loxP system [297-301]. All these models resemble to a certain extend the several stages of human prostate cancer. However, also differences are observed between the various models, which could be explained by differences in activity and cell specificity of the promoter used to drive Cre expression. As described previously in section 1.5 of this chapter also the genetic background of the mice could influence the onset, the spectrum, and the progression of tumorigenesis caused by *Pten* inactivation [220].

The MMTV promoter used by Backman et al., (2004) to drive Cre expression had limited tissue specificity since *Pten* recombination was also found in skin, thymus, and mammary gland [297]. In the *MMTV-Cre;Pten-loxP/loxP* mice hyperplastic prostates were already detected a few days after birth and by 2 weeks of age PIN lesions were present. Although at 14 weeks focal invasions were detected in half of the investigated prostates, the investigation of later stages of prostate cancer development was hampered in this model because the mice died from other malignancies [297].

Prostate specific recombination was achieved in the models in which Cre expression was regulated by either a modified rat probasin promoter (PB) or the human prostate specific antigen promoter (PSA) [298,300,301]. In the *PB-Cre;Pten-loxP/loxP* mice PIN lesions and invasive adenocarcinoma already occurred at respectively 6 and 9 week of age and in 50 percent of the older mice metastases were found [300,301]. In addition, *PB-Cre;Pten-loxP/loxP* mice showed specific gene expression changes that were also seen in human prostate cancers [301]. The process of prostate cancer development in the PSA-Cre based model was slower since massive hyperplasia and focal PIN were first observed at 4-5 months of age [298]. At older age more extensive PIN lesions were detected, followed by the development of invasive carcinoma and in only one mouse a lymph node metastasis was found [298].

Recently, a mouse model was described in which prostate carcinogenesis is initiated in the fully differentiated prostate of adult mice using the inducible tamoxifen dependent PSA-Cre-ER(T2) [299]. Within 4 weeks after *Pten* ablation these mice developed hyperplasia followed by PIN after 2-3 months. After 8-10 months of *Pten* ablation some PINs had progressed to adenocarcinomas but no metastases were found even up to 20 months after *Pten* inactivation [299].

To drive prostate tumorigenesis, Pten functions, at least partly, through Akt since key downstream targets such as Akt, mTOR, and FOXO are affected as a result of *Pten* loss [298,300,301]. To determine whether Akt activation was sufficient for prostate epithelial cell transformation the murine prostate restricted Akt kinase (MPAKT) model was developed [302]. In these mice Akt activation resulted in the development of PIN lesions but progression to invasive adenocarcinomas and metastases could not be detected, suggesting that in the MPAKT model additional oncogenic events are required for prostate cancer progression [302]. Chen et al., (2006), however,

showed that inactivation of Akt1 in *Pten* +/- mice is sufficient to significantly inhibit tumor development, including prostate neoplasia [224].

Whereas various data obtained from clinical prostate cancer specimens already suggested that PTEN might be haploinsufficient in tumor suppression, also several mouse models indicated that Pten haploinsufficiency contributed to tumorigenesis [300,303-308]. In the transgenic adenocarcinoma mouse prostate (TRAMP) model it was shown that loss of a single *Pten* allele significantly increased the rate of prostate cancer progression suggesting that haploinsufficiency of *Pten* promotes progression of prostate cancer [306]. In addition, Trotman et al., (2003) showed by the generation of a mouse mutant series with decreasing Pten activity, that *Pten* is haploinsufficient in tumor suppression, and that *Pten* dosage is an important determinant in cancer progression [300]. However, in the prostate specific *Pten* knock-out models it was shown that mono-allelic *Pten* inactivation is insufficient for prostate cancer development [297,298,300,301].

1.10 Prognostic value of PTEN/Akt pathway members in clinical prostate cancer

In the management of cancer, prognostic markers are important tools in predicting whether a patient is at risk of experiencing a cancer progression or recurrence. During the last several years a marked increase was observed in the early detection of prostate cancer due to large scale PSA based screening. To prevent possible overtreatment of the increased number of newly identified cases it is of utmost importance to identify markers that are able to recognize tumors with the potential to progress to metastatic disease. In several tumor types, like gasterointestinal tumors and hepatocellular carcinomas, loss of PTEN function can be of prognostic value, and in breast cancer PTEN inactivation is associated with tumor progression and lymph node metastasis [309-311]. Various groups reported that loss of PTEN expression in prostate cancer was correlated with tumor stage and grade [312,313]. An indication that loss of PTEN protein expression could serve as an early prognostic marker for prostate cancer metastasis came from a study by Schmitz et al., (2007) showing that loss of PTEN was found in 60 percent of the investigated lymph node metastases and that half of these cases already exhibited loss of PTEN expression at first diagnosis [314]. Despite the relatively small number of investigated patient samples, the study also indicated that patients with a low Gleason score and loss of PTEN expression were found to be potential candidates for the development of

metastatic disease [314]. Moreover, a study investigating the clinical impact of genomic *PTEN* deletions in prostate cancer demonstrated a significant association between *PTEN* deletion and earlier onset of disease recurrence [257].

At the other hand, however, several reports appeared that could not confirm a role for PTEN as a prognostic marker in prostate cancer [315,316] (Korsten et al., unpublished data). Fenic et al., (2004) assessed the PTEN mRNA and protein expression in PIN, primary prostate carcinomas as well as metastases and found that total or partial loss of PTEN protein occurred with tumour progression but this association was not statistically significant [316]. Others analyzed a panel formalin-fixed paraffin-embedded prostate cancer specimens for *PTEN* mutations and found that although patients with *PTEN* mutations had a significantly higher Gleason score and a higher rate of metastasis, the *PTEN* mutation itself is not an accurate factor in the prediction of the prognosis [315]. A study evaluating the prognostic value of *PTEN* inactivation in radical prostatectomies based on PSA recurrence data showed that although *PTEN* inactivation was more frequently found in tumors showing PSA progression, a statistical significant correlation between the absence of PTEN protein expression and PSA recurrence was not found (Korsten et al., unpublished data).

Recently, it was published that the degree of PTEN/PI3K pathway activation is directly related to the metastatic potential of the primary tumor for several carcinoma types, including prostate cancer [317]. That next to PTEN also other members of the PTEN/Akt pathway might serve as a progression marker in prostate cancer was previously suggested by various studies showing that overexpression and activation of Akt has been associated with higher Gleason grades and poor clinical outcome [318-321]. In addition, loss of PTEN expression, together with the Gleason score and the increased phosphorylation of Akt at the time of prostatectomy is of significant predictive value for determining the risk of prostate cancer recurrence [322]. Further, the absence or low expression of the Akt downstream target $p27^{kip1}$, is an important prognostic marker in a wide variety of tumors, including prostate cancer [323]. Several studies analyzing $p27^{kip1}$ expression in prostate adenocarcinoma by immunohistochemistry have shown that decreased $p27^{kip1}$ expression is associated with high tumor grade and poor prognosis [323-326]. However, the relationship between PTEN deficiency and decreased $p27^{kip1}$ expression in clinical prostate cancer is not completely clear [316,327,328]. Finally, overexpression of 4E-BP1, a downstream target of mTOR, was found to be

strongly associated with prostate cancer, especially when combined with PTEN and mTOR expression data [329].

1.11 Aim and scope of this thesis

The research described in this thesis started only a few years after the initial discovery of PTEN and much had to be learned about its specific genetic and cell biological aspects in prostate cancer. The aim of this thesis was to contribute to the understanding of PTEN function in prostate cancer, which will augment our understanding of tumorigenesis and may ultimately lead to the development of novel therapeutic options for prostate cancer. The chapters 2 and 3 describe the investigations of the cell biological and molecular aspects of PTEN in prostate cancer cell lines. Chapter 2 focusses on the role of PTEN in the regulation of the cell cycle inhibitor $p27^{kip1}$ in various prostate cancer cell lines. In chapter 3 the development of the novel cell line LNCaP/PTEN, which stably expresses PTEN under inducible control, is described. This cell line enabled us to obtain a broad view of the cell biological and molecular effects regulated by PTEN in prostate cancer. Further, in this chapter a novel mechanism by which PTEN can be involved in cell cycle regulation is defined.

Since it became clear that not only bi-allelic but also mono-allelic *PTEN* inactivation might contribute to tumorigenesis, we further investigated the mechanism of *PTEN* inactivation in clinical prostate cancer samples in chapter 4. Fourty locally progressive clinical prostate cancer specimens obtained by transurethral resection of the prostate were screened for *PTEN* inactivation by various, complementary techniques. During these investigations we developed a novel and accurate method, denoted quantitative wild-type/pseudogene ratio analysis (q-WPR), to detect gene copy number changes utilizing *PTEN* in prostate cancer as a model system, as described in chapter 5. Q-WPR is not restricted to *PTEN* but can be very valuable in prognostic studies of many genes due to its high sample throughput and its suitability to be used on low quality DNA and can be of high importance as a diagnostic tool. Finally, chapter 6 discusses the results described in this thesis and provides directions for future research.

References

1. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., et al. (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer *Science*, **275,** 1943-1947.
2. Li, D.M. and Sun, H. (1997) TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta *Cancer Res*, **57,** 2124-2129.
3. Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H., Langford, L.A., Baumgard, M.L., Hattier, T., Davis, T., et al. (1997) Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers *Nat Genet*, **15,** 356-362.
4. Maehama, T. and Dixon, J.E. (1999) PTEN: a tumour suppressor that functions as a phospholipid phosphatase *Trends Cell Biol*, **9,** 125-128.
5. Myers, M.P., Stolarov, J.P., Eng, C., Li, J., Wang, S.I., Wigler, M.H., Parsons, R.,Tonks, N.K. (1997) P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase *Proc Natl Acad Sci U S A*, **94,** 9052-9057.
6. Tamura, M., Gu, J., Matsumoto, K., Aota, S., Parsons, R.,Yamada, K.M. (1998) Inhibition of cell migration, spreading, and focal adhesions by tumor suppressor PTEN *Science*, **280,** 1614-1617.
7. Raftopoulou, M., Etienne-Manneville, S., Self, A., Nicholls, S.,Hall, A. (2004) Regulation of cell migration by the C2 domain of the tumor suppressor PTEN *Science*, **303,** 1179-1181.
8. Mahimainathan, L. and Choudhury, G.G. (2004) Inactivation of platelet-derived growth factor receptor by the tumor suppressor PTEN provides a novel mechanism of action of the phosphatase *J Biol Chem*, **279,** 15258-15268.
9. Maehama, T. and Dixon, J.E. (1998) The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate *J Biol Chem*, **273,** 13375-13378.
10. Myers, M.P., Pass, I., Batty, I.H., Van der Kaay, J., Stolarov, J.P., Hemmings, B.A., Wigler, M.H., Downes, C.P.,Tonks, N.K. (1998) The lipid phosphatase activity of PTEN is critical for its tumor supressor function *Proc Natl Acad Sci U S A*, **95,** 13513-13518.
11. Lee, J.O., Yang, H., Georgescu, M.M., Di Cristofano, A., Maehama, T., Shi, Y., Dixon, J.E., Pandolfi, P.,Pavletich, N.P. (1999) Crystal structure of the PTEN tumor suppressor: implications for its phosphoinositide phosphatase activity and membrane association *Cell*, **99,** 323-334.
12. Rizo, J. and Sudhof, T.C. (1998) C2-domains, structure and function of a universal Ca2+-binding domain *J Biol Chem*, **273,** 15879-15882.

13. Georgescu, M.M., Kirsch, K.H., Kaloudis, P., Yang, H., Pavletich, N.P.,Hanafusa, H. (2000) Stabilization and productive positioning roles of the C2 domain of PTEN tumor suppressor *Cancer Res*, **60,** 7033-7038.
14. Waite, K.A. and Eng, C. (2002) Protean PTEN: form and function *Am J Hum Genet*, **70,** 829-844.
15. Tamguney, T. and Stokoe, D. (2007) New insights into PTEN *J Cell Sci*, **120,** 4071-4079.
16. Wang, L., Wang, W.L., Zhang, Y., Guo, S.P., Zhang, J.,Li, Q.L. (2007) Epigenetic and genetic alterations of PTEN in hepatocellular carcinoma *Hepatol Res*, **37,** 389-396.
17. Wiencke, J.K., Zheng, S., Jelluma, N., Tihan, T., Vandenberg, S., Tamguney, T., Baumber, R., Parsons, R., Lamborn, K.R., Berger, M.S., et al. (2007) Methylation of the PTEN promoter defines low-grade gliomas and secondary glioblastoma *Neuro Oncol*, **9,** 271-279.
18. Zysman, M.A., Chapman, W.B.,Bapat, B. (2002) Considerations when analyzing the methylation status of PTEN tumor suppressor gene *Am J Pathol*, **160,** 795-800.
19. Salvesen, H.B., MacDonald, N., Ryan, A., Jacobs, I.J., Lynch, E.D., Akslen, L.A.,Das, S. (2001) PTEN methylation is associated with advanced stage and microsatellite instability in endometrial carcinoma *Int J Cancer*, **91,** 22-26.
20. Mirmohammadsadegh, A., Marini, A., Nambiar, S., Hassan, M., Tannapfel, A., Ruzicka, T.,Hengge, U.R. (2006) Epigenetic silencing of the PTEN gene in melanoma *Cancer Res*, **66,** 6546-6552.
21. Kawaguchi, K., Oda, Y., Saito, T., Takahira, T., Yamamoto, H., Tamiya, S., Iwamoto, Y.,Tsuneyoshi, M. (2005) Genetic and epigenetic alterations of the PTEN gene in soft tissue sarcomas *Hum Pathol*, **36,** 357-363.
22. Garcia, J.M., Silva, J., Pena, C., Garcia, V., Rodriguez, R., Cruz, M.A., Cantos, B., Provencio, M., Espana, P.,Bonilla, F. (2004) Promoter methylation of the PTEN gene is a common molecular change in breast cancer *Genes Chromosomes Cancer*, **41,** 117-124.
23. Goel, A., Arnold, C.N., Niedzwiecki, D., Carethers, J.M., Dowell, J.M., Wasserman, L., Compton, C., Mayer, R.J., Bertagnolli, M.M.,Boland, C.R. (2004) Frequent inactivation of PTEN by promoter hypermethylation in microsatellite instability-high sporadic colorectal cancers *Cancer Res*, **64,** 3014-3021.
24. Dahia, P.L., FitzGerald, M.G., Zhang, X., Marsh, D.J., Zheng, Z., Pietsch, T., von Deimling, A., Haluska, F.G., Haber, D.A.,Eng, C. (1998) A highly conserved processed PTEN pseudogene is located on chromosome band 9p21 *Oncogene*, **16,** 2403-2406.
25. Meng, F., Henson, R., Wehbe-Janek, H., Ghoshal, K., Jacob, S.T.,Patel, T. (2007) MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer *Gastroenterology*, **133,** 647-658.
26. Yang, H., Kong, W., He, L., Zhao, J.J., O'Donnell, J.D., Wang, J., Wenham, R.M., Coppola, D., Kruk, P.A., Nicosia, S.V., et al. (2008) MicroRNA expression profiling in

human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN *Cancer Res*, **68,** 425-433.

27. Xiao, C., Srinivasan, L., Calado, D.P., Patterson, H.C., Zhang, B., Wang, J., Henderson, J.M., Kutok, J.L.,Rajewsky, K. (2008) Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes *Nat Immunol*, **9,** 405-414.
28. Meister, G. (2007) miRNAs get an early start on translational silencing *Cell*, **131,** 25-28.
29. Gericke, A., Munson, M.,Ross, A.H. (2006) Regulation of the PTEN phosphatase *Gene*, **374,** 1-9.
30. Kim, R.H. and Mak, T.W. (2006) Tumours and tremors: how PTEN regulation underlies both *Br J Cancer*, **94,** 620-624.
31. Maccario, H., Perera, N.M., Davidson, L., Downes, C.P.,Leslie, N.R. (2007) PTEN is destabilized by phosphorylation on Thr366 *Biochem J*, **405,** 439-444.
32. Vazquez, F., Grossman, S.R., Takahashi, Y., Rokas, M.V., Nakamura, N.,Sellers, W.R. (2001) Phosphorylation of the PTEN tail acts as an inhibitory switch by preventing its recruitment into a protein complex *J Biol Chem*, **276,** 48627-48630.
33. Valiente, M., Andres-Pons, A., Gomar, B., Torres, J., Gil, A., Tapparel, C., Antonarakis, S.E.,Pulido, R. (2005) Binding of PTEN to specific PDZ domains contributes to PTEN protein stability and phosphorylation by microtubule-associated serine/threonine kinases *J Biol Chem*, **280,** 28936-28943.
34. Wu, W., Wang, X., Zhang, W., Reed, W., Samet, J.M., Whang, Y.E.,Ghio, A.J. (2003) Zinc-induced PTEN protein degradation through the proteasome pathway in human airway epithelial cells *J Biol Chem*, **278,** 28258-28263.
35. Torres, J. and Pulido, R. (2001) The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated degradation *J Biol Chem*, **276,** 993-998.
36. Wang, X., Trotman, L.C., Koppie, T., Alimonti, A., Chen, Z., Gao, Z., Wang, J., Erdjument-Bromage, H., Tempst, P., Cordon-Cardo, C., et al. (2007) NEDD4-1 is a proto-oncogenic ubiquitin ligase for PTEN *Cell*, **128,** 129-139.
37. Leslie, N.R., Lindsay, Y., Ross, S.H.,Downes, C.P. (2004) Redox regulation of phosphatase function *Biochem Soc Trans*, **32,** 1018-1020.
38. Salmeen, A. and Barford, D. (2005) Functions and mechanisms of redox regulation of cysteine-based phosphatases *Antioxid Redox Signal*, **7,** 560-577.
39. Seo, J.H., Ahn, Y., Lee, S.R., Yeol Yeo, C.,Chung Hur, K. (2005) The major target of the endogenously generated reactive oxygen species in response to insulin stimulation is phosphatase and tensin homolog and not phosphoinositide-3 kinase (PI-3 kinase) in the PI-3 kinase/Akt pathway *Mol Biol Cell*, **16,** 348-357.

40. Leslie, N.R., Bennett, D., Lindsay, Y.E., Stewart, H., Gray, A.,Downes, C.P. (2003) Redox regulation of PI 3-kinase signalling via inactivation of PTEN *Embo J*, **22,** 5501-5510.
41. Downes, C.P., Walker, S., McConnachie, G., Lindsay, Y., Batty, I.H.,Leslie, N.R. (2004) Acute regulation of the tumour suppressor phosphatase, PTEN, by anionic lipids and reactive oxygen species *Biochem Soc Trans*, **32,** 338-342.
42. Cho, S.H., Lee, C.H., Ahn, Y., Kim, H., Ahn, C.Y., Yang, K.S.,Lee, S.R. (2004) Redox regulation of PTEN and protein tyrosine phosphatases in H(2)O(2) mediated cell signaling *FEBS Lett*, **560,** 7-13.
43. Okumura, K., Mendoza, M., Bachoo, R.M., DePinho, R.A., Cavenee, W.K.,Furnari, F.B. (2006) PCAF modulates PTEN activity *J Biol Chem*, **281,** 26562-26568.
44. Deleris, P., Bacqueville, D., Gayral, S., Carrez, L., Salles, J.P., Perret, B.,Breton-Douillon, M. (2003) SHIP-2 and PTEN are expressed and active in vascular smooth muscle cell nuclei, but only SHIP-2 is associated with nuclear speckles *J Biol Chem*, **278,** 38884-38891.
45. Gimm, O., Perren, A., Weng, L.P., Marsh, D.J., Yeh, J.J., Ziebold, U., Gil, E., Hinze, R., Delbridge, L., Lees, J.A., et al. (2000) Differential nuclear and cytoplasmic expression of PTEN in normal thyroid tissue, and benign and malignant epithelial thyroid tumors *Am J Pathol*, **156,** 1693-1700.
46. Lachyankar, M.B., Sultana, N., Schonhoff, C.M., Mitra, P., Poluha, W., Lambert, S., Quesenberry, P.J., Litofsky, N.S., Recht, L.D., Nabi, R., et al. (2000) A role for nuclear PTEN in neuronal differentiation *J Neurosci*, **20,** 1404-1413.
47. Perren, A., Komminoth, P., Saremaslani, P., Matter, C., Feurer, S., Lees, J.A., Heitz, P.U.,Eng, C. (2000) Mutation and expression analyses reveal differential subcellular compartmentalization of PTEN in endocrine pancreatic tumors compared to normal islet cells *Am J Pathol*, **157,** 1097-1103.
48. Lindsay, Y., McCoull, D., Davidson, L., Leslie, N.R., Fairservice, A., Gray, A., Lucocq, J.,Downes, C.P. (2006) Localization of agonist-sensitive PtdIns(3,4,5)P3 reveals a nuclear pool that is insensitive to PTEN expression *J Cell Sci*, **119,** 5160-5168.
49. Liu, J.L., Sheng, X., Hortobagyi, Z.K., Mao, Z., Gallick, G.E.,Yung, W.K. (2005) Nuclear PTEN-mediated growth suppression is independent of Akt down-regulation *Mol Cell Biol*, **25,** 6211-6224.
50. Trotman, L.C., Wang, X., Alimonti, A., Chen, Z., Teruya-Feldstein, J., Yang, H., Pavletich, N.P., Carver, B.S., Cordon-Cardo, C., Erdjument-Bromage, H., et al. (2007) Ubiquitination regulates PTEN nuclear import and tumor suppression *Cell*, **128,** 141-156.
51. Gil, A., Andres-Pons, A., Fernandez, E., Valiente, M., Torres, J., Cervera, J.,Pulido, R. (2006) Nuclear localization of PTEN by a Ran-dependent mechanism enhances apoptosis: Involvement of an N-terminal nuclear localization domain and multiple nuclear exclusion motifs *Mol Biol Cell*, **17,** 4002-4013.

52. Radu, A., Neubauer, V., Akagi, T., Hanafusa, H.,Georgescu, M.M. (2003) PTEN induces cell cycle arrest by decreasing the level and nuclear localization of cyclin D1 *Mol Cell Biol*, **23,** 6139-6149.
53. Shen, W.H., Balajee, A.S., Wang, J., Wu, H., Eng, C., Pandolfi, P.P.,Yin, Y. (2007) Essential role for nuclear PTEN in maintaining chromosomal integrity *Cell*, **128,** 157-170.
54. Chung, J.H., Ginn-Pease, M.E.,Eng, C. (2005) Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) has nuclear localization signal-like sequences for nuclear import mediated by major vault protein *Cancer Res*, **65,** 4108-4116.
55. Baker, S.J. (2007) PTEN enters the nuclear age *Cell*, **128,** 25-28.
56. Eng, C. (2003) PTEN: one gene, many syndromes *Hum Mutat*, **22,** 183-198.
57. Dahia, P.L. (2000) PTEN, a unique tumor suppressor gene *Endocr Relat Cancer*, **7,** 115-129.
58. Ali, I.U., Schriml, L.M.,Dean, M. (1999) Mutational spectra of PTEN/MMAC1 gene: a tumor suppressor with lipid phosphatase activity *J Natl Cancer Inst*, **91,** 1922-1932.
59. Marsh, D.J., Dahia, P.L., Zheng, Z., Liaw, D., Parsons, R., Gorlin, R.J.,Eng, C. (1997) Germline mutations in PTEN are present in Bannayan-Zonana syndrome *Nat Genet*, **16,** 333-334.
60. Marsh, D.J., Coulon, V., Lunetta, K.L., Rocca-Serra, P., Dahia, P.L., Zheng, Z., Liaw, D., Caron, S., Duboue, B., Lin, A.Y., et al. (1998) Mutation spectrum and genotype-phenotype analyses in Cowden disease and Bannayan-Zonana syndrome, two hamartoma syndromes with germline PTEN mutation *Hum Mol Genet*, **7,** 507-515.
61. Nelen, M.R., van Staveren, W.C., Peeters, E.A., Hassel, M.B., Gorlin, R.J., Hamm, H., Lindboe, C.F., Fryns, J.P., Sijmons, R.H., Woods, D.G., et al. (1997) Germline mutations in the PTEN/MMAC1 gene in patients with Cowden disease *Hum Mol Genet*, **6,** 1383-1387.
62. Liaw, D., Marsh, D.J., Li, J., Dahia, P.L., Wang, S.I., Zheng, Z., Bose, S., Call, K.M., Tsou, H.C., Peacocke, M., et al. (1997) Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome *Nat Genet*, **16,** 64-67.
63. Albrecht, S., Haber, R.M., Goodman, J.C.,Duvic, M. (1992) Cowden syndrome and Lhermitte-Duclos disease *Cancer*, **70,** 869-876.
64. Eng, C., Murday, V., Seal, S., Mohammed, S., Hodgson, S.V., Chaudary, M.A., Fentiman, I.S., Ponder, B.A.,Eeles, R.A. (1994) Cowden syndrome and Lhermitte-Duclos disease in a family: a single genetic syndrome with pleiotropy? *J Med Genet*, **31,** 458-461.
65. Gorlin, R.J., Cohen, M.M., Jr., Condon, L.M.,Burke, B.A. (1992) Bannayan-Riley-Ruvalcaba syndrome *Am J Med Genet*, **44,** 307-314.
66. Zhou, X., Hampel, H., Thiele, H., Gorlin, R.J., Hennekam, R.C., Parisi, M., Winter, R.M.,Eng, C. (2001) Association of germline mutation in the PTEN tumour suppressor gene and Proteus and Proteus-like syndromes *Lancet*, **358,** 210-211.

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

68. Wang, S.I., Puc, J., Li, J., Bruce, J.N., Cairns, P., Sidransky, D.,Parsons, R. (1997) Somatic mutations of PTEN in glioblastoma multiforme *Cancer Res*, **57,** 4183-4186.

69. Risinger, J.I., Hayes, A.K., Berchuck, A.,Barrett, J.C. (1997) PTEN/MMAC1 mutations in endometrial cancers *Cancer Res*, **57,** 4736-4738.

70. Cairns, P., Okami, K., Halachmi, S., Halachmi, N., Esteller, M., Herman, J.G., Jen, J., Isaacs, W.B., Bova, G.S.,Sidransky, D. (1997) Frequent inactivation of PTEN/MMAC1 in primary prostate cancer *Cancer Res*, **57,** 4997-5000.

71. Cully, M., You, H., Levine, A.J.,Mak, T.W. (2006) Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis *Nat Rev Cancer*, **6,** 184-192.

72. Komander, D., Fairservice, A., Deak, M., Kular, G.S., Prescott, A.R., Peter Downes, C., Safrany, S.T., Alessi, D.R.,van Aalten, D.M. (2004) Structural insights into the regulation of PDK1 by phosphoinositides and inositol phosphates *Embo J*, **23,** 3918-3928.

73. Mora, A., Komander, D., van Aalten, D.M.,Alessi, D.R. (2004) PDK1, the master regulator of AGC kinase signal transduction *Semin Cell Dev Biol*, **15,** 161-170.

74. Hannigan, G., Troussard, A.A.,Dedhar, S. (2005) Integrin-linked kinase: a cancer therapeutic target unique among its ILK *Nat Rev Cancer*, **5,** 51-63.

75. Edwards, L.A., Thiessen, B., Dragowska, W.H., Daynard, T., Bally, M.B.,Dedhar, S. (2005) Inhibition of ILK in PTEN-mutant human glioblastomas inhibits PKB/Akt activation, induces apoptosis, and delays tumor growth *Oncogene*, **24,** 3596-3605.

76. Morimoto, A.M., Tomlinson, M.G., Nakatani, K., Bolen, J.B., Roth, R.A.,Herbst, R. (2000) The MMAC1 tumor suppressor phosphatase inhibits phospholipase C and integrin-linked kinase activity *Oncogene*, **19,** 200-209.

77. Persad, S., Attwell, S., Gray, V., Delcommenne, M., Troussard, A., Sanghera, J.,Dedhar, S. (2000) Inhibition of integrin-linked kinase (ILK) suppresses activation of protein kinase B/Akt and induces cell cycle arrest and apoptosis of PTEN-mutant prostate cancer cells *Proc Natl Acad Sci U S A*, **97,** 3207-3212.

78. Qian, Y., Zhong, X., Flynn, D.C., Zheng, J.Z., Qiao, M., Wu, C., Dedhar, S., Shi, X.,Jiang, B.H. (2005) ILK mediates actin filament rearrangements and cell migration and invasion through PI3K/Akt/Rac1 signaling *Oncogene*, **24,** 3154-3165.

79. Raftopoulou, M. and Hall, A. (2004) Cell migration: Rho GTPases lead the way *Dev Biol*, **265,** 23-32.

80. Filipenko, N.R., Attwell, S., Roskelley, C.,Dedhar, S. (2005) Integrin-linked kinase activity regulates Rac- and Cdc42-mediated actin cytoskeleton reorganization via alpha-PIX *Oncogene*, **24,** 5837-5849.

81. Han, J., Luby-Phelps, K., Das, B., Shu, X., Xia, Y., Mosteller, R.D., Krishna, U.M., Falck, J.R., White, M.A.,Broek, D. (1998) Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav *Science*, **279,** 558-560.

82. Liliental, J., Moon, S.Y., Lesche, R., Mamillapalli, R., Li, D., Zheng, Y., Sun, H.,Wu, H. (2000) Genetic deletion of the Pten tumor suppressor gene promotes cell motility by activation of Rac1 and Cdc42 GTPases *Curr Biol*, **10,** 401-404.

83. Vivanco, I., Palaskas, N., Tran, C., Finn, S.P., Getz, G., Kennedy, N.J., Jiao, J., Rose, J., Xie, W., Loda, M., et al. (2007) Identification of the JNK signaling pathway as a functional target of the tumor suppressor PTEN *Cancer Cell*, **11,** 555-569.

84. Bellacosa, A., Kumar, C.C., Di Cristofano, A.,Testa, J.R. (2005) Activation of AKT kinases in cancer: implications for therapeutic targeting *Adv Cancer Res*, **94,** 29-86.

85. Hanada, M., Feng, J.,Hemmings, B.A. (2004) Structure, regulation and function of PKB/AKT--a major therapeutic target *Biochim Biophys Acta*, **1697,** 3-16.

86. Hresko, R.C. and Mueckler, M. (2005) mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes *J Biol Chem*, **280,** 40406-40416.

87. Sarbassov, D.D., Guertin, D.A., Ali, S.M.,Sabatini, D.M. (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex *Science*, **307,** 1098-1101.

88. Yang, Z.Z., Tschopp, O., Baudry, A., Dummler, B., Hynx, D.,Hemmings, B.A. (2004) Physiological functions of protein kinase B/Akt *Biochem Soc Trans*, **32,** 350-354.

89. Brazil, D.P., Yang, Z.Z.,Hemmings, B.A. (2004) Advances in protein kinase B signalling: AKTion on multiple fronts *Trends Biochem Sci*, **29,** 233-242.

90. Greer, E.L. and Brunet, A. (2005) FOXO transcription factors at the interface between longevity and tumor suppression *Oncogene*, **24,** 7410-7425.

91. Arden, K.C. (2006) Multiple roles of FOXO transcription factors in mammalian cells point to multiple roles in cancer *Exp Gerontol*, **41,** 709-717.

92. Barr, F.G. (2001) Gene fusions involving PAX and FOX family members in alveolar rhabdomyosarcoma *Oncogene*, **20,** 5736-5746.

93. Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J.,Greenberg, M.E. (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor *Cell*, **96,** 857-868.

94. Kops, G.J., de Ruiter, N.D., De Vries-Smits, A.M., Powell, D.R., Bos, J.L.,Burgering, B.M. (1999) Direct control of the Forkhead transcription factor AFX by protein kinase B *Nature*, **398,** 630-634.

95. Jacobs, F.M., van der Heide, L.P., Wijchers, P.J., Burbach, J.P., Hoekman, M.F.,Smidt, M.P. (2003) FoxO6, a novel member of the FoxO class of transcription factors with distinct shuttling dynamics *J Biol Chem*, **278,** 35959-35967.

96. van der Heide, L.P., Jacobs, F.M., Burbach, J.P., Hoekman, M.F.,Smidt, M.P. (2005) FoxO6 transcriptional activity is regulated by Thr26 and Ser184, independent of nucleo-cytoplasmic shuttling *Biochem J*, **391,** 623-629.

97. Matsuzaki, H., Daitoku, H., Hatta, M., Tanaka, K.,Fukamizu, A. (2003) Insulin-induced phosphorylation of FKHR (Foxo1) targets to proteasomal degradation *Proc Natl Acad Sci U S A*, **100,** 11285-11290.

98. Plas, D.R. and Thompson, C.B. (2003) Akt activation promotes degradation of tuberin and FOXO3a via the proteasome *J Biol Chem*, **278,** 12361-12366.

99. Lin, H.K., Yeh, S., Kang, H.Y.,Chang, C. (2001) Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor *Proc Natl Acad Sci U S A*, **98,** 7200-7205.

100. Kane, S., Sano, H., Liu, S.C., Asara, J.M., Lane, W.S., Garner, C.C.,Lienhard, G.E. (2002) A method to identify serine kinase substrates. Akt phosphorylates a novel adipocyte protein with a Rab GTPase-activating protein (GAP) domain *J Biol Chem*, **277,** 22115-22118.

101. Yuan, Z.Q., Feldman, R.I., Sussman, G.E., Coppola, D., Nicosia, S.V.,Cheng, J.Q. (2003) AKT2 inhibition of cisplatin-induced JNK/p38 and Bax activation by phosphorylation of ASK1: implication of AKT2 in chemoresistance *J Biol Chem*, **278,** 23432-23440.

102. Kim, A.H., Khursigara, G., Sun, X., Franke, T.F.,Chao, M.V. (2001) Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1 *Mol Cell Biol*, **21,** 893-901.

103. Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y.,Greenberg, M.E. (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery *Cell*, **91,** 231-241.

104. Cardone, M.H., Roy, N., Stennicke, H.R., Salvesen, G.S., Franke, T.F., Stanbridge, E., Frisch, S.,Reed, J.C. (1998) Regulation of cell death protease caspase-9 by phosphorylation *Science*, **282,** 1318-1321.

105. Fujita, E., Jinbo, A., Matuzaki, H., Konishi, H., Kikkawa, U.,Momoi, T. (1999) Akt phosphorylation site found in human caspase-9 is absent in mouse caspase-9 *Biochem Biophys Res Commun*, **264,** 550-555.

106. Kato, S., Ding, J.,Du, K. (2007) Differential activation of CREB by Akt1 and Akt2 *Biochem Biophys Res Commun*, **354,** 1061-1066.

107. Fulton, D., Gratton, J.P., McCabe, T.J., Fontana, J., Fujio, Y., Walsh, K., Franke, T.F., Papapetropoulos, A.,Sessa, W.C. (1999) Regulation of endothelium-derived nitric oxide production by the protein kinase Akt *Nature*, **399,** 597-601.

108. Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M.,Hemmings, B.A. (1995) Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B *Nature*, **378,** 785-789.

109. Ozes, O.N., Mayo, L.D., Gustin, J.A., Pfeffer, S.R., Pfeffer, L.M.,Donner, D.B. (1999) NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase *Nature*, **401,** 82-85.

110. Luo, M., Langlais, P., Yi, Z., Lefort, N., De Filippis, E.A., Hwang, H., Christ-Roberts, C.Y.,Mandarino, L.J. (2007) Phosphorylation of Human IRS-1 at Ser629 Plays a Positive Role in Insulin Signaling *Endocrinology,* **148,** 4895-4905.

111. Tang, X., Jang, S.W., Wang, X., Liu, Z., Bahr, S.M., Sun, S.Y., Brat, D., Gutmann, D.H.,Ye, K. (2007) Akt phosphorylation regulates the tumour-suppressor merlin through ubiquitination and degradation *Nat Cell Biol*, **9,** 1199-1207.

112. Ashcroft, M., Ludwig, R.L., Woods, D.B., Copeland, T.D., Weber, H.O., MacRae, E.J.,Vousden, K.H. (2002) Phosphorylation of HDM2 by Akt *Oncogene*, **21,** 1955-1962.

113. Sekulic, A., Hudson, C.C., Homme, J.L., Yin, P., Otterness, D.M., Karnitz, L.M.,Abraham, R.T. (2000) A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells *Cancer Res*, **60,** 3504-3513.

114. Rossig, L., Jadidi, A.S., Urbich, C., Badorff, C., Zeiher, A.M.,Dimmeler, S. (2001) Akt-dependent phosphorylation of p21(Cip1) regulates PCNA binding and proliferation of endothelial cells *Mol Cell Biol*, **21,** 5644-5657.

115. Fujita, N., Sato, S., Katayama, K.,Tsuruo, T. (2002) Akt-dependent Phosphorylation of p27Kip1 Promotes Binding to 14-3-3 and Cytoplasmic Localization *J Biol Chem*, **277,** 28706-28713.

116. Kovacina, K.S., Park, G.Y., Bae, S.S., Guzzetta, A.W., Schaefer, E., Birnbaum, M.J.,Roth, R.A. (2003) Identification of a proline-rich Akt substrate as a 14-3-3 binding partner *J Biol Chem*, **278,** 10189-10194.

117. Zimmermann, S. and Moelling, K. (1999) Phosphorylation and regulation of Raf by Akt (protein kinase B) *Science*, **286,** 1741-1744.

118. Yamada, E., Okada, S., Saito, T., Ohshima, K., Sato, M., Tsuchiya, T., Uehara, Y., Shimizu, H.,Mori, M. (2005) Akt2 phosphorylates Synip to regulate docking and fusion of GLUT4-containing vesicles *J Cell Biol*, **168,** 921-928.

119. Inoki, K., Li, Y., Zhu, T., Wu, J.,Guan, K.L. (2002) TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling *Nat Cell Biol*, **4,** 648-657.

120. Vitari, A.C., Deak, M., Collins, B.J., Morrice, N., Prescott, A.R., Phelan, A., Humphreys, S.,Alessi, D.R. (2004) WNK1, the kinase mutated in an inherited high-blood-pressure syndrome, is a novel PKB (protein kinase B)/Akt substrate *Biochem J*, **378,** 257-268.

121. Dan, H.C., Sun, M., Kaneko, S., Feldman, R.I., Nicosia, S.V., Wang, H.G., Tsang, B.K.,Cheng, J.Q. (2004) Akt phosphorylation and stabilization of X-linked inhibitor of apoptosis protein (XIAP) *J Biol Chem*, **279,** 5405-5412.

122. Sutherland, B.W., Kucab, J., Wu, J., Lee, C., Cheang, M.C., Yorida, E., Turbin, D., Dedhar, S., Nelson, C., Pollak, M., et al. (2005) Akt phosphorylates the Y-box binding protein 1 at Ser102 located in the cold shock domain and affects the anchorage-independent growth of breast cancer cells *Oncogene*, **24,** 4281-4292.

123. Fingar, D.C., Richardson, C.J., Tee, A.R., Cheatham, L., Tsou, C.,Blenis, J. (2004) mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E *Mol Cell Biol*, **24,** 200-216.

124. Fingar, D.C., Salama, S., Tsou, C., Harlow, E.,Blenis, J. (2002) Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E *Genes Dev*, **16,** 1472-1487.

125. Gingras, A.C., Raught, B.,Sonenberg, N. (2004) mTOR signaling to translation *Curr Top Microbiol Immunol*, **279,** 169-197.

126. Schmelzle, T. and Hall, M.N. (2000) TOR, a central controller of cell growth *Cell*, **103,** 253-262.

127. Reynolds, T.H.t., Bodine, S.C.,Lawrence, J.C., Jr. (2002) Control of Ser2448 phosphorylation in the mammalian target of rapamycin by insulin and skeletal muscle load *J Biol Chem*, **277,** 17657-17662.

128. Scott, P.H., Brunn, G.J., Kohn, A.D., Roth, R.A.,Lawrence, J.C., Jr. (1998) Evidence of insulin-stimulated phosphorylation and activation of the mammalian target of rapamycin mediated by a protein kinase B signaling pathway *Proc Natl Acad Sci U S A*, **95,** 7772-7777.

129. Nave, B.T., Ouwens, M., Withers, D.J., Alessi, D.R.,Shepherd, P.R. (1999) Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-acid deficiency on protein translation *Biochem J*, **344 Pt 2,** 427-431.

130. Manning, B.D. and Cantley, L.C. (2003) United at last: the tuberous sclerosis complex gene products connect the phosphoinositide 3-kinase/Akt pathway to mammalian target of rapamycin (mTOR) signalling *Biochem Soc Trans*, **31,** 573-578.

131. Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J.,Cantley, L.C. (2002) Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway *Mol Cell*, **10,** 151-162.

132. Tee, A.R., Manning, B.D., Roux, P.P., Cantley, L.C.,Blenis, J. (2003) Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb *Curr Biol*, **13,** 1259-1268.

133. Inoki, K., Li, Y., Xu, T.,Guan, K.L. (2003) Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling *Genes Dev*, **17,** 1829-1834.

134. Hay, N. and Sonenberg, N. (2004) Upstream and downstream of mTOR *Genes Dev*, **18,** 1926-1945.

135. Rebholz, H., Panasyuk, G., Fenton, T., Nemazanyy, I., Valovka, T., Flajolet, M., Ronnstrand, L., Stephens, L., West, A.,Gout, I.T. (2006) Receptor association and tyrosine phosphorylation of S6 kinases *Febs J*, **273,** 2023-2036.

136. Balendran, A., Currie, R., Armstrong, C.G., Avruch, J.,Alessi, D.R. (1999) Evidence that 3-phosphoinositide-dependent protein kinase-1 mediates phosphorylation of p70 S6 kinase in vivo at Thr-412 as well as Thr-252 *J Biol Chem*, **274,** 37400-37406.

137. Biondi, R.M., Kieloch, A., Currie, R.A., Deak, M.,Alessi, D.R. (2001) The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB *Embo J*, **20,** 4380-4390.

138. Mothe-Satney, I., Brunn, G.J., McMahon, L.P., Capaldo, C.T., Abraham, R.T.,Lawrence, J.C., Jr. (2000) Mammalian target of rapamycin-dependent phosphorylation of PHAS-I in four (S/T)P sites detected by phospho-specific antibodies *J Biol Chem*, **275,** 33836-33843.

139. Jope, R.S. and Johnson, G.V. (2004) The glamour and gloom of glycogen synthase kinase-3 *Trends Biochem Sci*, **29,** 95-102.

140. Kim, L. and Kimmel, A.R. (2006) GSK3 at the edge: regulation of developmental specification and cell polarization *Curr Drug Targets*, **7,** 1411-1419.

141. Frame, S. and Cohen, P. (2001) GSK3 takes centre stage more than 20 years after its discovery *Biochem J*, **359,** 1-16.

142. Diehl, J.A., Cheng, M., Roussel, M.F.,Sherr, C.J. (1998) Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization *Genes Dev*, **12,** 3499-3511.

143. Pap, M. and Cooper, G.M. (1998) Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-Kinase/Akt cell survival pathway *J Biol Chem*, **273,** 19929-19932.

144. Cahilly-Snyder, L., Yang-Feng, T., Francke, U.,George, D.L. (1987) Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line *Somat Cell Mol Genet*, **13,** 235-244.

145. Momand, J., Jung, D., Wilczynski, S.,Niland, J. (1998) The MDM2 gene amplification database *Nucleic Acids Res*, **26,** 3453-3459.

146. Iwakuma, T. and Lozano, G. (2003) MDM2, an introduction *Mol Cancer Res*, **1,** 993-1000.

147. Mayo, L.D. and Donner, D.B. (2001) A phosphatidylinositol 3-kinase/Akt pathway promotes translocation of Mdm2 from the cytoplasm to the nucleus *Proc Natl Acad Sci U S A*, **98,** 11598-11603.

148. Zhou, B.P. and Hung, M.C. (2002) Novel targets of Akt, p21(Cipl/WAF1), and MDM2 *Semin Oncol*, **29,** 62-70.

149. Feng, J., Tamaskovic, R., Yang, Z., Brazil, D.P., Merlo, A., Hess, D.,Hemmings, B.A. (2004) Stabilization of Mdm2 via decreased ubiquitination is mediated by protein kinase B/Akt-dependent phosphorylation *J Biol Chem*, **279,** 35510-35517.

150. Blanco-Aparicio, C., Renner, O., Leal, J.F.,Carnero, A. (2007) PTEN, more than the AKT pathway *Carcinogenesis*, **28,** 1379-1386.

151. Blagosklonny, M.V. and Pardee, A.B. (2002) The restriction point of the cell cycle *Cell Cycle*, **1,** 103-110.

152. Weng, L.P., Brown, J.L.,Eng, C. (2001) PTEN coordinates G(1) arrest by down-regulating cyclin D1 via its protein phosphatase activity and up-regulating p27 via its lipid phosphatase activity in a breast cancer model *Hum Mol Genet*, **10,** 599-604.

153. Liang, J., Zubovitz, J., Petrocelli, T., Kotchetkov, R., Connor, M.K., Han, K., Lee, J.H., Ciarallo, S., Catzavelos, C., Beniston, R., et al. (2002) PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest *Nat Med*, **8,** 1153-1160.

154. Liang, J. and Slingerland, J.M. (2003) Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression *Cell Cycle*, **2,** 339-345.

155. Li, Y., Dowbenko, D.,Lasky, L.A. (2002) AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21Cip/WAF1 and promotes cell survival *J Biol Chem*, **277,** 11352-11361.

156. Shin, I., Yakes, F.M., Rojo, F., Shin, N.Y., Bakin, A.V., Baselga, J.,Arteaga, C.L. (2002) PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization *Nat Med*, **8,** 1145-1152.

157. Viglietto, G., Motti, M.L., Bruni, P., Melillo, R.M., D'Alessio, A., Califano, D., Vinci, F., Chiappetta, G., Tsichlis, P., Bellacosa, A., et al. (2002) Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer *Nat Med*, **8,** 1136-1144.

158. Seminario, M.C., Precht, P., Wersto, R.P., Gorospe, M.,Wange, R.L. (2003) PTEN expression in PTEN-null leukaemic T cell lines leads to reduced proliferation via slowed cell cycle progression *Oncogene*, **22,** 8195-8204.

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

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

161. Ramaswamy, S., Nakamura, N., Vazquez, F., Batt, D.B., Perera, S., Roberts, T.M.,Sellers, W.R. (1999) Regulation of G1 progression by the PTEN tumor suppressor protein is linked

to inhibition of the phosphatidylinositol 3-kinase/Akt pathway *Proc Natl Acad Sci U S A*, **96,** 2110-2115.

162. Weng, L., Brown, J.,Eng, C. (2001) PTEN induces apoptosis and cell cycle arrest through phosphoinositol-3-kinase/Akt-dependent and -independent pathways *Hum Mol Genet*, **10,** 237-242.

163. van Duijn, P.W. and Trapman, J. (2006) PI3K/Akt signaling regulates p27(kip1) expression via Skp2 in PC3 and DU145 prostate cancer cells, but is not a major factor in p27(kip1) regulation in LNCaP and PC346 cells *Prostate*, **66,** 749-760.

164. Motti, M.L., Califano, D., Troncone, G., De Marco, C., Migliaccio, I., Palmieri, E., Pezzullo, L., Palombini, L., Fusco, A.,Viglietto, G. (2005) Complex regulation of the cyclin-dependent kinase inhibitor p27kip1 in thyroid cancer cells by the PI3K/AKT pathway: regulation of p27kip1 expression and localization *Am J Pathol*, **166,** 737-749.

165. Mamillapalli, R., Gavrilova, N., Mihaylova, V.T., Tsvetkov, L.M., Wu, H., Zhang, H.,Sun, H. (2001) PTEN regulates the ubiquitin-dependent degradation of the CDK inhibitor p27(KIP1) through the ubiquitin E3 ligase SCF(SKP2) *Curr Biol*, **11,** 263-267.

166. Jonason, J.H., Gavrilova, N., Wu, M., Zhang, H.,Sun, H. (2007) Regulation of SCF(SKP2) ubiquitin E3 ligase assembly and p27(KIP1) proteolysis by the PTEN pathway and cyclin D1 *Cell Cycle*, **6,** 951-961.

167. Medema, R.H., Kops, G.J., Bos, J.L.,Burgering, B.M. (2000) AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1 *Nature*, **404,** 782-787.

168. Nakamura, N., Ramaswamy, S., Vazquez, F., Signoretti, S., Loda, M.,Sellers, W.R. (2000) Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN *Mol Cell Biol*, **20,** 8969-8982.

169. Dijkers, P.F., Medema, R.H., Pals, C., Banerji, L., Thomas, N.S., Lam, E.W., Burgering, B.M., Raaijmakers, J.A., Lammers, J.W., Koenderman, L., et al. (2000) Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1) *Mol Cell Biol*, **20,** 9138-9148.

170. Kurose, K., Zhou, X.P., Araki, T., Cannistra, S.A., Maher, E.R.,Eng, C. (2001) Frequent loss of PTEN expression is linked to elevated phosphorylated Akt levels, but not associated with p27 and cyclin D1 expression, in primary epithelial ovarian carcinomas *Am J Pathol*, **158,** 2097-2106.

171. Gao, N., Flynn, D.C., Zhang, Z., Zhong, X.S., Walker, V., Liu, K.J., Shi, X.,Jiang, B.H. (2004) G1 cell cycle progression and the expression of G1 cyclins are regulated by PI3K/AKT/mTOR/p70S6K1 signaling in human ovarian cancer cells *Am J Physiol Cell Physiol*, **287,** C281-291.

172. Schmidt, M., Fernandez de Mattos, S., van der Horst, A., Klompmaker, R., Kops, G.J., Lam, E.W., Burgering, B.M.,Medema, R.H. (2002) Cell cycle inhibition by FoxO forkhead transcription factors involves downregulation of cyclin D *Mol Cell Biol*, **22,** 7842-7852.

173. Cobrinik, D. (2005) Pocket proteins and cell cycle control *Oncogene*, **24,** 2796-2809.

174. Kops, G.J., Medema, R.H., Glassford, J., Essers, M.A., Dijkers, P.F., Coffer, P.J., Lam, E.W.,Burgering, B.M. (2002) Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors *Mol Cell Biol*, **22,** 2025-2036.

175. Kandel, E.S., Skeen, J., Majewski, N., Di Cristofano, A., Pandolfi, P.P., Feliciano, C.S., Gartel, A.,Hay, N. (2002) Activation of Akt/protein kinase B overcomes a G(2)/m cell cycle checkpoint induced by DNA damage *Mol Cell Biol*, **22,** 7831-7841.

176. Katayama, K., Fujita, N.,Tsuruo, T. (2005) Akt/protein kinase B-dependent phosphorylation and inactivation of WEE1Hu promote cell cycle progression at G2/M transition *Mol Cell Biol*, **25,** 5725-5737.

177. Lee, S.R., Park, J.H., Park, E.K., Chung, C.H., Kang, S.S.,Bang, O.S. (2005) Akt-induced promotion of cell-cycle progression at G2/M phase involves upregulation of NF-Y binding activity in PC12 cells *J Cell Physiol*, **205,** 270-277.

178. Jin, Z.H., Kurosu, T., Yamaguchi, M., Arai, A.,Miura, O. (2005) Hematopoietic cytokines enhance Chk1-dependent G2/M checkpoint activation by etoposide through the Akt/GSK3 pathway to inhibit apoptosis *Oncogene*, **24,** 1973-1981.

179. Shtivelman, E., Sussman, J.,Stokoe, D. (2002) A role for PI 3-kinase and PKB activity in the G2/M phase of the cell cycle *Curr Biol*, **12,** 919-924.

180. Downward, J. (2004) PI 3-kinase, Akt and cell survival *Semin Cell Dev Biol*, **15,** 177-182.

181. Koseoglu, S., Lu, Z., Kumar, C., Kirschmeier, P.,Zou, J. (2007) AKT1, AKT2 and AKT3-Dependent Cell Survival is Cell Line-specific and Knockdown of All Three Isoforms Selectively Induces Apoptosis in 20 Human Tumor Cell Lines *Cancer Biol Ther*, **6,** 755-762.

182. Davies, M.A., Koul, D., Dhesi, H., Berman, R., McDonnell, T.J., McConkey, D., Yung, W.K.,Steck, P.A. (1999) Regulation of Akt/PKB activity, cellular growth, and apoptosis in prostate carcinoma cells by MMAC/PTEN *Cancer Res*, **59,** 2551-2556.

183. Xu, Z., Stokoe, D., Kane, L.P.,Weiss, A. (2002) The inducible expression of the tumor suppressor gene PTEN promotes apoptosis and decreases cell size by inhibiting the PI3K/Akt pathway in Jurkat T cells *Cell Growth Differ*, **13,** 285-296.

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

185. 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) The PTEN/MMAC1/TEP tumor suppressor gene decreases

cell growth and induces apoptosis and anoikis in breast cancer cells *Oncogene*, **18,** 7034-7045.

186. 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) Adenoviral transgene expression of MMAC/PTEN in human glioma cells inhibits Akt activation and induces anoikis *Cancer Res*, **58,** 5285-5290.
187. Tamura, M., Gu, J., Danen, E.H., Takino, T., Miyamoto, S.,Yamada, K.M. (1999) PTEN interactions with focal adhesion kinase and suppression of the extracellular matrix-dependent phosphatidylinositol 3-kinase/Akt cell survival pathway *J Biol Chem*, **274,** 20693-20703.
188. Goberdhan, D.C. and Wilson, C. (2003) PTEN: tumour suppressor, multifunctional growth regulator and more *Hum Mol Genet*, **12 Spec No 2,** R239-248.
189. Huang, H., Potter, C.J., Tao, W., Li, D.M., Brogiolo, W., Hafen, E., Sun, H.,Xu, T. (1999) PTEN affects cell size, cell proliferation and apoptosis during Drosophila eye development *Development*, **126,** 5365-5372.
190. Goberdhan, D.C., Paricio, N., Goodman, E.C., Mlodzik, M.,Wilson, C. (1999) Drosophila tumor suppressor PTEN controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway *Genes Dev*, **13,** 3244-3258.
191. Scanga, S.E., Ruel, L., Binari, R.C., Snow, B., Stambolic, V., Bouchard, D., Peters, M., Calvieri, B., Mak, T.W., Woodgett, J.R., et al. (2000) The conserved PI3'K/PTEN/Akt signaling pathway regulates both cell size and survival in Drosophila *Oncogene*, **19,** 3971-3977.
192. Backman, S., Stambolic, V.,Mak, T. (2002) PTEN function in mammalian cell size regulation *Curr Opin Neurobiol*, **12,** 516-522.
193. Stiles, B., Groszer, M., Wang, S., Jiao, J.,Wu, H. (2004) PTENless means more *Dev Biol*, **273,** 175-184.
194. Giri, D. and Ittmann, M. (1999) Inactivation of the PTEN tumor suppressor gene is associated with increased angiogenesis in clinically localized prostate carcinoma *Hum Pathol*, **30,** 419-424.
195. Fang, J., Ding, M., Yang, L., Liu, L.Z.,Jiang, B.H. (2007) PI3K/PTEN/AKT signaling regulates prostate tumor angiogenesis *Cell Signal*, **19,** 2487-2497.
196. Koul, D., Shen, R., Garyali, A., Ke, L.D., Liu, T.J.,Yung, W.K. (2002) MMAC/PTEN tumor suppressor gene regulates vascular endothelial growth factor-mediated angiogenesis in prostate cancer *Int J Oncol*, **21,** 469-475.
197. Firtel, R.A. and Chung, C.Y. (2000) The molecular genetics of chemotaxis: sensing and responding to chemoattractant gradients *Bioessays*, **22,** 603-615.
198. Leslie, N.R., Yang, X., Downes, C.P.,Weijer, C.J. (2007) PtdIns(3,4,5)P(3)-dependent and -independent roles for PTEN in the control of cell migration *Curr Biol*, **17,** 115-125.

199. Comer, F.I. and Parent, C.A. (2007) Phosphoinositides specify polarity during epithelial organ development *Cell*, **128,** 239-240.

200. Martin-Belmonte, F., Gassama, A., Datta, A., Yu, W., Rescher, U., Gerke, V.,Mostov, K. (2007) PTEN-mediated apical segregation of phosphoinositides controls epithelial morphogenesis through Cdc42 *Cell*, **128,** 383-397.

201. Groszer, M., Erickson, R., Scripture-Adams, D.D., Dougherty, J.D., Le Belle, J., Zack, J.A., Geschwind, D.H., Liu, X., Kornblum, H.I.,Wu, H. (2006) PTEN negatively regulates neural stem cell self-renewal by modulating G0-G1 cell cycle entry *Proc Natl Acad Sci U S A*, **103,** 111-116.

202. Groszer, M., Erickson, R., Scripture-Adams, D.D., Lesche, R., Trumpp, A., Zack, J.A., Kornblum, H.I., Liu, X.,Wu, H. (2001) Negative regulation of neural stem/progenitor cell proliferation by the Pten tumor suppressor gene in vivo *Science*, **294,** 2186-2189.

203. Zhang, J., Grindley, J.C., Yin, T., Jayasinghe, S., He, X.C., Ross, J.T., Haug, J.S., Rupp, D., Porter-Westpfahl, K.S., Wiedemann, L.M., et al. (2006) PTEN maintains haematopoietic stem cells and acts in lineage choice and leukaemia prevention *Nature*, **441,** 518-522.

204. Yilmaz, O.H., Valdez, R., Theisen, B.K., Guo, W., Ferguson, D.O., Wu, H.,Morrison, S.J. (2006) Pten dependence distinguishes haematopoietic stem cells from leukaemia-initiating cells *Nature*, **441,** 475-482.

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

206. Tothova, Z. and Gilliland, D.G. (2007) FoxO transcription factors and stem cell homeostasis: insights from the hematopoietic system *Cell Stem Cell*, **1,** 140-152.

207. Tothova, Z., Kollipara, R., Huntly, B.J., Lee, B.H., Castrillon, D.H., Cullen, D.E., McDowell, E.P., Lazo-Kallanian, S., Williams, I.R., Sears, C., et al. (2007) FoxOs are critical mediators of hematopoietic stem cell resistance to physiologic oxidative stress *Cell*, **128,** 325-339.

208. Paik, J.H., Kollipara, R., Chu, G., Ji, H., Xiao, Y., Ding, Z., Miao, L., Tothova, Z., Horner, J.W., Carrasco, D.R., et al. (2007) FoxOs are lineage-restricted redundant tumor suppressors and regulate endothelial cell homeostasis *Cell*, **128,** 309-323.

209. Puc, J., Keniry, M., Li, H.S., Pandita, T.K., Choudhury, A.D., Memeo, L., Mansukhani, M., Murty, V.V., Gaciong, Z., Meek, S.E., et al. (2005) Lack of PTEN sequesters CHK1 and initiates genetic instability *Cancer Cell*, **7,** 193-204.

210. Mitra, P., Zhang, Y., Rameh, L.E., Ivshina, M.P., McCollum, D., Nunnari, J.J., Hendricks, G.M., Kerr, M.L., Field, S.J., Cantley, L.C., et al. (2004) A novel phosphatidylinositol(3,4,5)P3 pathway in fission yeast *J Cell Biol*, **166,** 205-211.

211. Ogg, S. and Ruvkun, G. (1998) The C. elegans PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway *Mol Cell*, **2,** 887-893.

212. Rouault, J.P., Kuwabara, P.E., Sinilnikova, O.M., Duret, L., Thierry-Mieg, D.,Billaud, M. (1999) Regulation of dauer larva development in Caenorhabditis elegans by daf-18, a homologue of the tumour suppressor PTEN *Curr Biol*, **9,** 329-332.

213. Gil, E.B., Malone Link, E., Liu, L.X., Johnson, C.D.,Lees, J.A. (1999) Regulation of the insulin-like developmental pathway of Caenorhabditis elegans by a homolog of the PTEN tumor suppressor gene *Proc Natl Acad Sci U S A*, **96,** 2925-2930.

214. Solari, F., Bourbon-Piffaut, A., Masse, I., Payrastre, B., Chan, A.M.,Billaud, M. (2005) The human tumour suppressor PTEN regulates longevity and dauer formation in Caenorhabditis elegans *Oncogene*, **24,** 20-27.

215. Gao, X., Neufeld, T.P.,Pan, D. (2000) Drosophila PTEN regulates cell growth and proliferation through PI3K-dependent and -independent pathways *Dev Biol*, **221,** 404-418.

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

217. Podsypanina, K., Ellenson, L.H., Nemes, A., Gu, J., Tamura, M., Yamada, K.M., Cordon-Cardo, C., Catoretti, G., Fisher, P.E.,Parsons, R. (1999) Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems *Proc Natl Acad Sci U S A*, **96,** 1563-1568.

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

219. Stambolic, V., Suzuki, A., de la Pompa, J.L., Brothers, G.M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J.M., Siderovski, D.P.,Mak, T.W. (1998) Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN *Cell*, **95,** 29-39.

220. Freeman, D., Lesche, R., Kertesz, N., Wang, S., Li, G., Gao, J., Groszer, M., Martinez-Diaz, H., Rozengurt, N., Thomas, G., et al. (2006) Genetic background controls tumor development in PTEN-deficient mice *Cancer Res*, **66,** 6492-6496.

221. Suzuki, A., Nakano, T., Mak, T.W.,Sasaki, T. (2008) Portrait of PTEN: messages from mutant mice *Cancer Sci*, **99,** 209-213.

222. Kishimoto, H., Hamada, K., Saunders, M., Backman, S., Sasaki, T., Nakano, T., Mak, T.W.,Suzuki, A. (2003) Physiological functions of Pten in mouse tissues *Cell Struct Funct*, **28,** 11-21.

223. Lu, T.L., Chang, J.L., Liang, C.C., You, L.R.,Chen, C.M. (2007) Tumor spectrum, tumor latency and tumor incidence of the Pten-deficient mice *PLoS ONE*, **2,** e1237.

224. Chen, M.L., Xu, P.Z., Peng, X.D., Chen, W.S., Guzman, G., Yang, X., Di Cristofano, A., Pandolfi, P.P.,Hay, N. (2006) The deficiency of Akt1 is sufficient to suppress tumor development in Pten+/- mice *Genes Dev*, **20,** 1569-1574.

225. Yoo, L.I., Liu, D.W., Le Vu, S., Bronson, R.T., Wu, H.,Yuan, J. (2006) Pten deficiency activates distinct downstream signaling pathways in a tissue-specific manner *Cancer Res*, **66,** 1929-1939.

226. Chow, L.M. and Baker, S.J. (2006) PTEN function in normal and neoplastic growth *Cancer Lett*, **241,** 184-196.

227. McNeal, J.E. (1981) The zonal anatomy of the prostate *Prostate*, **2,** 35-49.

228. De Marzo, A.M., Platz, E.A., Sutcliffe, S., Xu, J., Gronberg, H., Drake, C.G., Nakai, Y., Isaacs, W.B.,Nelson, W.G. (2007) Inflammation in prostate carcinogenesis *Nat Rev Cancer*, **7,** 256-269.

229. Nelson, W.G., De Marzo, A.M.,Isaacs, W.B. (2003) Prostate cancer *N Engl J Med*, **349,** 366-381.

230. Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J., Smigal, C.,Thun, M.J. (2006) Cancer statistics, 2006 *CA Cancer J Clin*, **56,** 106-130.

231. Bostwick, D.G., Burke, H.B., Djakiew, D., Euling, S., Ho, S.M., Landolph, J., Morrison, H., Sonawane, B., Shifflett, T., Waters, D.J., et al. (2004) Human prostate cancer risk factors *Cancer*, **101,** 2371-2490.

232. Ryan, C.J. and Small, E.J. (2004) Advances in prostate cancer *Curr Opin Oncol*, **16,** 242-246.

233. Ryan, C.J. and Small, E.J. (2006) Prostate cancer update: 2005 *Curr Opin Oncol*, **18,** 284-288.

234. Sonpavde, G. and Hutson, T.E. (2006) New approaches in hormone refractory prostate cancer *Am J Clin Oncol*, **29,** 196-201.

235. Petrylak, D. (2005) Therapeutic options in androgen-independent prostate cancer: building on docetaxel *BJU Int*, **96 Suppl 2,** 41-46.

236. Shand, R.L. and Gelmann, E.P. (2006) Molecular biology of prostate-cancer pathogenesis *Curr Opin Urol*, **16,** 123-131.

237. Rubin, M.A. and De Marzo, A.M. (2004) Molecular genetics of human prostate cancer *Mod Pathol*, **17,** 380-388.

238. Sun, J., Liu, W., Adams, T.S., Li, X., Turner, A.R., Chang, B., Kim, J.W., Zheng, S.L., Isaacs, W.B.,Xu, J. (2007) DNA copy number alterations in prostate cancers: a combined analysis of published CGH studies *Prostate*, **67,** 692-700.

239. Saramaki, O.R., Porkka, K.P., Vessella, R.L.,Visakorpi, T. (2006) Genetic aberrations in prostate cancer by microarray analysis *Int J Cancer*, **119,** 1322-1329.

240. Tomlins, S.A., Rhodes, D.R., Perner, S., Dhanasekaran, S.M., Mehra, R., Sun, X.W., Varambally, S., Cao, X., Tchinda, J., Kuefer, R., et al. (2005) Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer *Science*, **310,** 644-648.

241. Mehra, R., Tomlins, S.A., Shen, R., Nadeem, O., Wang, L., Wei, J.T., Pienta, K.J., Ghosh, D., Rubin, M.A., Chinnaiyan, A.M., et al. (2007) Comprehensive assessment of TMPRSS2 and ETS family gene aberrations in clinically localized prostate cancer *Mod Pathol*, **20,** 538-544.

242. Ittmann, M. (1996) Allelic loss on chromosome 10 in prostate adenocarcinoma *Cancer Res*, **56,** 2143-2147.

243. Komiya, A., Suzuki, H., Ueda, T., Yatani, R., Emi, M., Ito, H.,Shimazaki, J. (1996) Allelic losses at loci on chromosome 10 are associated with metastasis and progression of human prostate cancer *Genes Chromosomes Cancer*, **17,** 245-253.

244. Trybus, T.M., Burgess, A.C., Wojno, K.J., Glover, T.W.,Macoska, J.A. (1996) Distinct areas of allelic loss on chromosomal regions 10p and 10q in human prostate cancer *Cancer Res*, **56,** 2263-2267.

245. Gray, I.C., Phillips, S.M., Lee, S.J., Neoptolemos, J.P., Weissenbach, J.,Spurr, N.K. (1995) Loss of the chromosomal region 10q23-25 in prostate cancer *Cancer Res*, **55,** 4800-4803.

246. Nihei, N., Ichikawa, T., Kawana, Y., Kuramochi, H., Kugo, H., Oshimura, M., Killary, A.M., Rinker-Schaeffer, C.W., Barrett, J.C., Isaacs, J.T., et al. (1995) Localization of metastasis suppressor gene(s) for rat prostatic cancer to the long arm of human chromosome 10 *Genes Chromosomes Cancer*, **14,** 112-119.

247. Vlietstra, R.J., van Alewijk, D.C., Hermans, K.G., van Steenbrugge, G.J.,Trapman, J. (1998) Frequent inactivation of PTEN in prostate cancer cell lines and xenografts *Cancer Res*, **58,** 2720-2723.

248. Wang, S.I., Parsons, R.,Ittmann, M. (1998) Homozygous deletion of the PTEN tumor suppressor gene in a subset of prostate adenocarcinomas *Clin Cancer Res*, **4,** 811-815.

249. Suzuki, H., Freije, D., Nusskern, D.R., Okami, K., Cairns, P., Sidransky, D., Isaacs, W.B.,Bova, G.S. (1998) Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues *Cancer Res*, **58,** 204-209.

250. Pesche, S., Latil, A., Muzeau, F., Cussenot, O., Fournier, G., Longy, M., Eng, C.,Lidereau, R. (1998) PTEN/MMAC1/TEP1 involvement in primary prostate cancers *Oncogene*, **16,** 2879-2883.

251. Dong, J.T., Sipe, T.W., Hyytinen, E.R., Li, C.L., Heise, C., McClintock, D.E., Grant, C.D., Chung, L.W.,Frierson, H.F., Jr. (1998) PTEN/MMAC1 is infrequently mutated in pT2 and pT3 carcinomas of the prostate *Oncogene*, **17,** 1979-1982.

252. Dong, J.T., Li, C.L., Sipe, T.W.,Frierson, H.F., Jr. (2001) Mutations of PTEN/MMAC1 in primary prostate cancers from Chinese patients *Clin Cancer Res*, **7,** 304-308.

253. Feilotter, H.E., Nagai, M.A., Boag, A.H., Eng, C.,Mulligan, L.M. (1998) Analysis of PTEN and the 10q23 region in primary prostate carcinomas *Oncogene*, **16,** 1743-1748.

254. Gray, I.C., Stewart, L.M., Phillips, S.M., Hamilton, J.A., Gray, N.E., Watson, G.J., Spurr, N.K.,Snary, D. (1998) Mutation and expression analysis of the putative prostate tumour-suppressor gene PTEN *Br J Cancer*, **78,** 1296-1300.

255. Verhagen, P.C., van Duijn, P.W., Hermans, K.G., Looijenga, L.H., van Gurp, R.J., Stoop, H., van der Kwast, T.H.,Trapman, J. (2006) The PTEN gene in locally progressive prostate cancer is preferentially inactivated by bi-allelic gene deletion *J Pathol*, **208,** 699-707.

256. Yoshimoto, M., Cutz, J.C., Nuin, P.A., Joshua, A.M., Bayani, J., Evans, A.J., Zielenska, M.,Squire, J.A. (2006) Interphase FISH analysis of PTEN in histologic sections shows genomic deletions in 68% of primary prostate cancer and 23% of high-grade prostatic intra-epithelial neoplasias *Cancer Genet Cytogenet*, **169,** 128-137.

257. Yoshimoto, M., Cunha, I.W., Coudry, R.A., Fonseca, F.P., Torres, C.H., Soares, F.A.,Squire, J.A. (2007) FISH analysis of 107 prostate cancers shows that PTEN genomic deletion is associated with poor clinical outcome *Br J Cancer*, **97,** 678-685.

258. Teng, D.H., Hu, R., Lin, H., Davis, T., Iliev, D., Frye, C., Swedlund, B., Hansen, K.L., Vinson, V.L., Gumpper, K.L., et al. (1997) MMAC1/PTEN mutations in primary tumor specimens and tumor cell lines *Cancer Res*, **57,** 5221-5225.

259. Hermans, K.G., van Alewijk, D.C., Veltman, J.A., van Weerden, W., van Kessel, A.G.,Trapman, J. (2004) Loss of a small region around the PTEN locus is a major chromosome 10 alteration in prostate cancer xenografts and cell lines *Genes Chromosomes Cancer*, **39,** 171-184.

260. Konishi, N., Nakamura, M., Kishi, M., Nishimine, M., Ishida, E.,Shimada, K. (2002) Heterogeneous methylation and deletion patterns of the INK4a/ARF locus within prostate carcinomas *Am J Pathol*, **160,** 1207-1214.

261. Whang, Y.E., Wu, X., Suzuki, H., Reiter, R.E., Tran, C., Vessella, R.L., Said, J.W., Isaacs, W.B.,Sawyers, C.L. (1998) Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression *Proc Natl Acad Sci U S A*, **95,** 5246-5250.

262. Knudson, A.G. (2001) Two genetic hits (more or less) to cancer *Nat Rev Cancer*, **1,** 157-162.

263. Knudson, A.G., Jr. (1971) Mutation and cancer: statistical study of retinoblastoma *Proc Natl Acad Sci U S A*, **68,** 820-823.

264. Santarosa, M. and Ashworth, A. (2004) Haploinsufficiency for tumour suppressor genes: when you don't need to go all the way *Biochim Biophys Acta*, **1654,** 105-122.

265. Feilotter, H.E., Coulon, V., McVeigh, J.L., Boag, A.H., Dorion-Bonnet, F., Duboue, B., Latham, W.C., Eng, C., Mulligan, L.M.,Longy, M. (1999) Analysis of the 10q23 chromosomal region and the PTEN gene in human sporadic breast carcinoma *Br J Cancer*, **79,** 718-723.

266. Gottschalk, A.R., Doan, A., Nakamura, J.L., Haas-Kogan, D.A.,Stokoe, D. (2005) Inhibition of phosphatidylinositol-3-kinase causes cell death through a protein kinase B (PKB)-dependent mechanism and growth arrest through a PKB-independent mechanism *Int J Radiat Oncol Biol Phys*, **61,** 1183-1188.

267. Zhao, H., Dupont, J., Yakar, S., Karas, M.,LeRoith, D. (2004) PTEN inhibits cell proliferation and induces apoptosis by downregulating cell surface IGF-IR expression in prostate cancer cells *Oncogene*, **23,** 786-794.

268. Sharrard, R.M. and Maitland, N.J. (2000) Phenotypic effects of overexpression of the MMAC1 gene in prostate epithelial cells *Br J Cancer*, **83,** 1102-1109.

269. Lin, J., Adam, R.M., Santiestevan, E.,Freeman, M.R. (1999) The phosphatidylinositol 3'-kinase pathway is a dominant growth factor-activated cell survival pathway in LNCaP human prostate carcinoma cells *Cancer Res*, **59,** 2891-2897.

270. Tanaka, M., Rosser, C.J.,Grossman, H.B. (2005) PTEN gene therapy induces growth inhibition and increases efficacy of chemotherapy in prostate cancer *Cancer Detect Prev*, **29,** 170-174.

271. Carson, J.P., Kulik, G.,Weber, M.J. (1999) Antiapoptotic signaling in LNCaP prostate cancer cells: a survival signaling pathway independent of phosphatidylinositol 3'-kinase and Akt/protein kinase B *Cancer Res*, **59,** 1449-1453.

272. Davies, M.A., Kim, S.J., Parikh, N.U., Dong, Z., Bucana, C.D.,Gallick, G.E. (2002) Adenoviral-mediated Expression of MMAC/PTEN Inhibits Proliferation and Metastasis of Human Prostate Cancer Cells *Clin Cancer Res*, **8,** 1904-1914.

273. Xin, L., Lawson, D.A.,Witte, O.N. (2005) The Sca-1 cell surface marker enriches for a prostate-regenerating cell subpopulation that can initiate prostate tumorigenesis *Proc Natl Acad Sci U S A*, **102,** 6942-6947.

274. Wang, S., Garcia, A.J., Wu, M., Lawson, D.A., Witte, O.N.,Wu, H. (2006) Pten deletion leads to the expansion of a prostatic stem/progenitor cell subpopulation and tumor initiation *Proc Natl Acad Sci U S A*, **103,** 1480-1485.

275. Feldman, B.J. and Feldman, D. (2001) The development of androgen-independent prostate cancer *Nat Rev Cancer*, **1,** 34-45.

276. Bertram, J., Peacock, J.W., Fazli, L., Mui, A.L., Chung, S.W., Cox, M.E., Monia, B., Gleave, M.E.,Ong, C.J. (2006) Loss of PTEN is associated with progression to androgen independence *Prostate*, **66,** 895-902.

277. Gao, H., Ouyang, X., Banach-Petrosky, W.A., Gerald, W.L., Shen, M.M.,Abate-Shen, C. (2006) Combinatorial activities of Akt and B-Raf/Erk signaling in a mouse model of androgen-independent prostate cancer *Proc Natl Acad Sci U S A*, **103,** 14477-14482.

278. Gao, H., Ouyang, X., Banach-Petrosky, W.A., Shen, M.M.,Abate-Shen, C. (2006) Emergence of androgen independence at early stages of prostate cancer progression in nkx3.1; pten mice *Cancer Res*, **66,** 7929-7933.

279. Graff, J.R. (2002) Emerging targets in the AKT pathway for treatment of androgen-independent prostatic adenocarcinoma *Expert Opin Ther Targets*, **6,** 103-113.

280. Graff, J.R., Konicek, B.W., McNulty, A.M., Wang, Z., Houck, K., Allen, S., Paul, J.D., Hbaiu, A., Goode, R.G., Sandusky, G.E., et al. (2000) Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27Kip1 expression *J Biol Chem*, **275,** 24500-24505.

281. Jiao, J., Wang, S., Qiao, R., Vivanco, I., Watson, P.A., Sawyers, C.L.,Wu, H. (2007) Murine cell lines derived from Pten null prostate cancer show the critical role of PTEN in hormone refractory prostate cancer development *Cancer Res*, **67,** 6083-6091.

282. Li, B., Sun, A., Youn, H., Hong, Y., Terranova, P.F., Thrasher, J.B., Xu, P.,Spencer, D. (2007) Conditional Akt activation promotes androgen-independent progression of prostate cancer *Carcinogenesis*, **28,** 572-583.

283. Liao, Z., Wang, S., Boileau, T.W., Erdman, J.W., Jr.,Clinton, S.K. (2005) Increased phospho-AKT is associated with loss of the androgen receptor during the progression of N-methyl-N-nitrosourea-induced prostate carcinogenesis in rats *Prostate*, **64,** 186-199.

284. Murillo, H., Huang, H., Schmidt, L.J., Smith, D.I.,Tindall, D.J. (2001) Role of PI3K signaling in survival and progression of LNCaP prostate cancer cells to the androgen refractory state *Endocrinology*, **142,** 4795-4805.

285. Shen, M.M. and Abate-Shen, C. (2007) Pten inactivation and the emergence of androgen-independent prostate cancer *Cancer Res*, **67,** 6535-6538.

286. Wu, Z., Conaway, M., Gioeli, D., Weber, M.J.,Theodorescu, D. (2006) Conditional expression of PTEN alters the androgen responsiveness of prostate cancer cells *Prostate*, **66,** 1114-1123.

287. Ghosh, P.M., Malik, S., Bedolla, R.,Kreisberg, J.I. (2003) Akt in prostate cancer: possible role in androgen-independence *Curr Drug Metab*, **4,** 487-496.

288. Wang, Y., Kreisberg, J.I.,Ghosh, P.M. (2007) Cross-talk between the androgen receptor and the phosphatidylinositol 3-kinase/Akt pathway in prostate cancer *Curr Cancer Drug Targets*, **7,** 591-604.

289. Wen, Y., Hu, M.C., Makino, K., Spohn, B., Bartholomeusz, G., Yan, D.H.,Hung, M.C. (2000) HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway *Cancer Res*, **60,** 6841-6845.

290. Gioeli, D., Ficarro, S.B., Kwiek, J.J., Aaronson, D., Hancock, M., Catling, A.D., White, F.M., Christian, R.E., Settlage, R.E., Shabanowitz, J., et al. (2002) Androgen receptor

phosphorylation. Regulation and identification of the phosphorylation sites *J Biol Chem*, **277,** 29304-29314.

291. Li, P., Nicosia, S.V.,Bai, W. (2001) Antagonism between PTEN/MMAC1/TEP-1 and androgen receptor in growth and apoptosis of prostatic cancer cells *J Biol Chem*, **276,** 20444-20450.

292. Manin, M., Baron, S., Goossens, K., Beaudoin, C., Jean, C., Veyssiere, G., Verhoeven, G.,Morel, L. (2002) Androgen receptor expression is regulated by the phosphoinositide 3-kinase/Akt pathway in normal and tumoral epithelial cells *Biochem J*, **366,** 729-736.

293. Nan, B., Snabboon, T., Unni, E., Yuan, X.J., Whang, Y.E.,Marcelli, M. (2003) The PTEN tumor suppressor is a negative modulator of androgen receptor transcriptional activity *J Mol Endocrinol*, **31,** 169-183.

294. Falkenstein, E., Tillmann, H.C., Christ, M., Feuring, M.,Wehling, M. (2000) Multiple actions of steroid hormones--a focus on rapid, nongenomic effects *Pharmacol Rev*, **52,** 513-556.

295. Sun, M., Yang, L., Feldman, R.I., Sun, X.M., Bhalla, K.N., Jove, R., Nicosia, S.V.,Cheng, J.Q. (2003) Activation of phosphatidylinositol 3-kinase/Akt pathway by androgen through interaction of p85alpha, androgen receptor, and Src *J Biol Chem*, **278,** 42992-43000.

296. Baron, S., Manin, M., Beaudoin, C., Leotoing, L., Communal, Y., Veyssiere, G.,Morel, L. (2004) Androgen receptor mediates non-genomic activation of phosphatidylinositol 3-OH kinase in androgen-sensitive epithelial cells *J Biol Chem*, **279,** 14579-14586.

297. Backman, S.A., Ghazarian, D., So, K., Sanchez, O., Wagner, K.U., Hennighausen, L., Suzuki, A., Tsao, M.S., Chapman, W.B., Stambolic, V., et al. (2004) Early onset of neoplasia in the prostate and skin of mice with tissue-specific deletion of Pten *Proc Natl Acad Sci U S A*, **101,** 1725-1730.

298. Ma, X., Ziel-van der Made, A.C., Autar, B., van der Korput, H.A., Vermeij, M., van Duijn, P., Cleutjens, K.B., de Krijger, R., Krimpenfort, P., Berns, A., et al. (2005) Targeted biallelic inactivation of Pten in the mouse prostate leads to prostate cancer accompanied by increased epithelial cell proliferation but not by reduced apoptosis *Cancer Res*, **65,** 5730-5739.

299. Ratnacaram, C.K., Teletin, M., Jiang, M., Meng, X., Chambon, P.,Metzger, D. (2008) Temporally controlled ablation of PTEN in adult mouse prostate epithelium generates a model of invasive prostatic adenocarcinoma *Proc Natl Acad Sci U S A*, **105,** 2521-2526.

300. Trotman, L.C., Niki, M., Dotan, Z.A., Koutcher, J.A., Di Cristofano, A., Xiao, A., Khoo, A.S., Roy-Burman, P., Greenberg, N.M., Van Dyke, T., et al. (2003) Pten dose dictates cancer progression in the prostate *PLoS Biol*, **1,** E59.

301. Wang, S., Gao, J., Lei, Q., Rozengurt, N., Pritchard, C., Jiao, J., Thomas, G.V., Li, G., Roy-Burman, P., Nelson, P.S., et al. (2003) Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer *Cancer Cell*, **4,** 209-221.

302. Majumder, P.K., Yeh, J.J., George, D.J., Febbo, P.G., Kum, J., Xue, Q., Bikoff, R., Ma, H., Kantoff, P.W., Golub, T.R., et al. (2003) Prostate intraepithelial neoplasia induced by prostate restricted Akt activation: the MPAKT model *Proc Natl Acad Sci U S A*, **100,** 7841-7846.

303. Bai, F., Pei, X.H., Pandolfi, P.P.,Xiong, Y. (2006) p18 Ink4c and Pten constrain a positive regulatory loop between cell growth and cell cycle control *Mol Cell Biol*, **26,** 4564-4576.

304. Di Cristofano, A., De Acetis, M., Koff, A., Cordon-Cardo, C.,Pandolfi, P.P. (2001) Pten and p27KIP1 cooperate in prostate cancer tumor suppression in the mouse *Nat Genet*, **27,** 222-224.

305. Kim, M.J., Cardiff, R.D., Desai, N., Banach-Petrosky, W.A., Parsons, R., Shen, M.M.,Abate-Shen, C. (2002) Cooperativity of Nkx3.1 and Pten loss of function in a mouse model of prostate carcinogenesis *Proc Natl Acad Sci U S A*, **99,** 2884-2889.

306. Kwabi-Addo, B., Giri, D., Schmidt, K., Podsypanina, K., Parsons, R., Greenberg, N.,Ittmann, M. (2001) Haploinsufficiency of the Pten tumor suppressor gene promotes prostate cancer progression *Proc Natl Acad Sci U S A*, **98,** 11563-11568.

307. Ma, L., Teruya-Feldstein, J., Behrendt, N., Chen, Z., Noda, T., Hino, O., Cordon-Cardo, C.,Pandolfi, P.P. (2005) Genetic analysis of Pten and Tsc2 functional interactions in the mouse reveals asymmetrical haploinsufficiency in tumor suppression *Genes Dev*, **19,** 1779-1786.

308. You, M.J., Castrillon, D.H., Bastian, B.C., O'Hagan, R.C., Bosenberg, M.W., Parsons, R., Chin, L.,DePinho, R.A. (2002) Genetic analysis of Pten and Ink4a/Arf interactions in the suppression of tumorigenesis in mice *Proc Natl Acad Sci U S A*, **99,** 1455-1460.

309. Hu, T.H., Huang, C.C., Lin, P.R., Chang, H.W., Ger, L.P., Lin, Y.W., Changchien, C.S., Lee, C.M.,Tai, M.H. (2003) Expression and prognostic role of tumor suppressor gene PTEN/MMAC1/TEP1 in hepatocellular carcinoma *Cancer*, **97,** 1929-1940.

310. Chung, M.J., Jung, S.H., Lee, B.J., Kang, M.J.,Lee, D.G. (2004) Inactivation of the PTEN gene protein product is associated with the invasiveness and metastasis, but not angiogenesis, of breast cancer *Pathol Int*, **54,** 10-15.

311. Ricci, R., Maggiano, N., Castri, F., Rinelli, A., Murazio, M., Pacelli, F., Potenza, A.E., Vecchio, F.M.,Larocca, L.M. (2004) Role of PTEN in gastrointestinal stromal tumor progression *Arch Pathol Lab Med*, **128,** 421-425.

312. McMenamin, M.E., Soung, P., Perera, S., Kaplan, I., Loda, M.,Sellers, W.R. (1999) Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage *Cancer Res*, **59,** 4291-4296.

313. Koksal, I.T., Dirice, E., Yasar, D., Sanlioglu, A.D., Ciftcioglu, A., Gulkesen, K.H., Ozes, N.O., Baykara, M., Luleci, G.,Sanlioglu, S. (2004) The assessment of PTEN tumor suppressor gene in combination with Gleason scoring and serum PSA to evaluate progression of prostate carcinoma *Urol Oncol*, **22,** 307-312.

314. Schmitz, M., Grignard, G., Margue, C., Dippel, W., Capesius, C., Mossong, J., Nathan, M., Giacchi, S., Scheiden, R.,Kieffer, N. (2007) Complete loss of PTEN expression as a possible early prognostic marker for prostate cancer metastasis *Int J Cancer*, **120,** 1284-1292.

315. Pourmand, G., Ziaee, A.A., Abedi, A.R., Mehrsai, A., Alavi, H.A., Ahmadi, A.,Saadati, H.R. (2007) Role of PTEN gene in progression of prostate cancer *Urol J*, **4,** 95-100.

316. Fenic, I., Franke, F., Failing, K., Steger, K.,Woenckhaus, J. (2004) Expression of PTEN in malignant and non-malignant human prostate tissues: comparison with p27 protein expression *J Pathol*, **203,** 559-566.

317. Saal, L.H., Johansson, P., Holm, K., Gruvberger-Saal, S.K., She, Q.B., Maurer, M., Koujak, S., Ferrando, A.A., Malmstrom, P., Memeo, L., et al. (2007) Poor prognosis in carcinoma is associated with a gene expression signature of aberrant PTEN tumor suppressor pathway activity *Proc Natl Acad Sci U S A*, **104,** 7564-7569.

318. Ayala, G., Thompson, T., Yang, G., Frolov, A., Li, R., Scardino, P., Ohori, M., Wheeler, T.,Harper, W. (2004) High levels of phosphorylated form of Akt-1 in prostate cancer and non-neoplastic prostate tissues are strong predictors of biochemical recurrence *Clin Cancer Res*, **10,** 6572-6578.

319. Kreisberg, J.I., Malik, S.N., Prihoda, T.J., Bedolla, R.G., Troyer, D.A., Kreisberg, S.,Ghosh, P.M. (2004) Phosphorylation of Akt (Ser473) is an excellent predictor of poor clinical outcome in prostate cancer *Cancer Res*, **64,** 5232-5236.

320. Liao, Y., Grobholz, R., Abel, U., Trojan, L., Michel, M.S., Angel, P.,Mayer, D. (2003) Increase of AKT/PKB expression correlates with gleason pattern in human prostate cancer *Int J Cancer*, **107,** 676-680.

321. Malik, S.N., Brattain, M., Ghosh, P.M., Troyer, D.A., Prihoda, T., Bedolla, R.,Kreisberg, J.I. (2002) Immunohistochemical demonstration of phospho-Akt in high Gleason grade prostate cancer *Clin Cancer Res*, **8,** 1168-1171.

322. Bedolla, R., Prihoda, T.J., Kreisberg, J.I., Malik, S.N., Krishnegowda, N.K., Troyer, D.A.,Ghosh, P.M. (2007) Determining risk of biochemical recurrence in prostate cancer by immunohistochemical detection of PTEN expression and Akt activation *Clin Cancer Res*, **13,** 3860-3867.

323. Lloyd, R.V., Erickson, L.A., Jin, L., Kulig, E., Qian, X., Cheville, J.C.,Scheithauer, B.W. (1999) p27kip1: a multifunctional cyclin-dependent kinase inhibitor with prognostic significance in human cancers *Am J Pathol*, **154,** 313-323.

324. Tsihlias, J., Kapusta, L.R., DeBoer, G., Morava-Protzner, I., Zbieranowski, I., Bhattacharya, N., Catzavelos, G.C., Klotz, L.H.,Slingerland, J.M. (1998) Loss of cyclin-dependent kinase inhibitor p27Kip1 is a novel prognostic factor in localized human prostate adenocarcinoma *Cancer Res*, **58,** 542-548.

325. Yang, R.M., Naitoh, J., Murphy, M., Wang, H.J., Phillipson, J., deKernion, J.B., Loda, M.,Reiter, R.E. (1998) Low p27 expression predicts poor disease-free survival in patients with prostate cancer *J Urol*, **159,** 941-945.

326. Cheville, J.C., Lloyd, R.V., Sebo, T.J., Cheng, L., Erickson, L., Bostwick, D.G., Lohse, C.M.,Wollan, P. (1998) Expression of p27kip1 in prostatic adenocarcinoma *Mod Pathol*, **11,** 324-328.

327. Dreher, T., Zentgraf, H., Abel, U., Kappeler, A., Michel, M.S., Bleyl, U.,Grobholz, R. (2004) Reduction of PTEN and p27kip1 expression correlates with tumor grade in prostate cancer. Analysis in radical prostatectomy specimens and needle biopsies *Virchows Arch*, **444,** 509-517.

328. Halvorsen, O.J., Haukaas, S.A.,Akslen, L.A. (2003) Combined loss of PTEN and p27 expression is associated with tumor cell proliferation by Ki-67 and increased risk of recurrent disease in localized prostate cancer *Clin Cancer Res*, **9,** 1474-1479.

329. Kremer, C.L., Klein, R.R., Mendelson, J., Browne, W., Samadzedeh, L.K., Vanpatten, K., Highstrom, L., Pestano, G.A.,Nagle, R.B. (2006) Expression of mTOR signaling pathway markers in prostate cancer progression *Prostate*, **66,** 1203-1212.

Chapter 2

PI3K/Akt signaling regulates $p27^{kip1}$ expression via Skp2 in PC3 and DU145 prostate cancer cells, but is not a major factor in $p27^{kip1}$ regulation in LNCaP and PC346 cells

Petra W van Duijn and Jan Trapman

Department of Pathology, Josephine Nefkens Institute, Erasmus University Medical Center, Rotterdam, The Netherlands

Prostate 66: 749-760 (2006)

Abstract

We compared the involvement of PI3K/PTEN/Akt signaling in the regulation of the cell cycle regulator $p27^{kip1}$ and investigated the mechanism of PI3K/PTEN/Akt modulation of $p27^{kip1}$ in the prostate cancer cell lines LNCaP, PC346, PC3 and DU145. PI3K/PTEN/Akt signaling was manipulated by wortmannin or specific siRNA. The effects on PI3K/Akt downstream effectors and $p27^{kip1}$ expression were monitored on RNA and protein levels. PI3K/Akt inhibition in LNCaP and PC346 cells hardly affected $p27^{kip1}$ expression. As shown in LNCaP cells, $p27^{kip1}$ expression inversely correlated with Skp2 expression, but Skp2 was not regulated by Akt. Blocking PI3K/Akt signaling in PC3 cells resulted in decreased Skp2 protein expression and increased $p27^{kip1}$. Down-regulation of PTEN in DU145 cells also showed PTEN/Akt dependent regulation of Skp2 and $p27^{kip1}$. In PC3 and DU145 cells Skp2 is the main determinant in the PI3K/Akt dependent regulation of $p27^{kip1}$. In LNCaP and PC346 cells PI3K/Akt signaling is not a major factor in $p27^{kip1}$ regulation.

Introduction

Uncontrolled proliferation, enhanced migration and decreased apoptosis are important hallmarks of tumor cells [1]. The PI3K/Akt signaling pathway, which is negatively regulated by the tumor suppressor PTEN, is an important regulator of these biological processes. *PTEN* is frequently inactivated in human tumors including brain, endometrial and prostate cancer [2-5]. In approximately 15% of primary prostate tumors and in up to 60% of metastases, hetero transplants and cell lines *PTEN* defects have been found [6-8]. Absence of PTEN expression in primary prostate tumors is correlated with higher Gleason score and advanced pathological stage [9].

PTEN is a phosphatase that antagonizes PI3K by dephosphorylating the phospholipid PtdIns(3,4,5)P3 (PIP3) at position D3 of the inositol ring [10,11]. Although PTEN primarily functions as a PIP3 lipid phosphatase it can also dephosphorylate proteins at either serine/threonine or tyrosine residues [11]. PIP3 is required for the phosphorylation and activation of the serine/threonine kinase Akt, a key mediator of the PI3K/Akt signaling pathway.

The cell cycle inhibitor $p27^{kip1}$ causes cell cycle arrest at G_0/G_1 thereby inhibiting cell proliferation [12,13]. The $p27^{kip1}$ level can be regulated by various signaling pathways [14-16]. A role for PI3K/Akt signaling in $p27^{kip1}$ regulation was first

suggested by Li and Sun (1998)[17], who observed that PTEN induced cell cycle arrest was associated with altered $p27^{kip1}$ expression.

CDKN1B, encoding $p27^{kip1}$, has been identified as a direct target gene of FOXO transcription factors [18-20]. FOXO phosphorylation by phospho-Akt leads to their inactivation due to cytoplasmic sequestration [21,22]. A second important mechanism of $p27^{kip1}$ regulation is specific proteasomal degradation mediated by Skp2 dependent ubiquitination [23,24]. Skp2 has been identified as the specific substrate recognition subunit that targets $p27^{kip1}$ for degradation upon phosphorylation of Thr187 [23,25]. The PTEN/PI3K pathway could function through Skp2 to regulate ubiquitin dependent degradation of $p27^{kip1}$ [26-28].

Absence or low expression of $p27^{kip1}$ is an important prognostic marker in a wide variety of tumors, including prostate cancer [29]. Several studies analyzing $p27^{kip1}$ expression in prostate adenocarcinoma by immunohistochemistry have shown that decreased $p27^{kip1}$ expression is associated with high tumor grade and poor prognosis [14,30-32]. In several cancers including prostate cancer Skp2 is overexpressed and its expression is inversely correlated with the $p27^{kip1}$ level [33-35].

The importance of the complex process of cell cycle regulation prompted us to study the role of PI3K/Akt signaling in the regulation of $p27^{kip1}$ in prostate cancer cell lines. We observed a different effect of PI3K/Akt signaling on $p27^{kip1}$ in the prostate cancer cell lines. In PTEN negative LNCaP and PC346 cells modulation of PI3K/Akt signaling hardly affected $p27^{kip1}$ expression. The third PTEN negative cell line PC3 showed phospho-Akt dependent $p27^{kip1}$ degradation that was mediated by Skp2, whereas transcription regulation by FOXO did not significantly contribute to regulation of $p27^{kip1}$ expression in these cells. Additionally, PTEN positive DU145 cells also displayed a PI3K/Akt dependent regulation of $p27^{kip1}$ that was mediated by Skp2.

Results

PI3K/Akt signaling and $p27^{kip1}$ expression in prostate cancer cell lines

PI3K/Akt signaling was studied in the prostate cancer cell lines LNCaP, PC3, PC346, and DU145, cultured under comparable, optimal conditions. Expression levels of PTEN, phospho-Akt, and the direct phospho-Akt target phospho-FOXO3a were assayed in the four cell lines by immunoblotting (Figure 1A). High phospho-Akt expression was found in PTEN negative LNCaP, PC3, and PC346 cells. In contrast, phosphorylated Akt was undetectable in the PTEN positive DU145 cell

line. As expected, in LNCaP, PC3, and PC346 cells high expression of inactive, phosphorylated FOXO3a correlated with phospho-Akt expression (Figure 1A). In DU145 cells phospho-FOXO3a expression could not be detected, which correlated with the lack of phospho-Akt expression and the presence of active PTEN in this cell line (Figure 1A).

Next, expression of the cell cycle regulator $p27^{kip1}$ was determined (Figure 1B). The $p27^{kip1}$ level did not correlate with the PTEN status of a cell line. $P27^{kip1}$ expression was low in rapidly growing PC3 and DU145 cells, and high in slowly growing LNCaP and PC346 cell lines.

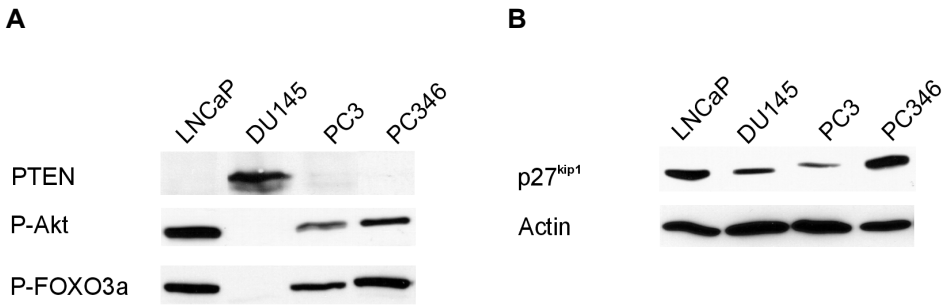


Figure 1. PTEN dependent PI3K/Akt signaling and $p27^{kip1}$ expression in prostate cancer cell lines. (A) Whole cell extracts were prepared from LNCaP, DU145, PC3, and PC346 cells and equal amounts of protein were subjected to Western Blot analysis, utilizing anti-PTEN, anti-phospho-Akt(Ser473) and anti-phospho-FOXO3a(Thr32) antibodies. (B) $P27^{kip1}$ expression was assayed in the prostate cancer cell lines by immunoblotting with an anti-$p27^{kip1}$ antibody. ß-Actin was used as an equal loading control.

Kinetics of regulation of PI3K/Akt signaling by wortmannin

To further study a presumed role of PI3K/Akt signaling in $p27^{kip1}$ expression, we first monitored the effect of PI3K inhibition by wortmannin on phospho-Akt and its downstream target phospho-FOXO3a in PC3, LNCaP, and PC346 cells. All three cell lines showed a strong reduction of both phospho-Akt and phospho-FOXO3a levels at 3 h after the start of the experiment, although within 24 h a renewed phosphorylation was observed (Figure 2A,B, and C). The kinetics of renewed Akt and FOXO3a phosphorylation markedly differed between the cell lines. In PC3 cells, at 24 h after addition of wortmannin phospho-Akt and phospho-FOXO3a had not yet returned to the original expression level, whereas in the LNCaP cell line

increased phospho-FOXO3a and phospho-Akt expression were detectable at 9 to 12 h after the start of the experiment (Figure 2A and B). Moreover, the expression of phospho-Akt and phospho-FOXO3a in PC346 cells was already restored at 6 to 9 h after wortmannin addition (Figure 2C). Repeated addition of wortmannin to PC346 cells at 3 and 6 h following the initial treatment did not result in an extended reduction of phosphorylated Akt expression (Figure 2D). No changes were observed in total Akt expression. These data indicated that the differential recovery of phospho-Akt expression was not due to degradation of wortmannin, but rather to a differential adaptation of PI3K/Akt signaling following blockade of PI3K activity.

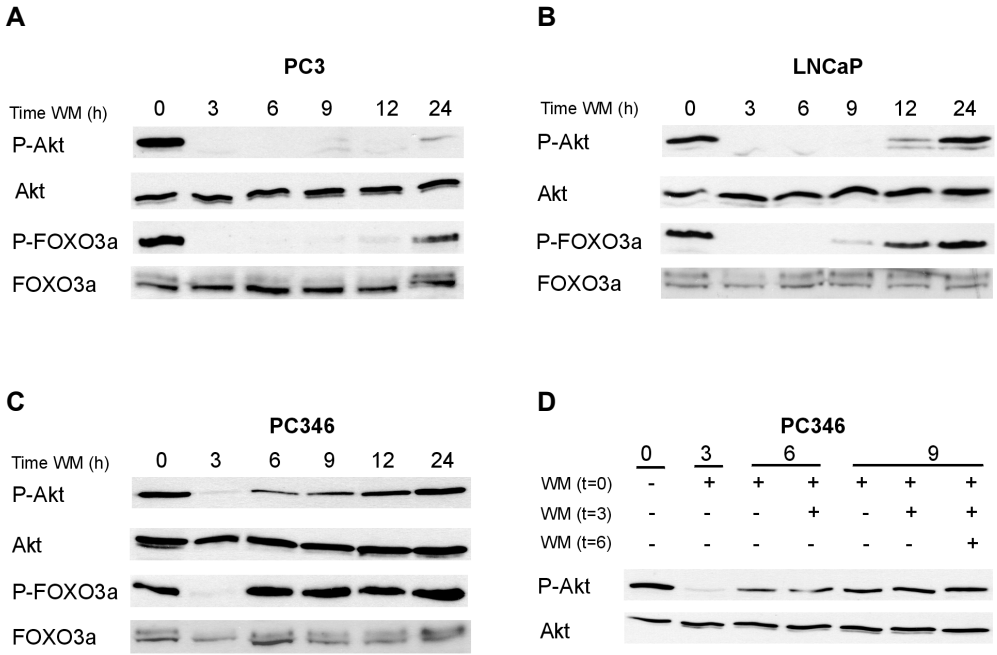


Figure 2. The effect of PI3K inactivation by wortmannin on the expression of phospho-Akt and phospho-FOXO3a in PTEN negative prostate cancer cell lines. Prostate cancer cell lines PC3 (A), LNCaP (B), and PC346 (C) were incubated with 200 nM wortmannin. Whole cell lysates were prepared at the indicated time points and equal amounts of total cell lysates were immunoblotted with anti-Akt, anti-phospho-Akt(Ser473), anti-FOXO3a and anti-phospho-FOXO3a(Thr32) antibodies, respectively. (D) Consecutive doses of wortmannin (200 nM end concentration) were added to PC346 cells at the indicated time points. Cells were harvested at 0, 3, 6 and 9 h and equal amounts of total cell lysates were immunoblotted with anti-phospho-Akt(Ser473). The total Akt expression remained unchanged throughout the experiment. Results of a representative experiment are shown.

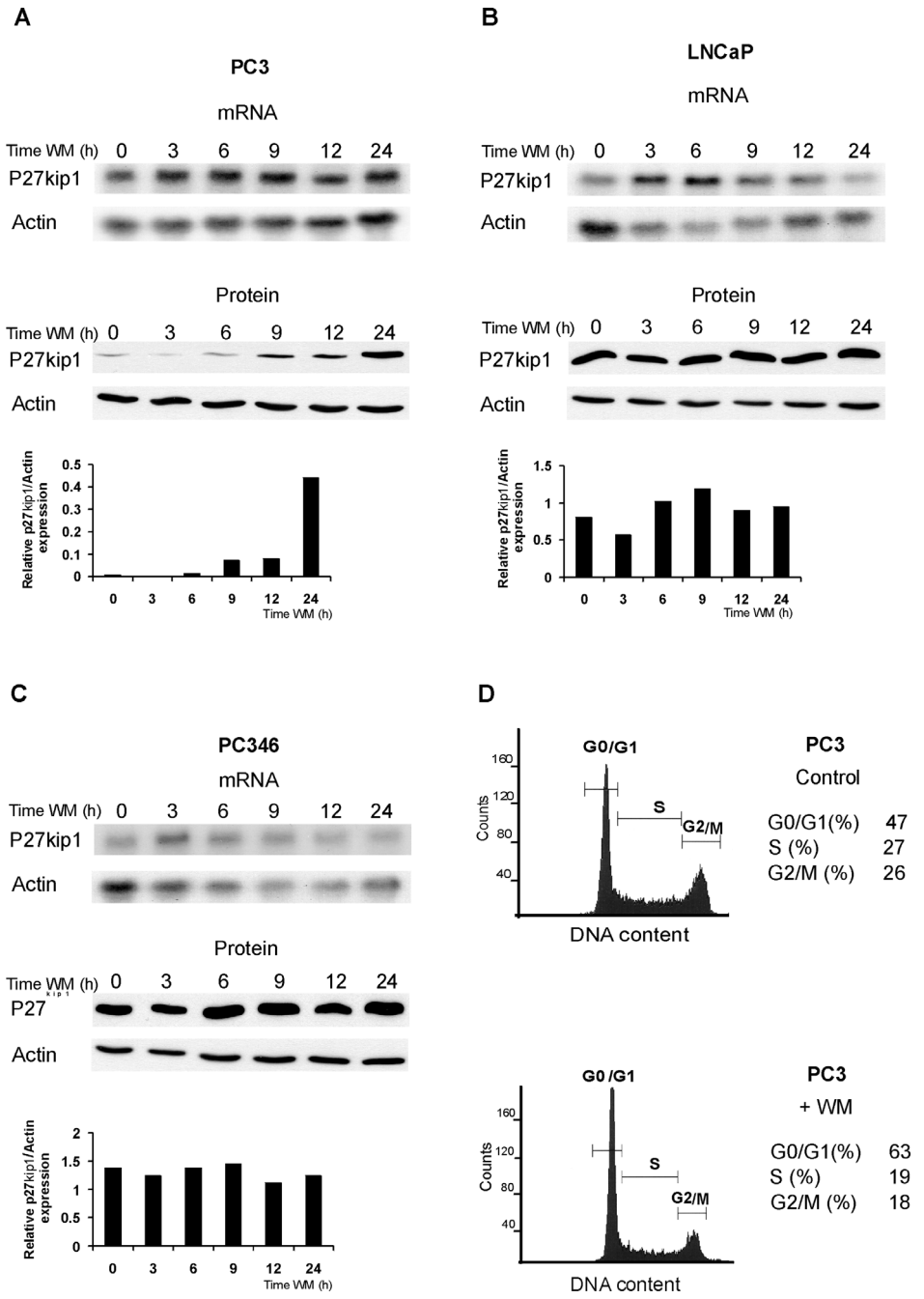


Figure 3. The effect of wortmannin on $p27^{kip1}$ mRNA and protein expression in PTEN negative prostate cancer cell lines. Protein and total RNA were isolated in parallel from wortmannin treated PC3 cells (A), LNCaP cells (B) and PC346 cells (C). The expression of $p27^{kip1}$ mRNA and protein

In all three cell lines, renewed phosphorylation of FOXO3a was more rapid than that of Akt itself, suggesting that a small, not yet measurable, increase in phospho-Akt had a major effect on the FOXO3a phosphorylation status. The expression of total Akt and FOXO3a remained constant throughout the experiment (Figure 2A,B and C). Taken together these data showed that, although the initial response to wortmannin was comparable between the cell lines, during time PC3, LNCaP, and PC346 cells all behaved differently.

Cell line specific effect of wortmannin on p27^{kip1} mRNA and protein expression

We utilized the differential effect of wortmannin on PC3, LNCaP, and PC346 cells to study regulation of the PI3K/Akt downstream target p27^{kip1}. To investigate whether FOXO3a activation due to inhibition of PI3K/Akt signaling (see Figure 2) contributed to the regulation of p27^{kip1} mRNA expression, Northern blot experiments were performed (Figure 3). In PC3 cells p27^{kip1} mRNA expression was hardly affected by wortmannin (Figure 3A), although in this cell line the most pronounced effect on phospho-FOXO3a was found (Figure 2A). Both LNCaP and PC346 cells showed a transient, small up-regulation of p27^{kip1} mRNA following dephosphorylation of FOXO3a (Figure 3B and C). However, a transient up-regulation of p27^{kip1} mRNA did not result in a measurable increase of the p27^{kip1} protein level in these cells (Figure 3B and C). Despite the absence of changes in its mRNA level, p27^{kip1} protein gradually accumulated in PC3 cells, starting 9 h after the addition of wortmannin (Figure 3A).

---

was determined by Northern and Western blotting, respectively. The Northern blots were hybridized with a ^{32}P - labeled p27^{kip1} cDNA probe. To illustrate equal loading of the samples the blots were reprobed with an actin cDNA probe. For protein analysis, equal amounts of total cell lysates were subjected to gel electrophoresis and immunoblotted with anti-p27^{kip1}. A duplicate blot was immunoblotted with anti-β-actin as a control for equal loading. Representative experiments are shown. The bottom panels of A, B and C represent a densitometric analysis of the expression level of p27^{kip1} protein, normalized to actin expression. Altered cell cycle distribution of PC3 cells after PI3K inhibition by wortmannin treatment was investigated by FACS analysis. (D) PC3 cells were either mock treated (DMSO) or treated with wortmannin (in DMSO) to an end concentration of 200 nM. Twenty-four hours after addition of wortmannin the cells were collected, stained with propidium iodide and subjected to cell cycle analysis. At the right the percentage of cells in each phase of the cell cycle is indicated.

Taken together these results indicate that transcription regulation of the $p27^{kip1}$ encoding *CDKN1B* gene, as mediated by PI3K/Akt dependent FOXO3a activation, does not significantly contribute to the $p27^{kip1}$ protein level in the three cell lines. In addition, these data suggest that in LNCaP and PC346 cells PI3K/Akt signaling is not a major factor in $p27^{kip1}$ protein regulation. The strong effect of inhibiting PI3K/Akt signaling by wortmannin on $p27^{kip1}$ protein expression in PC3 cells prompted us to study this cell line in more detail. We investigated whether the cell cycle distribution was affected upon wortmannin treatment. As shown in Figure 3D, blocking of PI3K activity in PC3 cells resulted in an accumulation of cells in the G_0/G_1 phase of the cell cycle and a concomitant reduction of the S and G_2/M phase. The increased number of cells in G_0/G_1 following wortmannin treatment correlated with the increased $p27^{kip1}$ protein expression shown in Figure 3A.

Skp2 protein and mRNA expression in wortmannin treated PC3 cells

One mechanism of regulation of $p27^{kip1}$ protein expression is by Skp2 mediated proteolysis [23,24]. Like FOXO mediated regulation of $p27^{kip1}$ mRNA expression, this process might be mediated by PI3K/Akt signaling [26]. We first determined the Skp2 protein level in the four prostate cancer cell lines. As depicted in Figure 4, Skp2 protein expression was found in all cell lines.

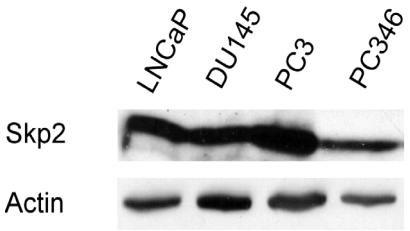


Figure 4. Skp2 protein expression in prostate cancer cell lines. Whole cell lysates were prepared from LNCaP, DU145, PC3 and PC346 cells and equal amounts of protein were immunoblotted with an anti-Skp2 antibody. β–Actin was used as an equal loading control.

Next, we analyzed the kinetics of Skp2 protein expression in PC3 cells following wortmannin incubation. As shown in Figure 5A, Skp2 expression gradually decreased due to PI3K inactivation. At 24 h, Skp2 was no longer detectable. This expression pattern inversely correlated with $p27^{kip1}$ protein accumulation as depicted in Figure 3A. This strongly suggests that in PC3 cells Skp2 mediated $p27^{kip1}$ degradation significantly contributed to the regulation of the $p27^{kip1}$ protein level via PI3K/Akt signaling. PI3K/Akt dependent Skp2 regulation occurred at the protein level because analysis of Skp2 mRNA by RT-PCR showed an unchanged expression during wortmannin treatment (Figure 5B).

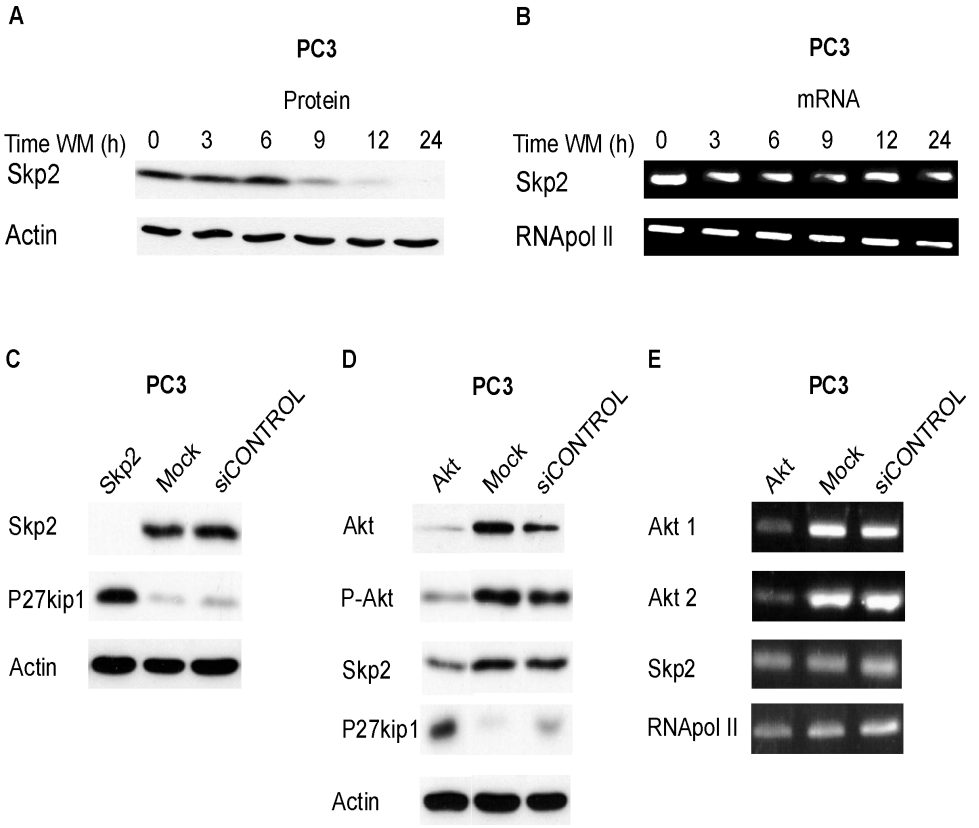


Figure 5. The effect of PI3K/Akt inhibition on Skp2 expression in PC3 cells. Protein and total RNA were isolated in parallel from PC3 cells treated with 200 nM wortmannin at the indicated time points. (A) Equal amounts of total cell lysates were immunoblotted with anti-Skp2 and anti-β-actin antibodies. (B) Skp2 mRNA expression was measured by semi-quantitative RT-PCR. RNApol II expression was used to show equal cDNA input. (C) PC3 cells were transfected with either Skp2 specific siRNA or siCONTROL non-targetting siRNA, or were mock treated. Cells were harvested 48 h after transfection and total cell lysates were subjected to immunoblotting with anti-Skp2 and anti-$p27^{kip1}$ antibodies. β-actin was used as equal loading control. D and E: Protein and total RNA were isolated in parallel from PC3 cells transfected with either Akt1,2 siRNA, or siCONTROL non-targetting siRNA and from mock treated cells. Cells were harvested 48 h after transfection. (D) Equal amounts of total cell lysates were analyzed by Western blotting using the indicated antibodies. (E) Semi-quantitative RT-PCR was used to assay Akt1,2 and Skp2 mRNA. RNApol II expression indicates equal cDNA input. Mock: cells treated with transfection reagent only. Representative experiments are shown.

Akt dependent regulation of Skp2 and p27^{kip1} in PC3 cells

We extended our knowledge of the relationship between PI3K/Akt signaling and Skp2/p27^{kip1} expression in PC3 cells by analyzing the effect of down-regulation of Skp2 and Akt by specific siRNAs on p27^{kip1} expression. First, we showed that down-regulation of Skp2 by siRNA resulted in up-regulation of p27^{kip1} expression (Figure 5C). Next, we applied Akt1,2 specific siRNA to reduce (phospho-)Akt expression. As depicted in Figure 5D a significant reduction of Akt and phospho-Akt expression was achieved. The reduction of phospho-Akt expression in PC3 cells resulted in down-regulation of Skp2 and up-regulation of p27^{kip1} protein expression indicating that the Skp2 expression is indeed regulated by an active PI3K/Akt pathway. Our previous wortmannin experiments indicated that PI3K/Akt signaling only affected Skp2 protein expression and not the Skp2 mRNA level in PC3 cells (see Figure 5A and B). An identical observation was made in the siRNA experiment. RT-PCR analysis showed that introduction of Akt1,2 siRNA in PC3 cells significantly reduced Akt1,2 mRNA expression, however, in contrast to Skp2 protein expression, Skp2 mRNA expression was unaltered (Figure 5E).

Akt independent regulation of Skp2 and p27^{kip1} expression in LNCaP cells

To further study the role of Skp2 in the regulation of p27^{kip1} in prostate cancer cells we reduced Skp2 expression by siRNA in the PTEN negative LNCaP cells. A significant increased expression of p27^{kip1} was found in these cells upon reduction of Skp2 protein expression, indicating a Skp2 dependent regulation of p27^{kip1} (Figure 6A). However, in contrast to PC3 cells, the expression of Skp2 in LNCaP cells remained unchanged after inhibition of Akt signaling by specific siRNAs (Figure 6B). Importantly, p27^{kip1} expression was also not affected by down-regulation of Akt. In agreement with the wortmannin experiments (Figure 3B), these results clearly indicate that in LNCaP cells, PI3K/Akt signaling is not a major factor in the regulation of p27^{kip1}.

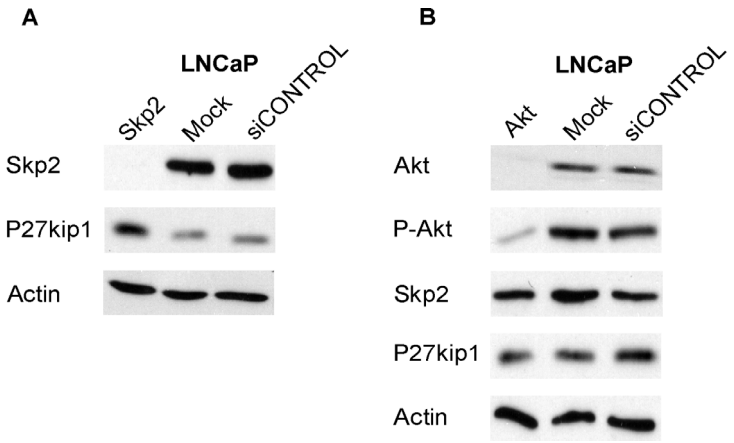


Figure. 6. Skp2 mediated regulation of $p27^{kip1}$ is independent of Akt in LNCaP cells. LNCaP cells were transfected with either Skp2 specific siRNA (A), or Akt1,2 specific siRNA (B)**.** Cells treated with transfection reagent only (mock) and siCONTROL non-targeting siRNA transfected cells were used as controls. Cells were harvested 48 h after transfection and equal amounts of total cell lysate were subjected to gel electrophoresis and immunoblotted with the indicated antibodies. Results of a representative experiment are shown.

PTEN dependent regulation of Skp2 expression in DU145 cells

In concordance with the Skp2 dependent regulation of $p27^{kip1}$ found in the PTEN negative cell lines PC3 and LNCaP, reduction of Skp2 expression by siRNA in PTEN positive DU145 prostate cancer cells also resulted in a strong increase of the $p27^{kip1}$ protein level (Figure 7A). To further define the role of PI3K/Akt signaling in Skp2 regulation we reduced PTEN expression by siRNA. Decreased PTEN expression resulted in increased phospho-Akt expression, whereas the Akt level remained unchanged (Figure 7B). As a result of activating PI3K signaling by lowering PTEN expression a significantly increased Skp2 and decreased $p27^{kip1}$ protein expression were observed (Figure 7B) indicating that, like in PC3 cells, in DU145 cells $p27^{kip1}$ is mainly regulated by PI3K/Akt dependent regulation of Skp2.

DU145 cells were also transfected with both PTEN and Skp2 specific siRNAs. If Skp2 is not the main determinant of the PTEN dependent regulation of $p27^{kip1}$ in these cells, the reduced $p27^{kip1}$ level observed after activation of Akt signaling would be counteracted by the increased $p27^{kip1}$ expression following the down regulation of Skp2. In that case, the total $p27^{kip1}$ expression might not change

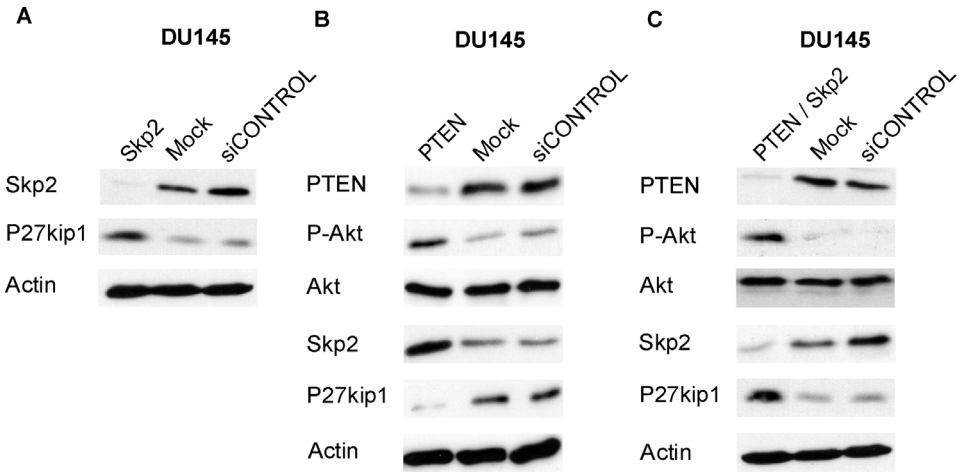


Figure 7. Skp2 dependent regulation of $p27^{kip1}$ is modulated by phospho-Akt in DU145 prostate cancer cells. Whole cell extracts were prepared from DU145 cells 48 h after transfection with Skp2 siRNA (A)**,** PTEN siRNA (B) or both Skp2 and PTEN siRNA (C). Cell lysates from siCONTROL non-targeting siRNA transfected DU145 cells or cells treated with transfection reagent only (mock) were used as controls**.** Equal amounts of total cell lysates were subjected to Western Blot analysis, utilizing the indicated antibodies. Representative experiments are shown.

upon transfection with both PTEN and Skp2 siRNA. However, reduction of both Skp2 and PTEN expression in DU145 cells resulted in a significantly increased expression of $p27^{kip1}$ suggesting that Skp2 is indeed the main determinant of PTEN/Akt dependent regulation of $p27^{kip1}$ in these cells (Figure 7C).

Taken together the results indicate that in PC3 and DU145 prostate cancer cell lines $p27^{kip1}$ expression is mainly regulated by PI3K/Akt dependent regulation of Skp2 protein expression. In LNCaP and PC346 cells PI3K/Akt signaling hardly contributes to the regulation of $p27^{kip1}$.

Discussion

$P27^{kip1}$ is an important negative regulator of cell cycle progression. Its cellular level and localization can be regulated by many different mechanisms [15]. One of the signaling pathways that can affect $p27^{kip1}$ is PI3K/Akt signaling [17,36-38]. The tumor suppressor PTEN, which is frequently inactivated in many tumors including prostate cancer, is a key factor in this signaling route by counteracting the function of PI3K via dephosphorylation of PIP3 [2,10]. In this study we compared the

involvement of the PI3K/Akt pathway in the regulation of $p27^{kip1}$ in four prostate cancer cell lines.

$P27^{kip1}$ function is controlled by the integration of several signaling pathways, including PI3K/Akt signaling, that could affect its mRNA and protein level, and its cellular localization (reviewed in refs 13, 15, 39). An important mechanism of $p27^{kip1}$ regulation is protein degradation mediated by the SCF^{Skp2} complex [39]. Phosphorylation of $p27^{kip1}$ affects its stability and cellular localization. Upon binding of $p27^{kip1}$ to the Cdk2/CyclinE complex $p27^{kip1}$ becomes phosphorylated at Thr187 by Cdk2 thereby creating a recognition site for the SCF^{Skp2} complex and promoting proteasomal degradation [23,25]. Phosphorylation of $p27^{kip1}$ at Ser10, mediated by hKIS, is necessary for its nuclear export and also contributes to the regulation of protein degradation [40-42]. The Ras signal transduction pathway affects the regulation of $p27^{kip1}$ translation (reviewed in 15, 16). Furthermore, several mechanisms of transcriptional regulation of $p27^{kip1}$ have been reported. $P27^{kip1}$ transcription can be stimulated by STAT, whereas c-Myc mediates repression of $p27^{kip1}$ promoter activity thereby inhibiting $p27^{kip1}$ transcription [18-20,43,44].

The effect of PI3K/Akt signaling on $p27^{kip1}$ is complex, and might occur at several levels. FOXO is able to regulate $p27^{kip1}$ transcription [18-20]. Phosphorylation by Akt sequesters these transcription factors to the cytoplasm, resulting in down-regulation of $p27^{kip1}$ mRNA expression [21,22]. Moreover, PI3K/Akt signaling can affect $p27^{kip1}$ protein degradation by regulating the expression of the E3 ubiquitin ligase Skp2 [26-28]. Additionally, by direct phosphorylation at different sites Akt might affect the cellular localization or the stability of $p27^{kip1}$ [45-48]. In the present study we focused on the role of both FOXO and Skp2 in $p27^{kip1}$ regulation.

In the four prostate cancer cell lines investigated in this study, $p27^{kip1}$ expression correlated with the growth rate (data not shown) and not with the PI3K/Akt status of a particular cell line (Figure 1). In two PTEN negative cell lines showing high $p27^{kip1}$ expression, LNCaP and PC346, no clear effect of inhibition of PI3K/Akt signaling on $p27^{kip1}$ was observed, whereas in the third PTEN deficient cell line PC3 and in PTEN positive DU145 cells a PTEN dependent regulation of $p27^{kip1}$ was found.

The three PTEN negative cell lines showed a different kinetics of recovery from the PI3K inhibitor wortmannin. However, it is unlikely that the more rapid renewed phospho-Akt expression in PC346 and LNCaP cells masks an increased $p27^{kip1}$ protein expression (compare Figures 2 and 3). The molecular mechanism that

underlies high $p27^{kip1}$ expression in LNCaP and PC346 cells seems to overrule PI3K/Akt mediated $p27^{kip1}$ regulation. It is tempting to speculate that an active androgen signaling pathway plays a role in this process, because growth of both cell lines is androgen regulated. In PC3 and DU145 cells, which both lack the androgen receptor, modulation of PI3K/Akt signaling strongly affects $p27^{kip1}$ expression.

In both PC3 and DU145 cells proteasomal degradation via Skp2 was the main determinant of the Akt mediated $p27^{kip1}$ regulation. Although Skp2 could regulate the $p27^{kip1}$ expression in LNCaP cells this regulation was independent of an active Akt pathway. In PC3 cells the Akt dependent regulation of Skp2 occurred at the protein level. PTEN dependent regulation of Skp2 has previously been described, but in contrast to our results in these studies PI3K/Akt signaling affected both Skp2 protein and mRNA expression [26,27]. The molecular background of this apparent discrepancy is not clear. It might be due to the difference in cellular context.

The PI3K/Akt induced increased Skp2 protein level might be caused by a stimulated synthesis or a decreased degradation. It has been reported that the Skp2 protein level can be regulated via proteasomal degradation mediated by the APC^{Cdh1} complex [49,50]. It has also been described that connexin43 affects Skp2 stability [51]. Recently, correlations were found between altered Skp2 expression and reduced FAS activity or COX-2 expression suggesting a role for these proteins in the regulation of Skp2 [52,53]. Furthermore, an inverse correlation was found between p107 and Skp2 protein expression [54]. Whether PI3K/Akt signaling plays a role in this regard, or whether other molecular mechanisms are involved in PI3K/Akt regulated Skp2 expression remains to be elucidated.

It is well established that low $p27^{kip1}$ expression is a marker of poor prognosis in human cancers including prostate cancer. However, a relationship between PTEN deficiency and decreased $p27^{kip1}$ expression in clinical prostate cancer is not clear [55-57]. Skp2 overexpression has been described in many tumors [33,35,58]. In the study of Yang and coworkers[32] an inverse correlation between Skp2 and $p27^{kip1}$ was reported in prostate tumors. A weak association between Skp2 overexpression and PTEN inactivation was also described in this study. In contrast, Drobnjak et al. (2003)[34] could not find a correlation between Skp2 overexpression and low $p27^{kip1}$. In summary, these data suggest heterogeneity of the effect of PTEN inactivation on $p27^{kip1}$ expression, which might or might not involve Skp2 overexpression, similar to that observed in the cell lines as described in the present study. These results

urge the further investigation of PI3K/Akt signaling and cell cycle regulators in both clinical prostate cancer specimens and model systems. The outcome of such studies is essential for prediction of the efficacy of targeted therapies affecting PI3K/Akt signaling.

Materials and Methods

Cell culture

Cell lines were cultured in RPMI 1640 (LNCaP) or DMEM (DU145 and PC3) (Bio-Whittaker Europe; Verviers, Belgium) supplemented with 5% FCS and 100 U/ml penicillin, 100 μg/ml streptomycin at 37 °C in a humidified, 5% CO_2 atmosphere. The PC346 cell line was maintained in DMEM/F12 supplemented with 10% FCS and antibiotics. This cell line, which was established from a transurethral resection of a primary prostate tumor, does not express a functional PTEN protein due to a nonsense mutation in exon 5 [7,59]. Culture medium was refreshed at 15 h prior to harvesting, or 15 h prior to the start of the time course experiments. The PI3K inhibitor wortmannin (Sigma Chemical, St Louis, MO) was added to the culture medium to a final concentration of 200 nM for the indicated time periods.

Western blot analysis

Cells were washed twice with ice cold PBS and directly lysed in 1 x Laemmli sample buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 10 mM DTT and 0.001% Bromophenolblue). The samples were sonicated for 30 sec in a sonication bath (Elma Transsonic 460H, Singen, Germany) to shear DNA and boiled for 5 min followed by 5 min centrifugation in an eppendorf centrifuge at 14 000 rpm.

Equal amounts of protein were subjected to 10% or 12% SDS-PAGE. After electrophoresis the proteins were transferred by electroblotting to Nitrocellulose Transfer Membrane (Protran; Schleicher and Schuell, Dassel, Germany). Membranes were blocked in 5% non-fat milk in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20) for 2 h at room temperature. Primary antibody incubation was performed for 1 h at room temperature. After incubation with a secondary horseradish peroxidase (HRP)-linked antibody proteins were visualized by enhanced chemiluminescence utilizing supersignal substrate from Perbio Science (Rockford, IL).

Densitometric analyses of Western blots were performed utilizing ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Antibodies

Rabbit polyclonal antibodies to Akt, phospho-Akt(Ser473) and phospho-FOXO1(Thr24)/FOXO3a(Thr32) were purchased from Cell Signaling Technology (Beverly, MA) and applied in a 1:1000 dilution. The mouse monoclonal anti-human PTEN (Clone 6H2.1; 1:1000) was from Cascade Bioscience (Winchester, MA) and the mouse monoclonals anti-β-actin (1:10,000) and anti-$p27^{kip1}$ (1:500) were obtained from Sigma Chemical and Novo Castra Laboratories (Newcastle upon Tyne, UK) respectively. The rabbit polyclonal antibodies anti-FOXO3a (1:1000) and anti-Skp2 (1:1000) were purchased from Upstate Biotechnologies (Lake Placid, NY) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Goat-anti-Rabbit HRP secondary antibody was from Cell Signaling Technology and the secondary antibody Goat-anti-Mouse HRP was from DAKO (Glostrup, Denmark); both were applied in a 1:2000 dilution.

Northern blot analysis

Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's description, including an on column DNaseI digestion using the RNAse free DNAse set from Qiagen.

From each sample, 10 μg RNA was separated on a 1% formaldehyde/agarose gel, blotted onto Hybond-N+ membrane (Amersham Biosciences UK, Little Chalfont, UK) and UV-crosslinked at 150 mJ UV utilizing the GS Gene linker (Biorad Laboratories, Hercules, CA). The blot was prehybridized in buffer containing 0.5 M NaPi, 7% SDS, 1% BSA, 10 mM EDTA, pH 7.2 and 10 mg haring sperm DNA/100 ml for 4 h at 65 °C. Probes were ^{32}P-ATP labeled and hybridization was carried out overnight at 65 °C. After hybridization blots were washed in 3 x SSC/0.1% SDS and 1 x SSC/0.1% SDS for 30 min each and subjected to standard autoradiography for an appropriate time period. A 350 bp PCR fragment from $p27^{kip1}$ cDNA was used as hybridization probe (forward primer 5'-CTGAGGACACGCATTTGGTG-3'; reverse primer 5'-CTTCCTTGCTTCATCAAGCAG-3'). Equal RNA loading was verified by reprobing the blots with an actin cDNA probe.

cDNA preparation and semi-quantitative RT-PCR analysis

One µg total RNA was incubated for 1 h at 37 °C in buffer containing 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP's and 200 U M-MLV-reverse transcriptase (Invitrogen, Carlsbad, CA), 40 U RNAguard (Amersham Biosciences) and 0.5 µg oligodT primer. Specific cDNA fragments were amplified by PCR and analyzed on a 1% agarose gel.

The following primers were used for the RT-PCR:

Akt1 forward 5'-CTGCGCTGGACGATAGCTTG-3'
Akt1 reverse 5'-CCTCAGAGACACGGCCTTAGTG-3'
Akt2 forward 5'-CTGACCGCTATGACAGCCTG-3'
Akt2 reverse 5'-GAGTCTGGGCACAAAGGTG-3'
Skp2 forward 5'-ACCTATCACTCAGTCGGTG-3'
Skp2 reverse 5'-ATGGTGGTGAAATGGGAGC-3'
RNApol II forward 5'-GCTGAGAGAGCCAAGGATAT-3'
RNApol II reverse 5'-CACCACCTCTTCCTCCTCTT-3'

siRNA and transfections

One day prior to transfection approximately $1.5x10^5$ cells per 60 mm dish were seeded in antibiotic free medium. Cells were washed in PBS and the medium was replaced by serum and antibiotics free medium. Transfection was carried out using JetSI transfection reagent from Eurogentec (Hampshire, UK) following the manufacturer's description. Cells were harvested at 48 h. Akt1 and Akt2 siRNAs and siControl non-targeting siRNA pool were obtained as ready-annealed, purified duplexes from Dharmacon Research (Lafayette, CO). Single-stranded siRNAs to target PTEN and Skp2 were purchased from Eurogentec and were annealed according to the manufacturer's description. PTEN and Skp2 siRNA sequences were:

PTEN sense 5'-AGGCACAAGAGGCCCUAGA-dTT-3'
anti-sense 5'-UCUAGGGCCUCUUGUGCCU-dTT-3'
Skp2 sense 5'-CCUUUCUGGGUGUUCUGGA-dTT-3'
anti-sense 5'-UCCAGAACACCCAGAAAGG-dTT-3'

All siRNA duplexes were used at a final concentration of 100 nM.

Cell cycle analysis

Cell cycle distribution was analyzed by flow cytometry. PC3 cells were incubated with wortmannin (200 nM) for 24 h. Next, cells were trypsinized and washed in ice-cold PBS. Subsequently, cells were fixed by drop-wise adding to ice-cold 70% ethanol. Fixed cells were washed twice in PBS, and incubated in PBS containing 100 µg/ml RNAseA and 10 µg/ml Propidium Iodide (Sigma) for 1 h at 37 °C. Stained cells were assayed in a FACScan (Becton Dickinson, San Jose, CA), and the data were analyzed utilizing CellQuest software. For each sample 1 x 10^4 cells were recorded.

Acknowledgements

We thank Hanneke Korsten and Marja Verleun-Mooijman for their technical support and helpful discussions.

References

1. Hanahan, D. and Weinberg, R.A. (2000) The hallmarks of cancer *Cell*, **100,** 57-70.
2. Dahia, P.L. (2000) PTEN, a unique tumor suppressor gene *Endocr Relat Cancer*, **7,** 115-129.
3. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., et al. (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer *Science*, **275,** 1943-1947.
4. Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H., Langford, L.A., Baumgard, M.L., Hattier, T., Davis, T., et al. (1997) Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers *Nat Genet*, **15,** 356-362.
5. Bonneau, D. and Longy, M. (2000) Mutations of the human PTEN gene *Hum Mutat*, **16,** 109-122.
6. Suzuki, H., Freije, D., Nusskern, D.R., Okami, K., Cairns, P., Sidransky, D., Isaacs, W.B.,Bova, G.S. (1998) Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues *Cancer Res*, **58,** 204-209.
7. Vlietstra, R.J., van Alewijk, D.C., Hermans, K.G., van Steenbrugge, G.J.,Trapman, J. (1998) Frequent inactivation of PTEN in prostate cancer cell lines and xenografts *Cancer Res*, **58,** 2720-2723.
8. Whang, Y.E., Wu, X., Suzuki, H., Reiter, R.E., Tran, C., Vessella, R.L., Said, J.W., Isaacs, W.B.,Sawyers, C.L. (1998) Inactivation of the tumor suppressor PTEN/MMAC1 in

advanced human prostate cancer through loss of expression *Proc Natl Acad Sci U S A*, **95,** 5246-5250.

9. McMenamin, M.E., Soung, P., Perera, S., Kaplan, I., Loda, M.,Sellers, W.R. (1999) Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage *Cancer Res*, **59,** 4291-4296.
10. Maehama, T. and Dixon, J.E. (1998) The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate *J Biol Chem*, **273,** 13375-13378.
11. Myers, M.P., Stolarov, J.P., Eng, C., Li, J., Wang, S.I., Wigler, M.H., Parsons, R.,Tonks, N.K. (1997) P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase *Proc Natl Acad Sci U S A*, **94,** 9052-9057.
12. Sgambato, A., Cittadini, A., Faraglia, B.,Weinstein, I.B. (2000) Multiple functions of p27(Kip1) and its alterations in tumor cells: a review *J Cell Physiol*, **183,** 18-27.
13. Slingerland, J. and Pagano, M. (2000) Regulation of the cdk inhibitor p27 and its deregulation in cancer *J Cell Physiol*, **183,** 10-17.
14. Macri, E. and Loda, M. (1998) Role of p27 in prostate carcinogenesis *Cancer Metastasis Rev*, **17,** 337-344.
15. Alkarain, A. and Slingerland, J. (2004) Deregulation of p27 by oncogenic signaling and its prognostic significance in breast cancer *Breast Cancer Res*, **6,** 13-21.
16. Pruitt, K. and Der, C.J. (2001) Ras and Rho regulation of the cell cycle and oncogenesis *Cancer Lett*, **171,** 1-10.
17. Li, D.M. and Sun, H. (1998) PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells *Proc Natl Acad Sci U S A*, **95,** 15406-15411.
18. Nakamura, N., Ramaswamy, S., Vazquez, F., Signoretti, S., Loda, M.,Sellers, W.R. (2000) Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN *Mol Cell Biol*, **20,** 8969-8982.
19. Medema, R.H., Kops, G.J., Bos, J.L.,Burgering, B.M. (2000) AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1 *Nature*, **404,** 782-787.
20. Dijkers, P.F., Medema, R.H., Pals, C., Banerji, L., Thomas, N.S., Lam, E.W., Burgering, B.M., Raaijmakers, J.A., Lammers, J.W., Koenderman, L., et al. (2000) Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1) *Mol Cell Biol*, **20,** 9138-9148.
21. Kops, G.J., de Ruiter, N.D., De Vries-Smits, A.M., Powell, D.R., Bos, J.L.,Burgering, B.M. (1999) Direct control of the Forkhead transcription factor AFX by protein kinase B *Nature*, **398,** 630-634.

22. Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J.,Greenberg, M.E. (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor *Cell*, **96,** 857-868.

23. Carrano, A.C., Eytan, E., Hershko, A.,Pagano, M. (1999) SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27 *Nat Cell Biol*, **1,** 193-199.

24. Pagano, M., Tam, S.W., Theodoras, A.M., Beer-Romero, P., Del Sal, G., Chau, V., Yew, P.R., Draetta, G.F.,Rolfe, M. (1995) Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27 *Science*, **269,** 682-685.

25. Tsvetkov, L.M., Yeh, K.H., Lee, S.J., Sun, H.,Zhang, H. (1999) p27(Kip1) ubiquitination and degradation is regulated by the SCF(SKP2) complex through phosphorylated Thr187 in p27 *Curr Biol*, **9,** 661-664.

26. Mamillapalli, R., Gavrilova, N., Mihaylova, V.T., Tsvetkov, L.M., Wu, H., Zhang, H.,Sun, H. (2001) PTEN regulates the ubiquitin-dependent degradation of the CDK inhibitor p27(KIP1) through the ubiquitin E3 ligase SCF(SKP2) *Curr Biol*, **11,** 263-267.

27. Andreu, E.J., Lledo, E., Poch, E., Ivorra, C., Albero, M.P., Martinez-Climent, J.A., Montiel-Duarte, C., Rifon, J., Perez-Calvo, J., Arbona, C., et al. (2005) BCR-ABL induces the expression of SKP2 through the PI3K pathway to promote p27Kip1 degradation and proliferation of chronic myelogenous leukemia cells *Cancer Res*, **65,** 3264-3272.

28. Motti, M.L., Califano, D., Troncone, G., De Marco, C., Migliaccio, I., Palmieri, E., Pezzullo, L., Palombini, L., Fusco, A.,Viglietto, G. (2005) Complex regulation of the cyclin-dependent kinase inhibitor p27kip1 in thyroid cancer cells by the PI3K/AKT pathway: regulation of p27kip1 expression and localization *Am J Pathol*, **166,** 737-749.

29. Lloyd, R.V., Erickson, L.A., Jin, L., Kulig, E., Qian, X., Cheville, J.C.,Scheithauer, B.W. (1999) p27kip1: a multifunctional cyclin-dependent kinase inhibitor with prognostic significance in human cancers *Am J Pathol*, **154,** 313-323.

30. Cheville, J.C., Lloyd, R.V., Sebo, T.J., Cheng, L., Erickson, L., Bostwick, D.G., Lohse, C.M.,Wollan, P. (1998) Expression of p27kip1 in prostatic adenocarcinoma *Mod Pathol*, **11,** 324-328.

31. Tsihlias, J., Kapusta, L.R., DeBoer, G., Morava-Protzner, I., Zbieranowski, I., Bhattacharya, N., Catzavelos, G.C., Klotz, L.H.,Slingerland, J.M. (1998) Loss of cyclin-dependent kinase inhibitor p27Kip1 is a novel prognostic factor in localized human prostate adenocarcinoma *Cancer Res*, **58,** 542-548.

32. Yang, R.M., Naitoh, J., Murphy, M., Wang, H.J., Phillipson, J., deKernion, J.B., Loda, M.,Reiter, R.E. (1998) Low p27 expression predicts poor disease-free survival in patients with prostate cancer *J Urol*, **159,** 941-945.

33. Ben-Izhak, O., Lahav-Baratz, S., Meretyk, S., Ben-Eliezer, S., Sabo, E., Dirnfeld, M., Cohen, S.,Ciechanover, A. (2003) Inverse relationship between SKP2 ubiquitin ligase and the cyclin dependent kinase inhibitor p27Kip1 in prostate cancer *J Urol*, **170,** 241-245.
34. Drobnjak, M., Melamed, J., Taneja, S., Melzer, K., Wieczorek, R., Levinson, B., Zeleniuch-Jacquotte, A., Polsky, D., Ferrara, J., Perez-Soler, R., et al. (2003) Altered expression of p27 and SKP2 proteins in prostate cancer of African-American patients *Clin Cancer Res*, **9,** 2613-2619.
35. Yang, G., Ayala, G., De Marzo, A., Tian, W., Frolov, A., Wheeler, T.M., Thompson, T.C.,Harper, J.W. (2002) Elevated SKP2 protein expression in human prostate cancer: association with loss of the cyclin-dependent kinase inhibitor p27 and PTEN and with reduced recurrence-free survival *Clin Cancer Res*, **8,** 3419-3426.
36. Collado, M., Medema, R.H., Garcia-Cao, I., Dubuisson, M.L., Barradas, M., Glassford, J., Rivas, C., Burgering, B.M., Serrano, M.,Lam, E.W. (2000) Inhibition of the phosphoinositide 3-kinase pathway induces a senescence-like arrest mediated by p27Kip1 *J Biol Chem*, **275,** 21960-21968.
37. Cheney, I.W., Neuteboom, S.T., Vaillancourt, M.T., Ramachandra, M.,Bookstein, R. (1999) Adenovirus-mediated gene transfer of MMAC1/PTEN to glioblastoma cells inhibits S phase entry by the recruitment of p27Kip1 into cyclin E/CDK2 complexes *Cancer Res*, **59,** 2318-2323.
38. Graff, J.R., Konicek, B.W., McNulty, A.M., Wang, Z., Houck, K., Allen, S., Paul, J.D., Hbaiu, A., Goode, R.G., Sandusky, G.E., et al. (2000) Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27Kip1 expression *J Biol Chem*, **275,** 24500-24505.
39. Bloom, J. and Pagano, M. (2003) Deregulated degradation of the cdk inhibitor p27 and malignant transformation *Semin Cancer Biol*, **13,** 41-47.
40. Boehm, M., Yoshimoto, T., Crook, M.F., Nallamshetty, S., True, A., Nabel, G.J.,Nabel, E.G. (2002) A growth factor-dependent nuclear kinase phosphorylates p27(Kip1) and regulates cell cycle progression *Embo J*, **21,** 3390-3401.
41. Ishida, N., Kitagawa, M., Hatakeyama, S.,Nakayama, K. (2000) Phosphorylation at serine 10, a major phosphorylation site of p27(Kip1), increases its protein stability *J Biol Chem*, **275,** 25146-25154.
42. Rodier, G., Montagnoli, A., Di Marcotullio, L., Coulombe, P., Draetta, G.F., Pagano, M.,Meloche, S. (2001) p27 cytoplasmic localization is regulated by phosphorylation on Ser10 and is not a prerequisite for its proteolysis *Embo J*, **20,** 6672-6682.
43. Kortylewski, M., Heinrich, P.C., Mackiewicz, A., Schniertshauer, U., Klingmuller, U., Nakajima, K., Hirano, T., Horn, F.,Behrmann, I. (1999) Interleukin-6 and oncostatin M-

induced growth inhibition of human A375 melanoma cells is STAT-dependent and involves upregulation of the cyclin-dependent kinase inhibitor p27/Kip1 *Oncogene*, **18,** 3742-3753.

44. Gartel, A.L. and Shchors, K. (2003) Mechanisms of c-myc-mediated transcriptional repression of growth arrest genes *Exp Cell Res*, **283,** 17-21.
45. Fujita, N., Sato, S., Katayama, K.,Tsuruo, T. (2002) Akt-dependent Phosphorylation of p27Kip1 Promotes Binding to 14-3-3 and Cytoplasmic Localization *J Biol Chem*, **277,** 28706-28713.
46. Liang, J., Zubovitz, J., Petrocelli, T., Kotchetkov, R., Connor, M.K., Han, K., Lee, J.H., Ciarallo, S., Catzavelos, C., Beniston, R., et al. (2002) PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest *Nat Med*, **8,** 1153-1160.
47. Shin, I., Yakes, F.M., Rojo, F., Shin, N.Y., Bakin, A.V., Baselga, J.,Arteaga, C.L. (2002) PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization *Nat Med*, **8,** 1145-1152.
48. Viglietto, G., Motti, M.L., Bruni, P., Melillo, R.M., D'Alessio, A., Califano, D., Vinci, F., Chiappetta, G., Tsichlis, P., Bellacosa, A., et al. (2002) Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer *Nat Med*, **8,** 1136-1144.
49. Wei, W., Ayad, N.G., Wan, Y., Zhang, G.J., Kirschner, M.W.,Kaelin, W.G., Jr. (2004) Degradation of the SCF component SKP2 in cell-cycle phase G1 by the anaphase-promoting complex *Nature*, **428,** 194-198.
50. Bashir, T., Dorrello, N.V., Amador, V., Guardavaccaro, D.,Pagano, M. (2004) Control of the SCF(SKP2-Cks1) ubiquitin ligase by the APC/C(Cdh1) ubiquitin ligase *Nature*, **428,** 190-193.
51. Zhang, Y.W., Nakayama, K.,Morita, I. (2003) A novel route for connexin 43 to inhibit cell proliferation: negative regulation of S-phase kinase-associated protein (Skp 2) *Cancer Res*, **63,** 1623-1630.
52. Honjo, S., Kase, S., Osaki, M., Ardyanto, T.D., Kaibara, N.,Ito, H. (2005) COX-2 correlates with F-box protein, SKP2 expression and prognosis in human gastric carcinoma *Int J Oncol*, **26,** 353-360.
53. Knowles, L.M., Axelrod, F., Browne, C.D.,Smith, J.W. (2004) A fatty acid synthase blockade induces tumor cell-cycle arrest by down-regulating SKP2 *J Biol Chem*, **279,** 30540-30545.
54. Rodier, G., Makris, C., Coulombe, P., Scime, A., Nakayama, K., Nakayama, K.I.,Meloche, S. (2005) p107 inhibits G1 to S phase progression by down-regulating expression of the F-box protein SKP2 *J Cell Biol*, **168,** 55-66.
55. Dreher, T., Zentgraf, H., Abel, U., Kappeler, A., Michel, M.S., Bleyl, U.,Grobholz, R. (2004) Reduction of PTEN and p27kip1 expression correlates with tumor grade in prostate

cancer. Analysis in radical prostatectomy specimens and needle biopsies *Virchows Arch*, **444,** 509-517.

56. Halvorsen, O.J., Haukaas, S.A.,Akslen, L.A. (2003) Combined loss of PTEN and p27 expression is associated with tumor cell proliferation by Ki-67 and increased risk of recurrent disease in localized prostate cancer *Clin Cancer Res*, **9,** 1474-1479.
57. Fenic, I., Franke, F., Failing, K., Steger, K.,Woenckhaus, J. (2004) Expression of PTEN in malignant and non-malignant human prostate tissues: comparison with p27 protein expression *J Pathol*, **203,** 559-566.
58. Chiarle, R., Budel, L.M., Skolnik, J., Frizzera, G., Chilosi, M., Corato, A., Pizzolo, G., Magidson, J., Montagnoli, A., Pagano, M., et al. (2000) Increased proteasome degradation of cyclin-dependent kinase inhibitor p27 is associated with a decreased overall survival in mantle cell lymphoma *Blood*, **95,** 619-626.
59. Marques, R.B., Erkens-Schulze, S., de Ridder, C.M., Hermans, K.G., Waltering, K., Visakorpi, T., Trapman, J., Romijn, J.C., van Weerden, W.M.,Jenster, G. (2005) Androgen receptor modifications in prostate cancer cells upon long-termandrogen ablation and antiandrogen treatment *Int J Cancer*, **117,** 221-229.

Chapter 3

PTEN mediated G_1 cell cycle arrest in LNCaP prostate cancer cells is associated with transcriptional regulation of cell cycle regulators

Petra W. van Duijn, Angelique C.J. Ziel-van der Made, Hetty A.G.M van der Korput, and Jan Trapman

Department of Pathology, Josephine Nefkens Institute, Erasmus University Medical Center, Rotterdam, The Netherlands

Submitted

Abstract

The tumor suppressor *PTEN* is the most frequently inactivated gene in prostate cancer. Loss of PTEN function leads to activation of the PI3K/Akt pathway. To better understand the biological function of PTEN in prostate cancer we stably transfected PTEN negative LNCaP cells with wild-type PTEN cDNA under inducible control, resulting in LNCaP/PTEN cells. Induced expression of PTEN in these cells significantly inhibited cell growth, at least partly due to cell cycle arrest at the G_1 phase. Expression profiling combined with pathway analysis revealed that PTEN dependent G_1 growth arrest was associated with an altered mRNA expression of the G_1 cell cycle regulators Cdc25a, E2F2, cyclin G2 and RBL2/p130. Specific inhibition of Akt signaling by siRNA resulted in transcriptional down-regulation of both E2F2 and Cdc25a and up-regulation of the FOXO target cyclin G2, comparable with the effect observed after PTEN induction. However, Akt did not mediate the PTEN dependent transcription regulation of RBL2/p130 in LNCaP/PTEN cells. Overall, these results indicated that PTEN dependent transcription regulation is important in cell cycle regulation. This PTEN regulated transcription was mediated by both Akt dependent and independent mechanisms.

Introduction

The tumor suppressor PTEN is inactivated in a wide variety of cancers [1]. In prostate cancer *PTEN* is the most frequently inactivated gene and loss of its expression is correlated with the later stages of the disease [2-4]. PTEN primarily acts as a lipid phosphatase by specifically catalyzing the removal of phosphates from the D3 position of phosphoinositides like PIP3, a lipid second messenger produced by PI3K (phosphatidylinositol-3 kinase) [5,6]. Thereby PTEN counteracts PI3K and functionally antagonizes signaling pathways that rely on the activity of PI3K, of which the Akt signal transduction route is the best studied. This pathway is involved in the regulation of many important cell biological processes, including proliferation and survival [7,8].

The PI3K/PTEN signaling pathway appears to regulate these cell biological processes through a large number of downstream effectors [8]. Many of these effectors are regulated by Akt mediated phosphorylation, but also Akt independent effects have been reported [8]. Well known downstream effectors of Akt signaling are mTOR that modulates translation and the FOXO transcription factors [9].

Previously, inducible expression systems have been used to study the cell biological and molecular functions of PTEN in several cell types [10-16]. It turned out that PTEN expression introduced variable, cell type specific biological effects. Data on inducible PTEN expression in prostate cancer cells are limited. Abrogation of PI3K/PTEN signaling in prostate cell lines by either constitutive overexpression of PTEN or by small molecules that inhibit PI3K function, resulted in growth inhibition, apoptosis or in both [17-22].

The aim of the present study was to obtain further insight in the role of PTEN in prostate cancer. We stably introduced PTEN cDNA under inducible control in the PTEN negative LNCaP prostate cancer cell line and investigated transcriptional changes related to PTEN signaling. We showed that in these cells PTEN expression specifically blocks cell cycle progression at G_1 phase. Our data revealed that the PTEN dependent cell cycle arrest correlated with the transcriptional regulation of various G_1 cell cycle regulators. This regulation was mediated by both Akt dependent and independent mechanisms indicating that PTEN dependent transcription can be modulated by several distinct mechanisms.

Results

Generation and characterization of LNCaP/PTEN cells

To specifically study the cell biological and molecular functions of PTEN in prostate cancer we introduced wild-type PTEN under inducible control in the PTEN null prostate cancer cell line LNCaP. First, LNCaP cells were stably transfected with the Tet-On vector, encoding a transactivator that is activated upon the addition of doxycycline (dox), resulting in the LNCaP/Tet-On parental control cells. These LNCaP/Tet-On cells were subsequently transfected with pBi-EGFP/wtPTEN, resulting in LNCaP/PTEN cells (see Materials and Methods for specific details). Western blot analysis showed that PTEN expression was undetectable in the absence of dox and induced after stimulation with 50-100 ng/ml dox with a maximum expression occurring after exposure to 500 ng/ml dox (Figure 1A).

The PTEN expression level that was reached after 24 h treatment with 250-500 ng/ml dox is comparable to the endogenous PTEN level in the DU145 prostate cancer cell line (Figure 1B), indicating that in LNCaP/PTEN cells PTEN is expressed at a physiological level. Time course experiments showed that PTEN expression was clearly detectable as soon as 6 h after treatment of the cells with

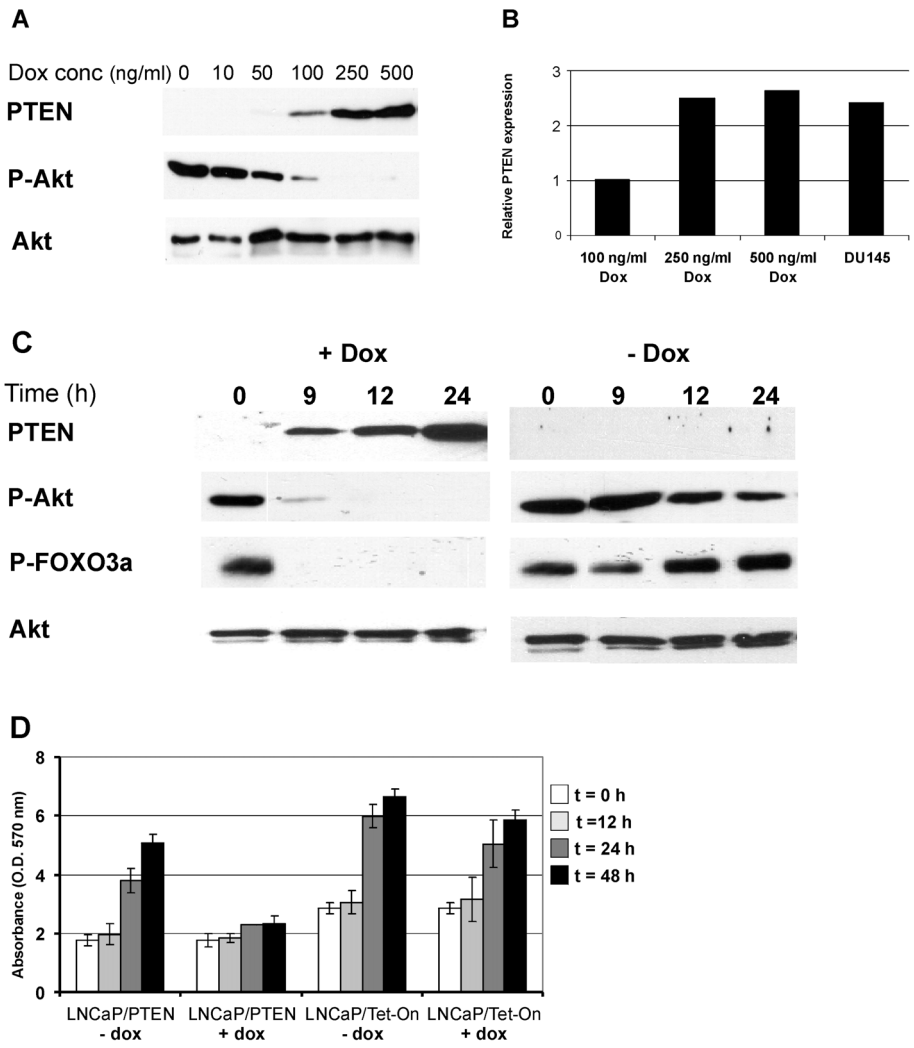


Figure 1. Characterization of LNCaP/PTEN prostate cancer cells. (A) Whole cell extracts were prepared from LNCaP/PTEN cells incubated with dox for 24 h at the indicated concentrations. Equal amounts of protein were subjected to Western blot analysis, utilizing anti-PTEN, anti-phospho-Akt(Ser473) and anti-Akt antibodies. (B) Densitometric analysis of PTEN expression in LNCaP/PTEN cells incubated with dox for 24 h at the indicated concentrations and control PTEN positive DU145 prostate cancer cells. (C) Whole cell lysates were prepared from LNCaP/PTEN cells, cultured in the presence or absence of 250 ng/ml dox, at the indicated time points. Equal amounts of total cell lysates were immunoblotted with anti-PTEN, anti-Akt, anti-phospho-Akt(Ser473) and anti-phospho-FOXO3a(Thr32) antibodies, respectively. (D) LNCaP/PTEN and LNCaP/Tet-On were cultured in the presence or absence of 250 ng/ml dox. At the indicated time points the cell concentrations were determined by the MTT assay (OD 570 nm).

dox (data not shown), and that in time the expression of PTEN significantly increased (Figure 1C). PTEN expression inversely correlated with Akt phosphorylation, showing that the introduced PTEN is functionally active (Figure 1A,C). Inhibition of PTEN/Akt signaling was further indicated by reduced phosphorylation of the Akt target FOXO3a upon PTEN induction (Figure 1C). Thethe cells with dox (data not shown), and that in time the expression of PTEN significantly increased (Figure 1C). PTEN expression inversely correlated with Akt phosphorylation, showing that the introduced PTEN is functionally active (Figure 1A,C). Inhibition of PTEN/Akt signaling was further indicated by reduced phosphorylation of the Akt target FOXO3a upon PTEN induction (Figure 1C). The lack of detectable PTEN protein expression in the absence of dox and at early time points during dox incubation indicates that the system is tightly controlled.

To study the effect of induced PTEN expression on cell growth, LNCaP/PTEN and control LNCaP/Tet-On cells were cultured in the presence or absence of dox. At the indicated time points cell growth was assessed, using the MTT assay (Figure 1D). Dox treatment strongly inhibited growth of LNCaP/PTEN cells compared to control LNCaP/Tet-On cells, indicating that this effect was indeed mediated by PTEN expression.

PTEN dependent gene expression profiles in LNCaP/PTEN cells

To gain further insight into the underlying mechanisms affected by PTEN expression in LNCaP/PTEN cells expression profiling was performed. RNA was isolated from cells grown in the presence or absence of 250 ng/ml dox for 12 and 24 h, respectively, and was subsequently analyzed on Affymetrix Human Genome U133A plus 2.0 gene chips to determine the global gene expression profiles. Approximately 80 genes showed an at least 2-fold difference in mRNA expression upon PTEN induction. In Figure 2A the 25 strongest up-regulated genes are depicted. A list of down-regulated genes is provided as supplementary data (Figure S1). To validate the micro-array data the expression level of DRE1, RIOK3 and MST1 was verified by Q-PCR. The expression of all three genes was already increased as early as 9 h after incubation with dox, indicating that up-regulation of these genes is a rapid effect (Figure 2B). The down-regulation of two randomly selected genes, MCM10 and TNFRSF12a, was also confirmed by Q-PCR (Figure S1).

PTEN suppresses cell growth in LNCaP/PTEN cells by cell cycle arrest in G_1

To assess the cellular processes that were most prominently affected by PTEN induction the micro-array data were subjected to Ingenuity Pathway Analysis (IPA). Functional category and pathway analysis revealed three canonical pathways that were most clearly affected by PTEN induction in LNCaP/PTEN cells: G_1/S checkpoint regulation of the cell cycle, and Insulin Receptor Signaling and IGF-1 signaling (Figure 3A). These predictions were based on the number of focus genes with an altered expression level, which are characteristic for these functional categories, including cyclin G2, Cdc25a, RBL2 and E2F2 for G_1 arrest and IRS2 and IGFR for Insulin and IGF signaling.

PTEN dependent cell cycle arrest in G_1 would explain growth inhibition of LNCaP/PTEN cells upon dox treatment (Figure 1D). G_1 arrest was functionally tested by assaying the cell cycle distribution of LNCaP/PTEN cells in the presence or absence of dox. Indeed incubation of LNCaP/PTEN cells with dox for 24 h resulted in an accumulation of cells in the G_0/G_1 phase and a concomitant decrease of cells in the S phase (Figure 3B). In Figure 3C the cell cycle distribution at additional time points of both dox- treated and non-treated LNCaP/PTEN cells is summarized. In the absence of PTEN expression the cell cycle distribution of LNCaP/PTEN cells remained constant throughout the whole experiment, but PTEN expression rapidly induced an accumulation of cells in G_0/G_1. The first effects on the cell cycle are already apparent at 12 h, corresponding with the inactivation of the Akt pathway as represented by the down-regulation of phospho-Akt (Figure 1C). At later time points a strong decrease of the number of cells in S phase was observed, followed by a reduced number of cells in G_2/M phase (Figure 3C). Overall, these data indicate that induced PTEN expression initially caused G_1 arrest thereby indirectly affecting the other phases of the cell cycle.

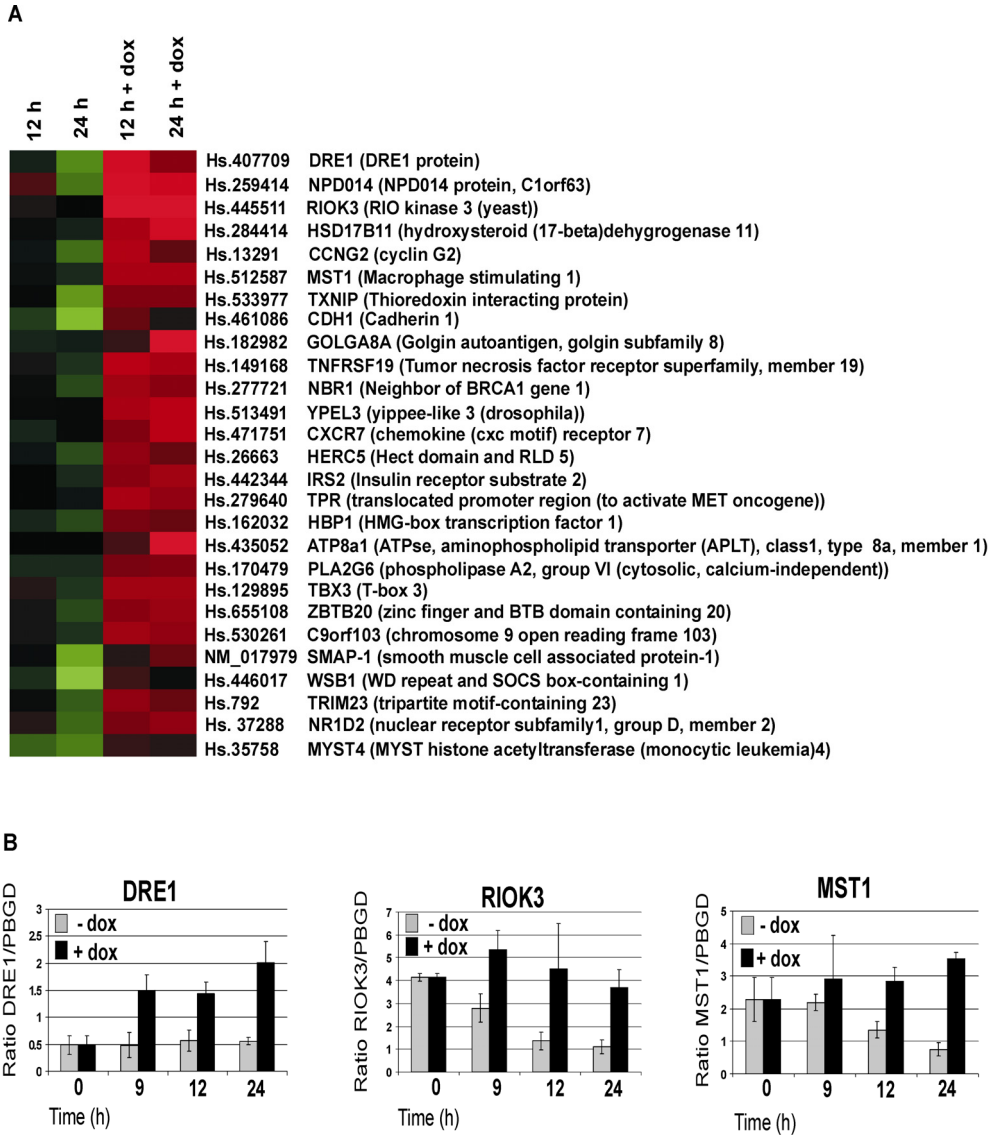
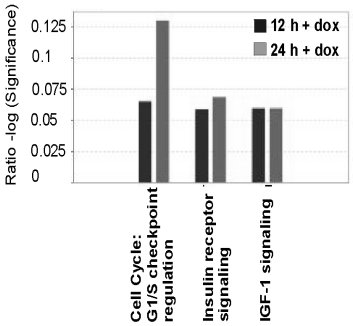
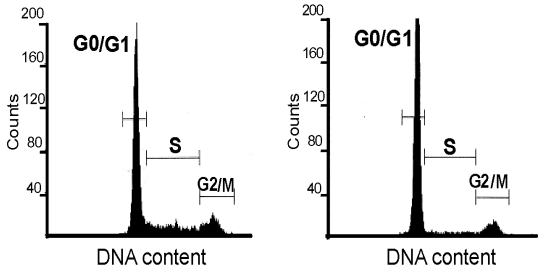


Figure 2. Strongest up-regulated genes by PTEN induction in LNCaP/PTEN cells.
(A) Overview of the 25 strongest up-regulated genes in LNCaP/PTEN cells after treatment with 250 ng/ml dox for 12 h and 24 h. (B) Total RNA was isolated from LNCaP/PTEN cells incubated with 250 ng/ml dox for the indicated time periods. DRE1, RIOK3 and MST1 mRNA expression was measured by Q-PCR, using PBGD expression as a reference. Representative Q-PCR analyses of several independent RNA isolations are shown. Data are shown as average ± SD.

A



B



LNCaP/PTEN
t = 0

G0/G1(%)	72
S (%)	16
G2/M (%)	12

LNCaP/PTEN
t = 24 + dox

G0/G1(%)	86
S (%)	4
G2/M (%)	10

C

LNCaP/PTEN + dox

	G_0/G_1 phase (%)	S phase (%)	G_2/M phase (%)
t = 0 h	72	16	12
t = 12 h	76	14	10
t = 24 h	86	4	10
t = 48 h	91	4	5

LNCaP/PTEN - dox

	G_0/G_1 phase (%)	S phase (%)	G_2/M phase (%)
t = 0 h	72	16	12
t = 12 h	73	17	10
t = 24 h	77	12	11
t = 48 h	75	15	10

Figure 3. PTEN dependent cell cycle regulation of LNCaP/PTEN cells.
(A) The average Log2 ratios of both the dox treated and non-treated samples were compared and differentially expressed genes (Log2 ratio >0.8) were studied by IPA to identify underlying pathways and biological processes. The graph represents the IPA assigned global functional categories for the differentially expressed genes upon PTEN induction. The -log(significance) indicates the rate of confidence for each category. (B) Cell cycle distribution of LNCaP/PTEN cells following dox incubation (250 ng/ml). At t=0 and t=24 h cells were collected, stained with propidium iodide and subjected to cell cycle analysis. Below the figures the percentage of cells in each phase of the cell cycle is indicated. (C) Overview of cell cycle distribution of LNCaP/PTEN cells cultured in the presence or absence of 250 ng/ml dox for the indicated time periods.

PTEN mediated cell cycle arrest in G_1 is associated with transcriptional regulation of important cell cycle regulators

Progress through the cell cycle is orchestrated by tightly regulated expression and activity of a many proteins, including cyclins and cyclin dependent kinases. Most knowledge about PTEN dependent cell cycle regulation has been derived from studies that investigated the role of PTEN/Akt signaling on phosphorylation and stability of cell cycle regulatory proteins [11,14,23-27]. In the present study we focused on the PTEN mediated transcription regulation of cell cycle modulators. As mentioned above the predicted inhibition of G_1/S cell cycle progression upon PTEN induction was based on the significantly altered expression of the cell cycle regulators E2F2, Cdc25a, cyclin G2 and RBL2. Both cyclin G2 and Cdc25a belonged to the 25 strongest up- and down-regulated genes, respectively, following PTEN induction (Figures 2A and S1). Q-PCR analysis confirmed the observed down-regulation of E2F2 and Cdc25a mRNA and the up-regulation of both RBL2 and cyclin G2 mRNA upon induced PTEN expression in LNCaP/PTEN cells (Figure 4A).

To investigate whether the Akt/FOXO pathway was involved in the transcriptional regulation of these cell cycle genes we blocked Akt signaling by specific siRNAs. Transfection of LNCaP/PTEN cells with Akt specific siRNA resulted in a decreased expression of phospho-Akt and also decreased the expression of phospho-FOXO3a, indicative of its activation (Figure 4B). In concordance with FOXO activation a significant up-regulation of cyclin G2 was found (Figure 4C). However, despite FOXO activation the expression level of RBL2 remained constant, suggesting that in these LNCaP/PTEN cells PTEN dependent up-regulation of RBL2 mRNA expression was not mediated by Akt/FOXO (Figure 4C). Q-PCR further showed that blocking of Akt signaling significantly inhibited E2F2 and Cdc25a mRNA expression, indicating that inactivation of Akt is not only correlated with up-regulation of gene expression but also with down-regulation of gene expression. (Figure 4C). Taken together, our results showed that PTEN dependent transcription regulation could be mediated by distinct Akt dependent and independent processes.

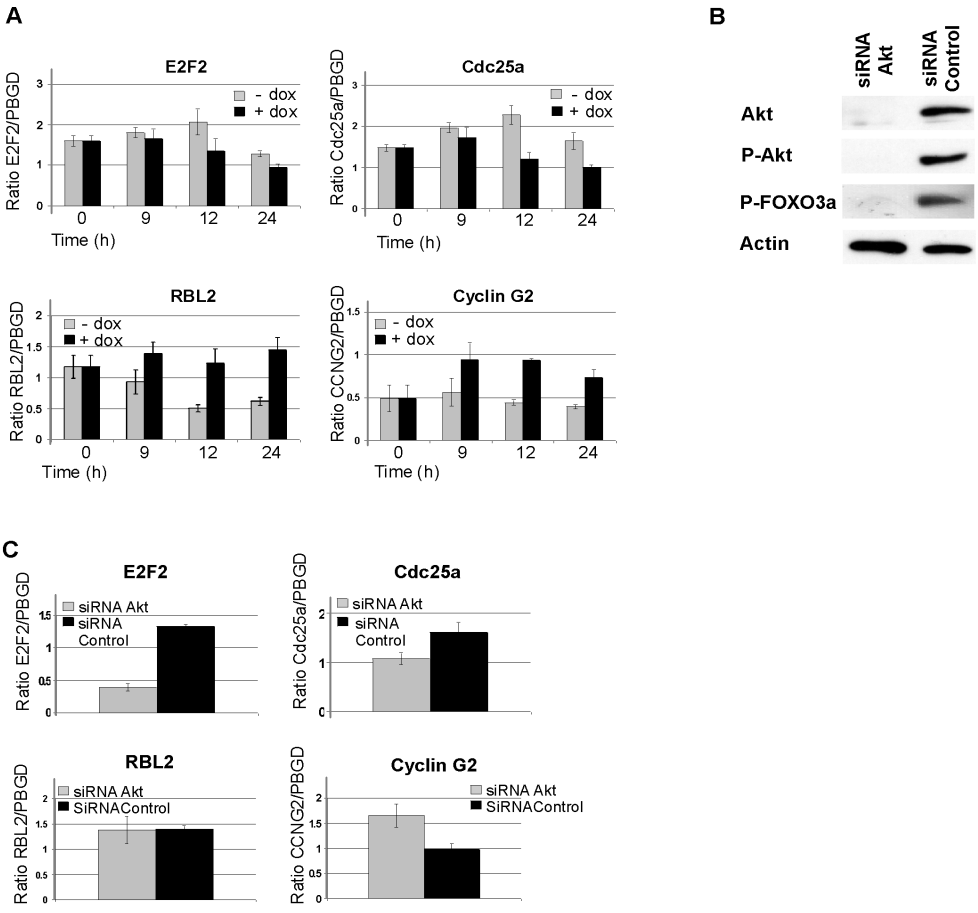


Figure 4. PTEN dependent transcriptional regulation of cell cycle genes in LNCaP/PTEN cells. (A) Total RNA was isolated from LNCaP/PTEN cells incubated with 250 ng/ml dox for the indicated time periods. Cdc25a, E2F2, RBL2 and cyclin G2 mRNA expression was measured by Q-PCR. PBGD expression was used as a Q-PCR reference. Representative Q-PCR analyses of several independent RNA isolations are shown. Data are resented as average ± SD. (B and C) Protein and total RNA were isolated in parallel from LNCaP/PTEN cells cultured in the absence of dox and transfected with either Akt specific siRNA or siCONTROL non-targeting siRNA. (B) Equal amounts of total cell lysates were analysed by Western blotting utilizing the indicated antibodies. C: Q-PCR of Cdc25a, E2F2, RBL2 and cyclin G2 transcripts. Expression of PBGD was used as a Q-PCR reference.

Discussion

PTEN is the most frequently inactivated gene in prostate cancer and it is therefore of high interest to understand the molecular mechanisms of PTEN signaling in this tumor type [3,28]. To study PTEN function in detail we stably transfected a PTEN expression vector under dox inducible control in the PTEN negative prostate cancer cell line LNCaP, resulting in the novel cell line LNCaP/PTEN. To understand the function of PTEN and to identify downstream effectors of PTEN signaling RNA was isolated from LNCaP/PTEN cells grown in the absence or presence of dox, and subjected to global expression profiling. IPA indicated that G_1 checkpoint regulation and Insulin and IGF signaling were the processes most prominently affected by PTEN induction in this novel cell line. Moreover, expression profiling and IPA indicated that the PTEN dependent G_1 checkpoint regulation was associated with altered transcription of the cell cycle modulators E2F2, Cdc25a, RBL2 and cyclin G2. This transcriptional regulation was found to be selectively mediated by either Akt dependent or independent mechanisms.

Several approaches varying from the application of small molecule inhibitors to the use of inducible expression systems have been applied to study PTEN function in different cell types, however, manipulating PTEN signaling turned out to have variable effects that seem to be cell type specific [10-16,29]. In prostate cancer cells, abrogation of PI3k/Akt signaling by overexpression of PTEN or by specific PI3K inhibitors resulted in both growth inhibition and apoptosis [17-22]. Infection of LNCaP and PC3 cells with an adenoviral expression vector of PTEN resulted in significantly reduced cell growth [18,21]. PTEN expression in LNCaP cells also affected apoptosis [21]. Treatment with the PI3K inhibitor Ly294002 caused increased apoptosis as early as 3 h after addition to the culture medium [22]. In contrast, overexpression of wildtype PTEN has also been reported to induce cell shrinkage and rounding without classical apoptosis [19]. In agreement with our findings a growth inhibitory effect of PTEN, due to G_0/G_1 arrest, was observed in C4-2 cells, a hormone independent LNCaP subline [15]. The variable effects of PTEN expression even within comparable cell lines prompted us to develop a dox-inducible expression system. The tightly controlled PTEN expression that was achieved with this system, which approached a physiological PTEN expression level, minimizes the risk of nonspecific effects due to constitutive overexpression or the use of small molecule inhibitors.

Together with the G_1 checkpoint regulation IPA indicated Insulin and IGF signaling as prominent PTEN regulated processes in LNCaP/PTEN cells. Important focus genes that showed an altered expression upon PTEN induction were IGFR and IRS2. These data extended previous findings indicating that in prostate cancer cells PTEN could inhibit cell proliferation and apoptosis by down-regulation of cell surface IGF-IR and experiments showing implication of PTEN in expression regulation of IRS2 in breast cancer cells [30,31].

Expression profiling also indicated down-regulation of genes, such as CDT1, CDC6 and MCM10, involved in DNA replication upon PTEN expression (Figure S1). However, this seemed a more indirect process since further Q-PCR analyses showed that the expression of these genes first started to decrease at 24 h after PTEN induction (data not shown). Little is known about a role of PTEN in DNA replication, however, in line with our findings Groszer et al., (2006) showed a significantly altered mRNA expression of cell cycle and DNA replication related genes in Pten null neurosphere cultures [32]. CDC6, CDT1 and MCM proteins, including MCM10, are involved in the formation of the pre-replication complex and their activity is controlled by multiple mechanisms [33]. Moreover, several reports showed that E2F transcription factors have been implicated in the transcriptional regulation of CDC6 and CDT1 [34-36]. It is tempting to speculate that PTEN dependent down-regulation of DNA replication genes in LNCaP/PTEN cells is due to PTEN/Akt mediated down-regulation of E2F2 as described here (Figure 4).

In the present study we focused on PTEN mediated transcription regulation of cell cycle genes. Besides the above mentioned down-regulation of E2F2, IPA also indicated that PTEN mediated G_1 checkpoint control was associated with altered expression of Cdc25a, RBL1 and cyclin G2. So far, most studies on the role of the PTEN/Akt pathway in cell cycle regulation described the effects of PTEN signaling on the expression of cell cycle regulators at the protein level by altered stability, distribution or phosphorylation [11,14,23-27]. Here we showed that also PTEN dependent transcription regulation can correlate with cell cycle control.

PTEN is known to modulate transcription by affecting the activity of the FOXO family of transcription factors. The significance of FOXO in prostate cancer was previously indicated by a study in LaPC4 prostate cancer cells overexpressing either FOXO1 or FOXO3a [37]. Modur et al., (2002) found that FOXO overexpression affected the expression of several genes involved in cellular proliferation or survival, including cyclin G2. In our micro-arrays we detected

besides cyclin G2 also the up-regulation of other FOXO targets that had been identified before in the LaPC4 cells, like ATP8a and JAK1, emphasizing that FOXO is an important downstream effector of PTEN signaling in LNCaP/PTEN cells (data not shown). In several cell types a contribution of FOXO to G_1/S cell-cycle arrest by regulating the expression of negative cell-cycle regulators, such as $p27^{kip1}$ and RBL2, has been described [38-43]. However, previously we found that in prostate cancer cells Akt/FOXO signaling is not a major regulator of $p27^{kip1}$ mRNA expression [44]. Here, we showed that although RBL2 was previously described as a FOXO target gene [41], specific inhibition of Akt signaling by siRNA did not affect its expression (Figure 4C), indicating a PTEN dependent but Akt/FOXO independent regulation of RBL2 in LNCaP/PTEN cells.

In addition to the up-regulation of cyclin G2 and RBL2 we observed PTEN dependent down-regulation of Cdc25a and E2F2 expression that was mediated by the Akt signaling pathway (Figure 4). Although best known as transcription activators, FOXO proteins may also repress transcription by competing with activatory transcription factors for a common binding site or by functioning as co-repressors thereby regulating transcription through promoters which lack FOXO binding sites [45-47]. However, a role of other, as yet unidentified, transcription factors in PTEN induced down-regulation of gene expression cannot be excluded.

Although it has been suggested that the Akt signal transduction pathway might have a role in regulation of the E2F protein level [48], little is known about the PTEN/Akt dependent transcriptional regulation of E2F2. Myc is known as an important activator of E2F2 expression [49,50]. However, we can exclude the involvement of Myc, because no evidence was found in LNCaP/PTEN cells that expression of other Myc target genes was modulated by PTEN induction. Recently, it has been described that $p21^{Cip1/Waf}$, a downstream target of Akt that becomes activated upon PTEN induction, functions as a transcriptional repressor of Cdc25a [51], suggesting a possible mechanism by which PTEN/Akt signaling could modulate down-regulation of gene expression.

Taken together we can conclude that G_1 growth arrest is one of the most prominent processes affected by PTEN signaling in LNCaP/PTEN cells. We showed that in addition to the relatively well studied effect of PTEN on the phosphorylation status and protein stability of particular cell cycle regulators, also PTEN dependent transcriptional regulation of cell cycle modulators is correlated with PTEN dependent cell cycle arrest. As summarized in Figure 5, we could

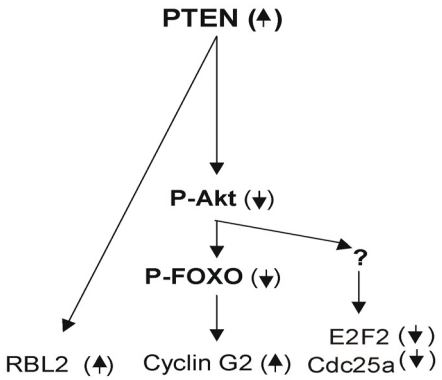


Figure 5. Overview of the various mechanisms of PTEN dependent transcription regulation in LNCaP/PTEN cells. By regulating the Akt/FOXO pathway PTEN causes transcriptional up-regulation of cyclin G2. The Akt signaling pathway also mediates PTEN dependent down-regulation of Cdc25a and E2F2. In contrast, up-regulation of RBL2 is PTEN but not Akt dependent.

discriminate at least three different mechanisms by which PTEN regulates transcription. PTEN dependent transcriptional activation was either independent of Akt activity (RBL2) or was mediated in an Akt dependent manner, most likely via the regulation of FOXO (cyclin G2). Besides activating transcription PTEN also repressed the expression of genes (E2F2 and Cdc25a) by as yet unidentified Akt dependent mechanisms

Materials and Methods

Plasmid construction

The pUB6-EM7-Zeo-BGH plasmid was generated by integrating the UBC promoter from pUB6HisA (Invitrogen, Carlsbad CA) into the BglII site of the EM7-Zeo vector (Invitrogen), resulting in pUB6-EM7-Zeo. Next, the BGH 3'UTR from pcDNA3.1 was cloned into the PvuII site of pUB6-EM7-Zeo, resulting in the pUB6-EM7-Zeo-BGH construct.

Generation of LNCaP/PTEN cells

LNCaP cells were transfected with the pTet-On vector (Clontech-Takara Bio Europe, Saint Germain-en-laye, France). Neomycin resistant clones were transiently transfected with pBi-Luc (Clontech) to test their induction capacity. LNCaP/Tet-On cells with a high induction capacity and low background were selected and subsequently transfected with pBi-EGFP/wtPTEN and pUB6-EM7-Zeo-BGH. Zeocin resistant clones were screened for rapid PTEN expression upon stimulation and low background. LNCaP/Tet-On and LNCaP/PTEN clones were maintained in RPMI 1640 (Bio-Whittaker Europe; Verviers, Belgium) supplemented with 5% TET system-proved FCS (Clontech), 800 μg/ml Neomycin,

100 U/ml penicillin, 100 µg/ml streptomycin, and 200 µg/ml Zeocin (Invitrogen) for LNCaP/PTEN cells, at 37 °C in a humidified, 5 % CO2 atmosphere. A final concentration of 250 ng/ml dox (Clontech) was used to induce PTEN expression.

Cell growth assay

LNCaP/PTEN and LNCaP/Tet-On cells were seeded into 96-well flat-bottom culture plates at a density of $5x10^3$ cells per well. After overnight attachment, medium was replaced by medium containing dox (250 ng/ml) and cells were further incubated for the indicated time periods. Control cells received medium without dox. At the end of the indicated incubation times 30 µl MTT reagent (5 mg/ml in PBS) (AppliChem GmbH, Darmstadt, Germany) was added and the incubation was extended for 4 h at 37 ^oC. After carefully removing the medium, 100 µl buffered DMSO (DMSO:Buffer (0.1 M glycine, 0.1M NaCl) ratio 8:1) was added to each well. Absorbance at 570 nm was determined with a microplate reader (BioRad, Richmond, CA).

Western Blot Analysis

Cells were washed twice with ice cold PBS and directly lysed in Laemmli sample buffer (50 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 10 mM DTT and 0.001% Bromophenolblue). The samples were boiled for 5 min, followed by 5 min centrifugation in an eppendorf centrifuge at 14 000 rpm.

Equal amounts of protein were subjected to 10% or 12% SDS-PAGE. After electrophoresis the proteins were transferred by electroblotting to Nitrocellulose Transfer Membrane (Protran; Schleicher and Schuell, Dassel, Germany). Membranes were blocked in 5% non-fat milk in TBST (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween-20) for 1 h at room temperature. Primary antibody incubation was performed overnight at 4 ºC. After incubation with a secondary horseradish peroxidase (HRP)-linked antibody, proteins were visualized by enhanced chemiluminescence utilizing supersignal substrate from Perbio Science (Rockford, IL). Densitometric analyses of Western blots were performed utilizing ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Antibodies

Rabbit polyclonal antibodies to Akt, phospho-Akt(Ser473) and phospho-FOXO3a(Thr32) were purchased from Cell Signaling Technology (Beverly, MA),

and applied in a 1:1000 dilution. The mouse monoclonals anti-human PTEN (Clone 6H2.1; 1:1000) and anti-β-actin (1:10,000) were from Cascade Bioscience (Winchester, MA) and Sigma Chemical, respectively. Goat-anti-Rabbit HRP secondary antibody was from Cell Signaling Technology and the secondary antibody Goat-anti-Mouse HRP was from DAKO (Glostrup, Denmark); both antibodies were applied in a 1:2000 dilution.

cDNA preparation and Quantitative RT-PCR analysis

Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's description, including an on column DNaseI digestion using the RNAse free DNAse set from Qiagen. cDNA was prepared from total RNA (2 μg) by reverse transcription using 400 units M-MLV RT (Invitrogen) and an oligo-dT12 primer.

Q-PCR analysis was performed in an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). QPCR reactions were performed in Power SYBR green PCR Master Mix (Applied Biosystems) supplemented with Forward and Reverse primer (330 nM) in a total volume of 25 μl. Thermocycling conditions were according to the recommendations of the manufacturer. RNA levels were determined relative to phorphobilinogen deaminase (PBGD) by the standard curve method (Applied Biosystems). Primer combinations used were:

	Forward 5'- 3'	Reverse 5'- 3'
PBGD	5'-CATGTCTGGTAACGGCAATG-3'	5'-GTACGAGGCTTTCAATGTTG-3'
DRE1	5'-GAGTGCTACGATCCTGTAAC-3'	5'-CATCACGGCTGTTGATTCTTC-3'
RIOK3	5'-TGTCAGTCAGTCAGTAGAAC-3'	5'-GCTTCATTATCTGCTGTGATG-3'
MST1	5'-GACCCCATTCGACTACTGTG-3'	5'-CCACTGCTCCTTCACTAG-3'
CCNG2	5'-GAGCTGCCAACGATACCTG-3'	5'-TCTAAGATGGAAAGCACAGTG-3'
E2F2	5'-CTCCGCAGACGAGACTGG-3'	5'- CTAGGGTCGGTGCTGCTG-3'
Cdc25a	5'-GTTGAAGAGACCAGAACGATC-3'	5'-AGATGCCAGGGATAAAGACTG-3'
RBL2	5'-CAACAACATCTACATCAAACAG-3'	5'-GAGTTTCTCCTGTGCGTATC-3'

siRNA and Transfection studies in LNCaP/PTEN cells

One day prior to transfection approximately $1.5x10^5$ LNCaP/PTEN cells were seeded per 60 mm dish. Next day, cells were washed in PBS and the medium was replaced by serum and antibiotics free medium. Transfection was carried out using Dharmafect3 transfection reagent (Dharmacon Research, Lafayette, CO) following the manufacturer's description. Cells were harvested at 48 h. Akt1 and Akt2

siRNAs and siControl non-targeting siRNA pool were obtained as ready-annealed, purified duplexes from Dharmacon Research. All siRNA duplexes were used at a final concentration of 100 nM.

Cell Cycle Analysis

Cell cycle distribution was analyzed by flow cytometry. LNCaP/PTEN cells were incubated with 250 ng/ml dox for the indicated time points. Next, cells were trypsinized and washed in ice-cold PBS. Subsequently, cells were fixed by drop-wise adding to ice-cold 70% ethanol. Fixed cells were washed twice in PBS, and incubated in PBS containing 100 µg/ml RNAseA and 10 µg/ml Propidium Iodide (Sigma) for 1 h at 37 °C. Stained cells were assayed in a FACScan (Becton Dickinson, San Jose, CA), and the data were analyzed utilizing CellQuest software. For each sample $1x10^4$ cells were recorded.

Affymetrix micro-array hybridization

All samples were hybridized to Affymetrix GeneChip Human Genome U133A plus 2.0 arrays containing 54 614 probe sets, representing approximately 47 000 transcripts (30 000 genes). Antisense biotinylated RNA was prepared from 1 ug total RNA according to the Affymetrix GeneChip eukaryotic one-cycle target preparation protocol (Affymetrix, Santa Clara, CA). Briefly, single-stranded cDNA was synthesized using a T7-Oligo(dT) Promoter Primer, followed by RNase H–facilitated second-strand cDNA synthesis. The cDNA served as a template in the subsequent *in vitro* transcription (IVT) reaction utilizing T7 RNA polymerase and a biotinylated nucleotide analogue/ribonucleotide mix for cRNA. The biotinylated cRNA targets were then cleaned up and fragmented. The amount of fragmented-labeled cRNA was determined by standard spectrophotometric analysis, and the quality was checked on an Agilent 2100 Bioanalyzer (Agilent, Amstelveen, the Netherlands), using an RNA 6000 NANO assay. Affymetrix GeneChips were hybridized with 15 ug of fragmented biotinylated cRNA (45 °C for 16 h) and subsequently washed, stained with streptavidin phycoerythrin and imaged according to the GeneChip Expression Analysis Technical Manual (Affymetrix). All GeneChips were visually inspected for irregularities. Average Log2 ratios of dox treated and non-treated samples were compared and selected genes (Log2 ratio > 0.8) were analyzed for their functions and networks using Ingenuity Pathway Analysis (IPA, Ingenuity Systems Inc., Redwood City, CA).

References

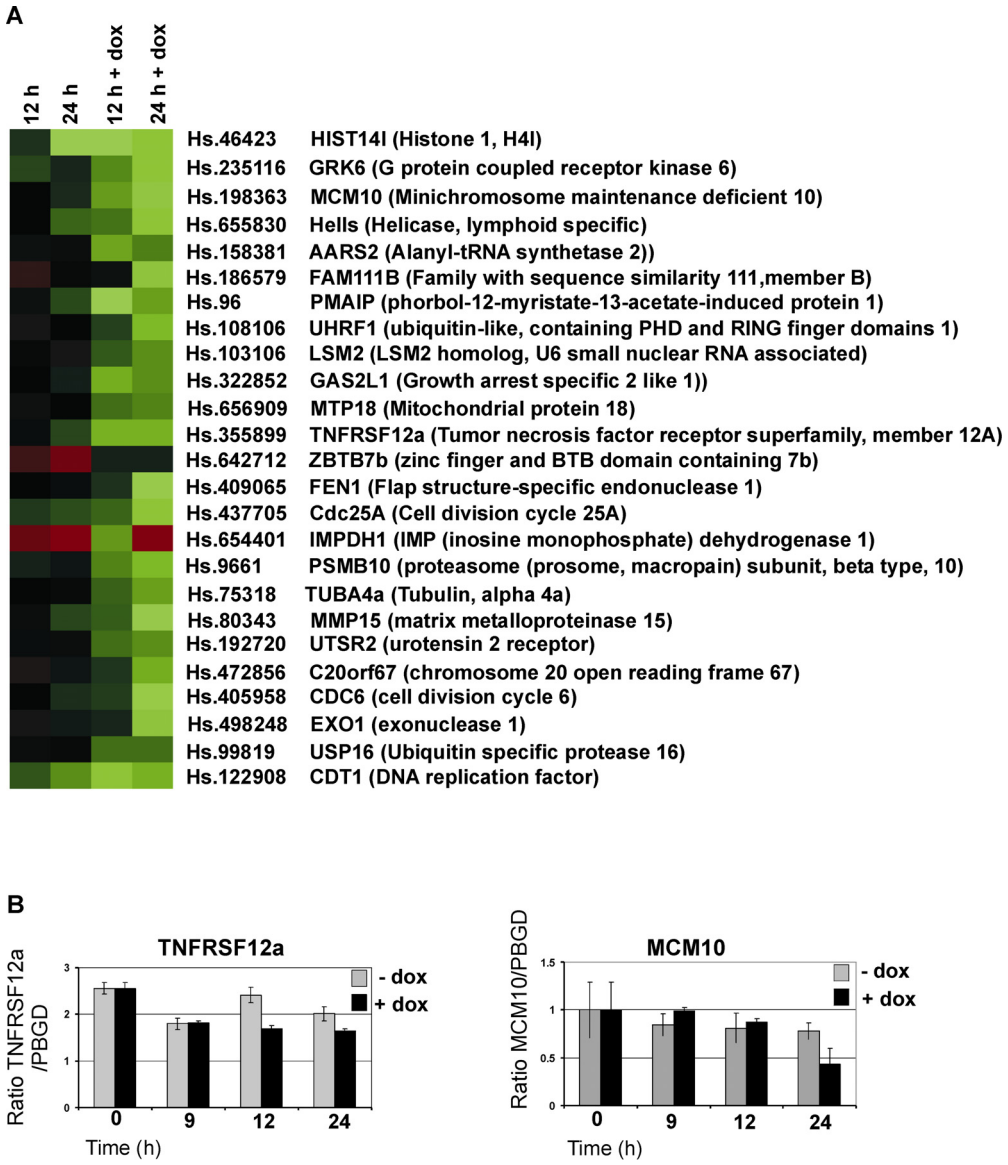
1. Dahia, P.L. (2000) PTEN, a unique tumor suppressor gene *Endocr Relat Cancer*, **7,** 115-129.
2. Verhagen, P.C., van Duijn, P.W., Hermans, K.G., Looijenga, L.H., van Gurp, R.J., Stoop, H., van der Kwast, T.H.,Trapman, J. (2006) The PTEN gene in locally progressive prostate cancer is preferentially inactivated by bi-allelic gene deletion *J Pathol*, **208,** 699-707.
3. Vlietstra, R.J., van Alewijk, D.C., Hermans, K.G., van Steenbrugge, G.J.,Trapman, J. (1998) Frequent inactivation of PTEN in prostate cancer cell lines and xenografts *Cancer Res*, **58,** 2720-2723.
4. Yoshimoto, M., Cutz, J.C., Nuin, P.A., Joshua, A.M., Bayani, J., Evans, A.J., Zielenska, M.,Squire, J.A. (2006) Interphase FISH analysis of PTEN in histologic sections shows genomic deletions in 68% of primary prostate cancer and 23% of high-grade prostatic intra-epithelial neoplasias *Cancer Genet Cytogenet*, **169,** 128-137.
5. Maehama, T. and Dixon, J.E. (1998) The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate *J Biol Chem*, **273,** 13375-13378.
6. Myers, M.P., Stolarov, J.P., Eng, C., Li, J., Wang, S.I., Wigler, M.H., Parsons, R.,Tonks, N.K. (1997) P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase *Proc Natl Acad Sci U S A*, **94,** 9052-9057.
7. Li, L., Ittmann, M.M., Ayala, G., Tsai, M.J., Amato, R.J., Wheeler, T.M., Miles, B.J., Kadmon, D.,Thompson, T.C. (2005) The emerging role of the PI3-K-Akt pathway in prostate cancer progression *Prostate Cancer Prostatic Dis*, **8,** 108-118.
8. Cully, M., You, H., Levine, A.J.,Mak, T.W. (2006) Beyond PTEN mutations: the PI3K pathway as an integrator of multiple inputs during tumorigenesis *Nat Rev Cancer*, **6,** 184-192.
9. Greer, E.L. and Brunet, A. (2005) FOXO transcription factors at the interface between longevity and tumor suppression *Oncogene*, **24,** 7410-7425.
10. Levitt, R.J., Georgescu, M.M.,Pollak, M. (2005) PTEN-induction in U251 glioma cells decreases the expression of insulin-like growth factor binding protein-2 *Biochem Biophys Res Commun*, **336,** 1056-1061.
11. Radu, A., Neubauer, V., Akagi, T., Hanafusa, H.,Georgescu, M.M. (2003) PTEN induces cell cycle arrest by decreasing the level and nuclear localization of cyclin D1 *Mol Cell Biol*, **23,** 6139-6149.
12. Seminario, M.C., Precht, P., Wersto, R.P., Gorospe, M.,Wange, R.L. (2003) PTEN expression in PTEN-null leukaemic T cell lines leads to reduced proliferation via slowed cell cycle progression *Oncogene*, **22,** 8195-8204.
13. Stolarov, J., Chang, K., Reiner, A., Rodgers, L., Hannon, G.J., Wigler, M.H.,Mittal, V. (2001) Design of a retroviral-mediated ecdysone-inducible system and its application to the expression profiling of the PTEN tumor suppressor *Proc Natl Acad Sci U S A*, **98,** 13043-13048.

14. Weng, L.P., Brown, J.L.,Eng, C. (2001) PTEN coordinates G(1) arrest by down-regulating cyclin D1 via its protein phosphatase activity and up-regulating p27 via its lipid phosphatase activity in a breast cancer model *Hum Mol Genet*, **10,** 599-604.
15. Wu, Z., Conaway, M., Gioeli, D., Weber, M.J.,Theodorescu, D. (2006) Conditional expression of PTEN alters the androgen responsiveness of prostate cancer cells *Prostate*, **66,** 1114-1123.
16. Xu, Z., Stokoe, D., Kane, L.P.,Weiss, A. (2002) The inducible expression of the tumor suppressor gene PTEN promotes apoptosis and decreases cell size by inhibiting the PI3K/Akt pathway in Jurkat T cells *Cell Growth Differ*, **13,** 285-296.
17. Zhao, H., Dupont, J., Yakar, S., Karas, M.,LeRoith, D. (2004) PTEN inhibits cell proliferation and induces apoptosis by downregulating cell surface IGF-IR expression in prostate cancer cells *Oncogene*, **23,** 786-794.
18. Tanaka, M., Rosser, C.J.,Grossman, H.B. (2005) PTEN gene therapy induces growth inhibition and increases efficacy of chemotherapy in prostate cancer *Cancer Detect Prev*, **29,** 170-174.
19. Sharrard, R.M. and Maitland, N.J. (2000) Phenotypic effects of overexpression of the MMAC1 gene in prostate epithelial cells *Br J Cancer*, **83,** 1102-1109.
20. Davies, M.A., Kim, S.J., Parikh, N.U., Dong, Z., Bucana, C.D.,Gallick, G.E. (2002) Adenoviral-mediated Expression of MMAC/PTEN Inhibits Proliferation and Metastasis of Human Prostate Cancer Cells *Clin Cancer Res*, **8,** 1904-1914.
21. Davies, M.A., Koul, D., Dhesi, H., Berman, R., McDonnell, T.J., McConkey, D., Yung, W.K.,Steck, P.A. (1999) Regulation of Akt/PKB activity, cellular growth, and apoptosis in prostate carcinoma cells by MMAC/PTEN *Cancer Res*, **59,** 2551-2556.
22. Carson, J.P., Kulik, G.,Weber, M.J. (1999) Antiapoptotic signaling in LNCaP prostate cancer cells: a survival signaling pathway independent of phosphatidylinositol 3'-kinase and Akt/protein kinase B *Cancer Res*, **59,** 1449-1453.
23. Li, Y., Dowbenko, D.,Lasky, L.A. (2002) AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21Cip/WAF1 and promotes cell survival *J Biol Chem*, **277,** 11352-11361.
24. Liang, J. and Slingerland, J.M. (2003) Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression *Cell Cycle*, **2,** 339-345.
25. Liang, J., Zubovitz, J., Petrocelli, T., Kotchetkov, R., Connor, M.K., Han, K., Lee, J.H., Ciarallo, S., Catzavelos, C., Beniston, R., et al. (2002) PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest *Nat Med*, **8,** 1153-1160.
26. Shin, I., Yakes, F.M., Rojo, F., Shin, N.Y., Bakin, A.V., Baselga, J.,Arteaga, C.L. (2002) PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization *Nat Med*, **8,** 1145-1152.
27. Viglietto, G., Motti, M.L., Bruni, P., Melillo, R.M., D'Alessio, A., Califano, D., Vinci, F., Chiappetta, G., Tsichlis, P., Bellacosa, A., et al. (2002) Cytoplasmic relocalization and

inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer *Nat Med*, **8,** 1136-1144.

28. Hermans, K.G., van Alewijk, D.C., Veltman, J.A., van Weerden, W., van Kessel, A.G.,Trapman, J. (2004) Loss of a small region around the PTEN locus is a major chromosome 10 alteration in prostate cancer xenografts and cell lines *Genes Chromosomes Cancer*, **39,** 171-184.
29. Herlevsen, M., Oxford, G., Ptak, C., Shabanowitz, J., Hunt, D.F., Conaway, M.,Theodorescu, D. (2007) A novel model to identify interaction partners of the PTEN tumor suppressor gene in human bladder cancer *Biochem Biophys Res Commun*, **352,** 549-555.
30. Zhao, X., Li, C., Paez, J.G., Chin, K., Janne, P.A., Chen, T.H., Girard, L., Minna, J., Christiani, D., Leo, C., et al. (2004) An integrated view of copy number and allelic alterations in the cancer genome using single nucleotide polymorphism arrays *Cancer Res*, **64,** 3060-3071.
31. Simpson, L., Li, J., Liaw, D., Hennessy, I., Oliner, J., Christians, F.,Parsons, R. (2001) PTEN expression causes feedback upregulation of insulin receptor substrate 2 *Mol Cell Biol*, **21,** 3947-3958.
32. Groszer, M., Erickson, R., Scripture-Adams, D.D., Dougherty, J.D., Le Belle, J., Zack, J.A., Geschwind, D.H., Liu, X., Kornblum, H.I.,Wu, H. (2006) PTEN negatively regulates neural stem cell self-renewal by modulating G0-G1 cell cycle entry *Proc Natl Acad Sci U S A*, **103,** 111-116.
33. Bell, S.P. and Dutta, A. (2002) DNA replication in eukaryotic cells *Annu Rev Biochem*, **71,** 333-374.
34. Hateboer, G., Wobst, A., Petersen, B.O., Le Cam, L., Vigo, E., Sardet, C.,Helin, K. (1998) Cell cycle-regulated expression of mammalian CDC6 is dependent on E2F *Mol Cell Biol*, **18,** 6679-6697.
35. Yan, Z., DeGregori, J., Shohet, R., Leone, G., Stillman, B., Nevins, J.R.,Williams, R.S. (1998) Cdc6 is regulated by E2F and is essential for DNA replication in mammalian cells *Proc Natl Acad Sci U S A*, **95,** 3603-3608.
36. Yoshida, K. and Inoue, I. (2004) Regulation of Geminin and Cdt1 expression by E2F transcription factors *Oncogene*, **23,** 3802-3812.
37. Modur, V., Nagarajan, R., Evers, B.M.,Milbrandt, J. (2002) FOXO proteins regulate tumor necrosis factor-related apoptosis inducing ligand expression. Implications for PTEN mutation in prostate cancer *J Biol Chem*, **277,** 47928-47937.
38. Nakamura, N., Ramaswamy, S., Vazquez, F., Signoretti, S., Loda, M.,Sellers, W.R. (2000) Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN *Mol Cell Biol*, **20,** 8969-8982.
39. Medema, R.H., Kops, G.J., Bos, J.L.,Burgering, B.M. (2000) AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1 *Nature*, **404,** 782-787.

40. Kops, G.J., de Ruiter, N.D., De Vries-Smits, A.M., Powell, D.R., Bos, J.L.,Burgering, B.M. (1999) Direct control of the Forkhead transcription factor AFX by protein kinase B *Nature*, **398,** 630-634.
41. Kops, G.J., Medema, R.H., Glassford, J., Essers, M.A., Dijkers, P.F., Coffer, P.J., Lam, E.W.,Burgering, B.M. (2002) Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors *Mol Cell Biol*, **22,** 2025-2036.
42. Collado, M., Medema, R.H., Garcia-Cao, I., Dubuisson, M.L., Barradas, M., Glassford, J., Rivas, C., Burgering, B.M., Serrano, M.,Lam, E.W. (2000) Inhibition of the phosphoinositide 3-kinase pathway induces a senescence-like arrest mediated by p27Kip1 *J Biol Chem*, **275,** 21960-21968.
43. Brunet, A., Bonni, A., Zigmond, M.J., Lin, M.Z., Juo, P., Hu, L.S., Anderson, M.J., Arden, K.C., Blenis, J.,Greenberg, M.E. (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor *Cell*, **96,** 857-868.
44. van Duijn, P.W. and Trapman, J. (2006) PI3K/Akt signaling regulates p27(kip1) expression via Skp2 in PC3 and DU145 prostate cancer cells, but is not a major factor in p27(kip1) regulation in LNCaP and PC346 cells *Prostate*, **66,** 749-760.
45. Zhao, H.H., Herrera, R.E., Coronado-Heinsohn, E., Yang, M.C., Ludes-Meyers, J.H., Seybold-Tilson, K.J., Nawaz, Z., Yee, D., Barr, F.G., Diab, S.G., et al. (2001) Forkhead homologue in rhabdomyosarcoma functions as a bifunctional nuclear receptor-interacting protein with both coactivator and corepressor functions *J Biol Chem*, **276,** 27907-27912.
46. Kitamura, T., Nakae, J., Kitamura, Y., Kido, Y., Biggs, W.H., 3rd, Wright, C.V., White, M.F., Arden, K.C.,Accili, D. (2002) The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic beta cell growth *J Clin Invest*, **110,** 1839-1847.
47. Hirota, K., Daitoku, H., Matsuzaki, H., Araya, N., Yamagata, K., Asada, S., Sugaya, T.,Fukamizu, A. (2003) Hepatocyte nuclear factor-4 is a novel downstream target of insulin via FKHR as a signal-regulated transcriptional inhibitor *J Biol Chem*, **278,** 13056-13060.
48. Lim, J.H., Chang, Y.C., Park, Y.B., Park, J.W.,Kwon, T.K. (2004) Transcriptional repression of E2F gene by proteasome inhibitors in human osteosarcoma cells *Biochem Biophys Res Commun*, **318,** 868-872.
49. Leone, G., DeGregori, J., Sears, R., Jakoi, L.,Nevins, J.R. (1997) Myc and Ras collaborate in inducing accumulation of active cyclin E/Cdk2 and E2F *Nature*, **387,** 422-426.
50. Sears, R., Ohtani, K.,Nevins, J.R. (1997) Identification of positively and negatively acting elements regulating expression of the E2F2 gene in response to cell growth signals *Mol Cell Biol*, **17,** 5227-5235.
51. Vigneron, A., Cherier, J., Barre, B., Gamelin, E.,Coqueret, O. (2006) The cell cycle inhibitor p21waf1 binds to the myc and cdc25A promoters upon DNA damage and induces transcriptional repression *J Biol Chem*, **281,** 34742-34750.



Supplementary Figure S1. Strongest down-regulated genes by PTEN induction in LNCaP/PTEN cells. (A) Overview of the 25 strongest down-regulated genes in LNCaP/PTEN cells after treatment with 250 ng/ml dox for 12 h and 24 h. (B) Total RNA was isolated from LNCaP/PTEN cells incubated with 250 ng/ml dox for the indicated time periods. MCM10 and TNFRSF12a mRNA expression was measured by Q-PCR, using PBGD expression as a reference. Representative Q-PCR analyses of several independent RNA isolations are shown. Data are shown as average ± SD.

Chapter 4

The *PTEN* gene in locally progressive prostate cancer is preferentially inactivated by bi-allelic gene deletion

Paul C.M.S. Verhagen[1], Petra W. van Duijn[2], Karin G.L. Hermans[2], Leendert H.J. Looijenga[3], Ruud J.H.L.M. van Gurp[3], Hans Stoop[3], Theo H. van der Kwast[2] and Jan Trapman[2].

[1]Department of Urology, [2]Department of Pathology, and [3]Department of Pathology, Laboratory for Experimental Patho-oncology, Josephine Nefkens Institute, Erasmus University Medical Center, Rotterdam, the Netherlands

Journal of Pathology 208: 699-707 (2006)

Abstract

PTEN is frequently inactivated during the development of many cancers, including prostate cancer, and both bi-allelic and mono-allelic *PTEN* inactivation may contribute to tumorigenesis. *PTEN* mutations in clinical cancer specimens can easily be recorded but mono- or bi-allelic gene deletions are often difficult to assess. We performed a comprehensive study to detect *PTEN* inactivation in 40 locally progressive clinical prostate cancer specimens obtained by transurethral resection of the prostate, utilizing a variety of complementary technical approaches. The methods to detect *PTEN* deletion included allelotype analysis, dual colour FISH and array-based CGH. We also applied a novel semi-quantitative approach, assessing the *PTEN* (wild-type) / *ψPTEN* (pseudogene) ratio (WPR). Structural analysis of *PTEN* was done by single-strand conformational polymorphism (PCR-SSCP) and sequencing. PTEN protein expression was assessed by immunohistochemistry. Our data predict complete *PTEN* inactivation in 12 samples (30%), nine of these by bi-allelic deletion. Loss of one *PTEN* copy was also detected by several methodologies but the number could not be accurately assessed. Immunohistochemistry indicated the absence of PTEN protein in 15 samples, and heterogeneous expression of the protein in eight tumors. Taken together, the data show that bi-allelic deletion is a major mechanism of *PTEN* inactivation in locally progressive prostate cancer.

Introduction

Prostate cancer is the most common cancer in men in the Western world and the second leading cause of male cancer death [1]. Effective treatments exist for localized prostate cancer, but when metastases develop no curative therapy is available. Hormonal therapy gives a response in most patients with bone metastases, but after an average period of 18 months hormone refractory disease develops, after which only palliative procedures remain. A better understanding of the molecular mechanisms underlying prostate cancer is important for development of new, targeted therapies. In addition, there is an urgent need for improved prediction of the clinical course of prostate cancer to better select patients for treatment options [2].

Germ-line mutations of *PTEN* are the cause of Cowden disease, characterized by hamartomas at multiple sites and a predisposition to various tumor types [3]. Many sporadic tumors contain aberrant *PTEN*, in particular neuroblastomas, endometrial cancer and prostate cancer [4,5]. PTEN dephosphorylates inositol phospholipids (PtdIns-3-P) by removal of the D3 phosphate from the inositol ring in this way counteracting

phosphoinositide 3-kinase (PI3K). Absence of PTEN leads to increased phosphorylation and thereby activation of the serine/threonine kinase phospho-Akt, a downstream target of phosphorylated inositols, which connects PTEN to cell cycle regulation, cell survival, cell size and cell polarity [6]. Prostate-targeted *Pten* knockout mice develop prostate hyperplasia, high grade prostatic intraepithelial neoplasia (PIN) and invasive cancer [7-9].

The frequency and mode of *PTEN* inactivation reported at various stages of clinical prostate cancer are variable. Homozygous deletions of *PTEN* have been detected in up to 10 percent of locally confined cancers and metastases [10-14], and in 30 percent of prostate cancer cell lines and xenografts [15]. *PTEN* point mutations have been described in 2 to 15 percent of primary prostate cancers, 20 to 30 percent of metastases, and 25 percent of xenografts and cell lines [12,14-17]. Immunohistochemical studies showed absence of PTEN expression in 20 to 25 percent of primary tumors [18,19]. No data are available on *PTEN* inactivation in locally progressive prostate cancer.

Although the high frequency of *PTEN* inactivation by bi-allelic deletion in prostate cancer xenografts and cell lines, as compared to clinical tumor specimens, might be due to selection, an alternative explanation is the underestimation of the frequency of gene deletions in clinical samples. The finding that loss at 10q23 might concern only *PTEN* and a few directly flanking genes indicates that sensitive methods are needed to assess the *PTEN* copy number [20].

To obtain information on *PTEN* alterations in locally progressive prostate cancer tissues collected by transurethral resection (TURP), we used several complementary techniques, including classical allelotype analysis, dual color FISH, and a novel PCR approach, denoted WPR (Wild-type/Pseudogene Ratio analysis). Selected samples were assayed by array-CGH. Mutation analysis by PCR-SSCP and sequencing, and PTEN expression studies by immunohistochemistry completed the study. Our data show preferential bi-allelic deletion of *PTEN* in locally progressive prostate cancer. They also indicate frequent loss of one *PTEN* gene copy in these tumor specimens.

Results

Because *PTEN* inactivation is the most frequent genetic alteration in late stage prostate cancer, PTEN downstream effectors are important candidates for therapeutic targeting of prostate cancer. In order to select patients for targeted therapies, knowledge of the status of the *PTEN* gene is of utmost importance. Exon sequencing can easily assess the structure of *PTEN* in tumor DNA. However, detection of *PTEN* deletions is more challenging since loss of *PTEN* may concern a small genomic fragment [15,20]. We applied a variety of complementary methods to investigate *PTEN* inactivation in locally progressive prostate cancer obtained by TURP.

Structural analysis of PTEN

The structure of *PTEN* was assessed in 40 TUR DNAs by PCR-SSCP analysis of all exons. PCR-SSCP was abnormal in three DNAs (T1-8, T2-2 and T10-7). In all three samples inactivating mutations of *PTEN* were found by subsequent sequence analysis (summarized in Figure 1). In T1-8 a C deletion in exon 7 gave rise to a frame shift and a truncated protein. In T2-2 a TTAC deletion in exon 8 also introduced a frame shift and a shorter protein. T10-7 contained a nonsense mutation (CAG>TAG) in exon 7.

Allelotype analysis

Allelotype analysis was performed with 4 highly polymorphic markers (*D10S1687, D10S1765, D10S541* and *D10S583*), mapping directly upstream or downstream of *PTEN* (See Materials and Methods, Figure 6). Nineteen DNAs from tumors obtained by TUR, for which corresponding normal blood DNA was available, were investigated. Eleven samples showed loss of at least one of the markers in the *PTEN* region (55%) (Figures 1 and 2). Among these are T1-8, T2-2 and T10-7, which contain an inactivating mutation in the second *PTEN* copy (see above). In two DNAs (T8-4, T10-12) markers directly adjacent to *PTEN* showed the presence of 2 alleles, whereas more distal markers showed loss of 10q, indicative of a homozygous deletion of *PTEN*. In T1-1, T3-7 and T10-2 markers directly flanking *PTEN* were not lost, but a more proximal or distal marker showed allelic imbalance, indicating bi-allelic *PTEN* loss, or no loss of *PTEN* at all.

	Clinical Stage (1)				Mutation	LOH	Ratio	FISH	Immuno	
Tumor	**T**	**N**	**M**	Horm Ther (2)		predicted	PTEN/pseud.		PTEN	expr. 0 (%)
T1-1	2	2	0	no	n	○◒●	●	●	0	100
T1-2	4	x	0	yes	n	-	○	●	2	0
T1-4	x	x	x	no	n	○	○	○	3	0
T1-7	3	1	0	yes	n	-	●	●	0-3	10
T1-8	3	0	0	yes	mutation	◒	◒	◒	0-3	10
T1-14	3	x	1	yes	n	-	◒	◒	0	100
T2-2	3	0	1	yes	mutation	◒	◒	◒	0	100
T2-4	4	0	0	yes	n	-	-	○	1	0
T2-9	3	x	1	yes	n	○◒●	◒	○	0-3	70
T2-11	4	2	0	yes	n	◒	○	◒	0	0
T2-14	2	x	x	yes	n	-	-	◒	0-3	80
T3-1	3	2	0	no	n	-	○	○	0-3	10
T3-7	3	0	0	yes	n	○◒●	●	●	0	100
T4-1	3	x	1	yes	n	◒	○	○	0	100
T4-5	4	0	0	yes	n	○	◒	○	2	0
T4-10	3	x	0	no	n	-	◒	○	3	0
T5-5	3	x	x	yes	n	-	○	○	2	0
T5-8	3	x	x	yes	n	○	○	○	-	-
T6-1	x	x	x	no	n	-	○	○	0-2	20
T6-9	3	0	0	yes	n	-	◒	●	0	100
T6-14	3	x	0	yes	n	-	●	○	0	100
T7-1	3	x	x	yes	n	-	○	○	1	0
T7-8	4	x	0	no	n	-	●	○	0	100
T7-11	3	0	0	yes	n	○	○	○	0-2	10
T8-4	3	x	0	no	n	●	●	●	0	100
T8-9	x	x	x	no	n	-	○	○	2	0
T8-13	4	x	1	yes	n	-	◒	○	0	100
T8-14	4	x	0	yes	n	-	◒	○	3	0
T9-4	4	0	0	yes	n	-	◒	◒	1	0
T9-8	2	2	0	?	n	-	◒	○	0	100
T9-11	4	x	0	yes	n	○	○	○	2	0
T10-2	3	x	0	no	n	○◒●	○	○	1	0
T10-3	3	x	0	yes	n	-	●	●	0	100
T10-4	3	0	x	yes	n	-	◒	○	0-1	10
T10-7	4	0	1	no	mutation	◒	◒	◒	0	100
T10-12	3	x	0	no	n	●	●	●	0	100
T10-15	3	x	1	yes	n	-	◒	○	2	0
T29-4	2	0	0	yes	n	○	◒	-	3	0
T30-0	4	0	0	no	n	○	○	-	1	0
T40-9	4	0	0	yes	n	○	○	-	-	-

○ 2 alleles
◒ 1 allele
● homozygous deletion
n normal
- not done
1 TNM 1997
2 hormonal therapy at time of TUR

immunohistochemistry
0 absent
1 weak
2 normal
3 strong

Figure 1. Overview of alterations of *PTEN* structure as obtained by mutation analysis, allelotype analysis (LOH), WPR analysis and FISH, and of PTEN expression by immunohistochemistry of prostate tumors obtained by TURP.

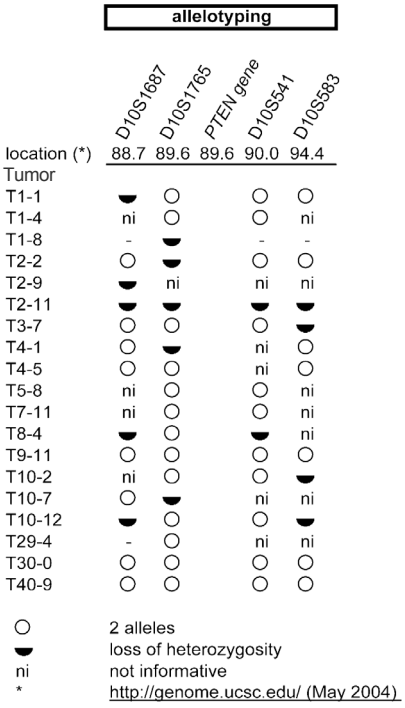


Figure 2. Allelotype analysis of the *PTEN* region of DNA from locally progressive prostate cancers. See Figure 6 for the positions of the markers.

PTEN analysis by dual color FISH

In 37 cases, frozen tissue sections with representative tumor were available for dual color FISH. Results are summarized in Figure 1. In 22 samples a normal *PTEN* signal was obtained. Eight cases showed complete absence of *PTEN* signals in cancer cells, in contrast to neighboring stromal cells, indicative of complete *PTEN* deletion (T1-1, T1-2, T1-7, T3-7, T6-9, T8-4, T10-3, T10-12). Seven tumor sections indicated loss of one *PTEN* copy.

PTEN analysis by the WPR assay

WPR is a semi-quantitative method to assess the *PTEN* gene copy number. In the WPR assay the *PTEN* pseudogene (*ψPTEN)* is used as an internal control (see Materials and Methods for details).

WPR was applied on thirty-eight TUR DNAs. Examples of relevant parts of sequence plots from tumor DNAs with either 2, 1 or 0 wild-type *PTEN* copies, as

confirmed by other methods, are shown in Figure 3. The results of all TUR DNAs are summarized in Figure 1. In 15/38 tumor DNA samples two *PTEN* alleles were detected; WPR indicated also loss of one allele in 15 samples. WPR predicted homozygous deletions of *PTEN* in eight tumor DNA's (T1-1, T1-7, T3-7, T6-14, T7-8, T8-4, T10-3 and T10-12).

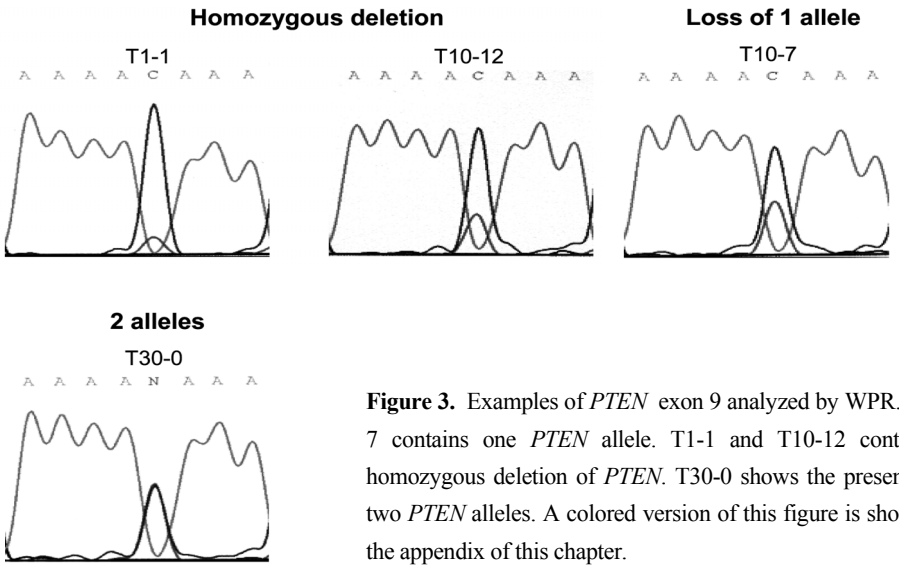


Figure 3. Examples of *PTEN* exon 9 analyzed by WPR. T10-7 contains one *PTEN* allele. T1-1 and T10-12 contain a homozygous deletion of *PTEN*. T30-0 shows the presence of two *PTEN* alleles. A colored version of this figure is shown in the appendix of this chapter.

PTEN copy number analysis by array-CGH

For comparison with allelotype analysis, FISH, and WPR, two tumor samples of which sufficient DNA was available (T1-1, T10-12) were analyzed by array-CGH. FISH and WPR predicted bi-allelic *PTEN* loss for T1-1; in case T10-12 all three assays indicated complete *PTEN* loss (Figure 1). The array-CGH pattern of T1-1 clearly showed losses of many chromosome regions (Figure 4A). Such a pattern is representative for DNA from a homogeneous high-grade tumor, containing a low percentage of normal cells. Tumor T10-12 contained fewer chromosomal alterations; one of the most prominent changes was loss of chromosome 10 (Figure 4B). In both tumor DNAs, but most clearly in T1-1, the chromosomal region with the lowest T/R ratio is 10q23, where *PTEN* is located, indicative of bi-allelic *PTEN* deletion. In T1-1 the predicted homozygous deletion (T/R ratio approx. –2) is located in a small 1 Mbp region of 10q23 loss (see Figure 4C). T10-12 has lost one copy of chromosome 10 and contains a small deletion in the second copy (Figure 4D). T10-12 seems to

contain a considerable number of cells without chromosome 10 losses, because the log2 T/R ratio of chromosome 10 is approx. -0.5; the log2 T/R ratio over the homozygous deletion is approximately -1 (Figure 4D). Even in the presence of DNA from cells with a normal chromosome 10 copy number (non-cancer cells) the deletion can be visualized.

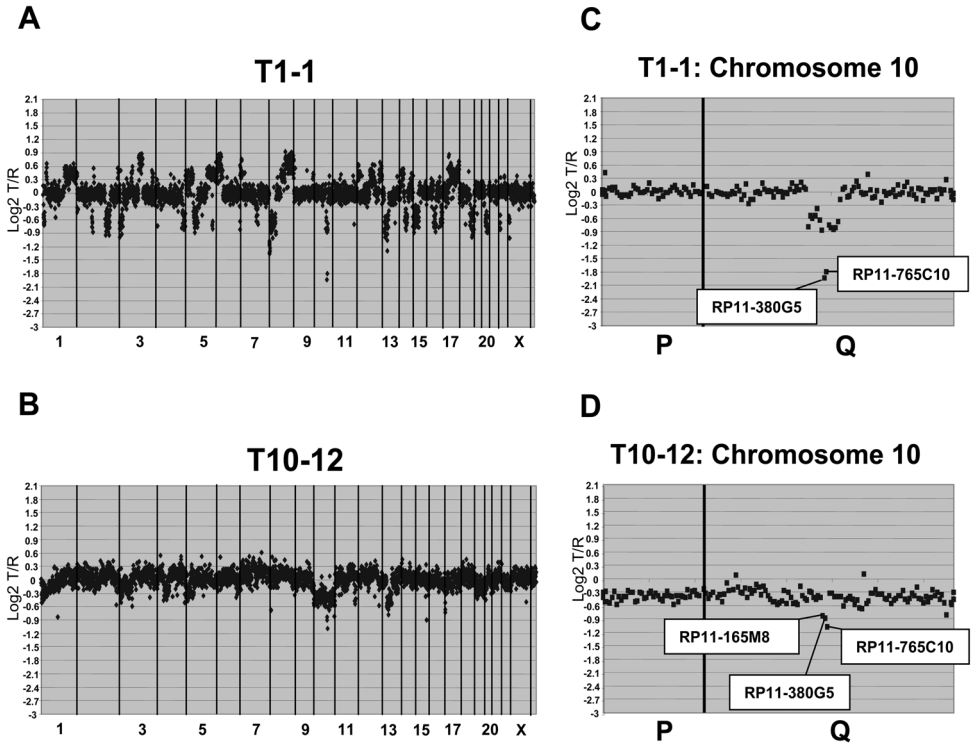


Figure 4. Array-CGH patterns of T1-1 and T10-12. (A, B) whole genome array-CGH. (C, D) Chromosome 10 profiles. Both tumors contain a homozygous deletion of *PTEN*. In T10-12 one complete copy of chromosome 10 is lost. In T1-1 the *PTEN* homozygous deletion is in a small region of 10q loss.

Expression of PTEN in locally progressive prostate cancer

Immunohistochemistry showed cytoplasmic and nuclear PTEN staining in normal prostatic epithelium and stromal cells (intensity 2) (Figure 5A). Intraprostatic nerves and urothelial cells showed strong cytoplasmic staining. As summarized in Figure 1, 15 out of 38 evaluable tumors were completely negative for PTEN (39%) (See e.g. T10-12, Figure 5B). Heterogeneous cytoplasmic staining with locally complete absence of staining was noted in eight tumors (Figure 5C).

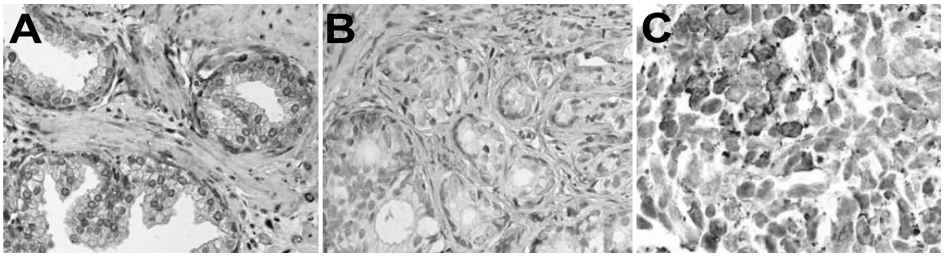


Figure 5. Immunohistochemical staining of PTEN (brown) in (A) a positive control prostate. (B) Absence of PTEN expression in the tumor cells in T10-12. Note PTEN expression in stromal cells. (C) Heterogeneous cytoplasmic staining with locally complete absence of PTEN expression in prostate tumor T1-8. A colored version of this figure is shown in the appendix of this chapter.

Discussion

PTEN inactivation is the most frequent genetic alteration in prostate cancer. Previously, we have shown that *PTEN* can be inactivated by bi-allelic deletion or mutation in over 60% of prostate cancer xenografts and cell lines, derived from prostate tumors of different clinical background [15]. In clinical prostate cancer *PTEN* inactivation is reported at a lower frequency [10,12,13,17,21]. In the most complete clinical study [10] loss of (part of) chromosome 10q was reported in 23 out of 80 prostate cancer specimens obtained by radical prostatectomy (60/80) or from regional lymph node metastases (20/80). In 10 of these samples, 7 lymph node metastases and 3 primary tumors, *PTEN* was found to be completely inactivated by bi-allelic deletion or by point mutation. Recently, we observed that *PTEN* inactivation by deletion frequently occurs by loss of a small chromosomal region [20]. Such small deletions might be overlooked in clinical prostate samples.

In the present study we analyzed PTEN alterations in 40 locally progressive prostate cancers obtained by TUR. In three samples structural analysis detected nonsense mutations or frameshifts in exons 7 or 8, causing the synthesis of a truncated protein. Furthermore, we utilized a variety of complementary methodologies, including allelotype analysis, dual color FISH, and the WPR method, to accurately assess the *PTEN* status in clinical prostate cancer samples. Overall, the various genetic approaches gave consistent results (Figure 1). In six samples both WPR and FISH indicated the presence of a homozygous deletion. The two homozygous deletions detected by allelotype analysis, and the two homozygous deletions found by array-CGH were observed by both WPR analysis and FISH (see Figure 1). In the three tumors containing inactivating point mutations (T1-8, T2-2 and T10-7) both allelotype

analysis and WPR experiments indicated loss of the second allele. In two cases, FISH indicated homozygous deletions that were not found by WPR (T1-2 and T6-9); also in 2 cases results were the opposite (T6-14 and T7-8). In three of four discrepant cases, immunohistochemistry confirmed the absence of PTEN expression, indicating a likely total number of nine specimens containing a homozygous deletion of *PTEN*. Discrepancies might be due to small deletions, precluding detection by FISH or array-CGH (see Figure 6). Further, tumors might be heterogeneous, affecting results of FISH experiments. Both tumor heterogeneity and presence of normal cell DNA can affect WPR data, array-CGH and allelotype analysis. WPR has the advantage of producing a quick result in every sample, requiring only 50 ng of DNA. The number of six to ten homozygous deletions in forty clinical tumor specimens (15-25%) is lower than reported in xenografts and cell lines (35%), but higher than previous reports in clinical samples (0-15%), utilizing different methodologies [10,14].

Fifteen samples (40%) were completely negative for PTEN expression by immunostaining, among those are 10 showing complete genetic inactivation of *PTEN* by one or more genetical analyses. Eight samples (20%) stained heterogeneously with fields of complete absence of PTEN, of which six could not be explained by genetic defects. Our data therefore indicate, in addition to genetic alterations, down-regulation of PTEN protein expression. Down-regulation might be by promoter hypermethylation, other epigenetic mechanisms or by mutations in regulatory gene sequences. Evidence for promoter hypermethylation has been reported in prostate cancer xenografts [22], however, others observed that *PTEN* promoter hypermethylation is rare or absent in clinical prostate cancer [15,23].

The higher frequency of loss of 10q23, as compared to complete *PTEN* inactivation gave rise to the speculation that 10q23 might contain a second tumor suppressor gene or that mono-allelic *PTEN* inactivation might contribute to tumorigenesis. The latter hypothesis is supported by studies in mouse models of prostate cancer, although we have recently shown that in a prostate targeted *Pten* knock-out model, mono-allelic *Pten* inactivation by itself is insufficient for tumorigenesis [8]. However, there is ample evidence that a *Pten+/-* genetic background can induce tumorigenesis or accelerate tumor progression in compound mouse prostate cancer models including *Nkx3.1*, *Ink4a/Arf* or *Cdkn1b* inactivation, and in TRAMP transgenic mice [24-27]. In the present study we postulate many tumor specimens with mono-allelic loss of *PTEN* (Figure 1). However, the limited accuracy of our data shows the complexity in discriminating between 2 copies and 1 copy of *PTEN*. More quantitative experiments are essential to

assess the frequency of mono-allelic *PTEN* inactivation in prostate cancer. We hypothesize that mono-allelic *PTEN* inactivation in late stage prostate cancer will synergistically cooperate with genetic or epigenetic alterations in other signaling pathways. Complete *PTEN* inactivation might further accelerate tumor progression.

In our study, aberrant PTEN expression appeared not to be predictive of survival, neither did it correlate with clinical stage. However, the sample size is limited. The TURP specimens were generally taken from patients progressive under hormonal therapy. Even if absence of PTEN expression would be predictive of a more aggressive course of the disease, this might not have become apparent because only aggressive cancers were included in this study.

In conclusion, we show aberrant PTEN expression in up to 58% of locally progressive prostate cancers, with bi-allelic deletion being the predominant mechanism of genetic inactivation.

Materials and Methods

Tumor samples

The study was approved by the institutional ethical committee. Tumor tissues collected by TURP were obtained from 40 symptomatic prostate cancer patients after informed consent. These patients had clinical signs of bladder outlet obstruction and/or haematuria (locally progressive prostate cancer). Twenty-seven tumors were progressive under hormone treatment. Twelve patients underwent TUR while hormonal therapy was not yet initiated; the status of one patient was unknown. All specimens were reviewed by a clinical pathologist (ThHvdK). From approximately half of the patients DNA from blood cells was available. The tissue specimens were snap-frozen and stored in liquid nitrogen until use. In addition paraffin-embedded tissue sections were available from all patients.

DNA isolation

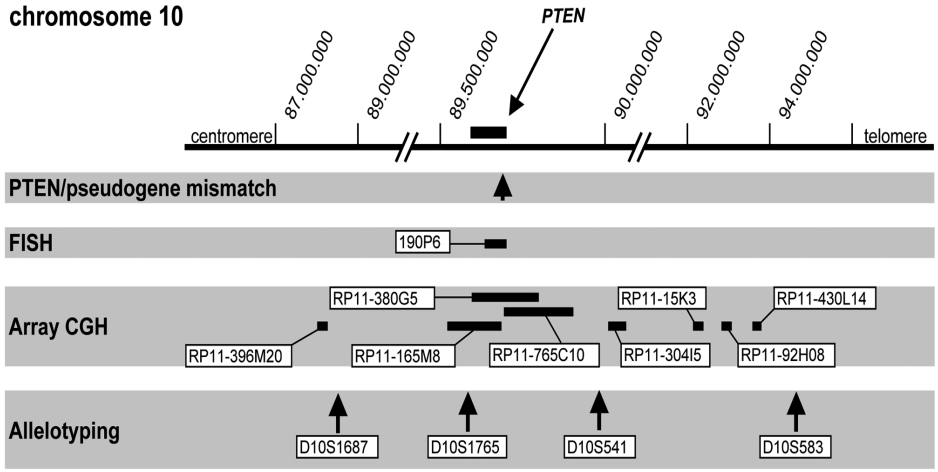
Standard protocols were used to isolate genomic DNA from TURPs and blood [28]. Tumor DNA was isolated by macrodissection from five consecutive 5 μm cryostat tissue sections containing at least 80% tumor according to standard procedures, including overnight proteinase K incubation at 55 ºC, phenol extraction and ethanol precipitation. DNAs were dissolved in TE buffer (10 mM Tris HCl, pH 7.8; 1 mM EDTA). For array-CGH DNA was further purified by RNAse treatment.

Allelotype analysis

For allelotype analysis, 100 ng tumor DNA and normal DNA were analyzed. Typical PCR settings were 30 cycles of 1 min at 95 ºC, 1 min at 55 ºC and 1 min at 72 ºC in 15 µ1 standard reaction buffer, supplemented with 1 µCi α^{32}PdATP (Amersham Biosciences, Buckinhamshire, UK). Four polymorphic markers flanking the *PTEN* gene were used: *D10S1687, D10S1765, D10S541* and *D10S583* (see Figure 6 for map positions). Products were separated on a denaturing polyacrylamide gel and visualized by exposure to X ray film.

Array-based-CGH

The human 3600 BAC/PAC genomic clone set, covering the full genome at 1 Mb-spacing for array production was obtained from the Sanger Institute (see Figure 6 for BACs mapping in and close to *PTEN*). Degenerated oligonucleotide PCR-products were prepared for spotting on CodeLink® slides (Amersham Biosciences) according to Fiegler [29] with some modifications [30]. DNA labeling and hybridization were performed essentially as described [29]. Briefly, 450 ng of test or reference DNA, 60 µl1x Random primer solution (Invitrogen) and water was heated for 10 min at 100 °C, and subsequently cooled on ice. After the addition of 15 µl dNTP mix (1 mM dCTP, 2 mM dATP, 2 mM dGTP and 2 mM dTTP), 2µl of 1mM Cy3- or Cy5-dCTP (test and reference respectively) (Amersham Biosciences) and 120 U Klenow fragment (Invitrogen), the mixture was gently mixed and incubated overnight at 37 °C. In one tube the Cy3-test sample and Cy5-reference sample were mixed with 125 µg Cot-I DNA (Invitrogen), 42.5 µl 3 M NaAc. Next, 1 ml cold 100% ethanol was added. In a second tube, 40 µg denatured herring sperm was mixed with 125 µg human Cot-I DNA, 16.5 µl 3 M NaAc and 400 µl cold 100% ethanol. After gentle mixing, the mixes were precipitated for 3 h at –20 °C. Hybridizations of arrays were performed as described [29]. Following hybridization, slides were washed for 15 min at 48 °C in 50% formamide/2x SSC, for 30 min at 48 °C in 2 x SSC/0.1% SDS, for 10-15 min at room temperature in 0.1 M sodium phosphate, 0,1% NP40, and dipped in ddH_2O, before drying by centrifugation for 3 min at 750 x *g*. The arrays were scanned in a ScanArray Express HT (Perkin Elmer, Boston, MA). The resulting TIFF images were analyzed with GenePix Pro 5.0 software (Axon Instruments) and subsequently analyzed and visualized with an excel macro [30].



Positions from http://genome.ucsc.edu/ (May 2004) and http://www.ensembl.org/

Figure 6. Overview of the *PTEN* region on chromosome 10q23.3. The upper part of the figure shows the position of *PTEN* in Mb from the top of 10p. Below are indicated the positions of the PAC (190P6) utilized for FISH, the BAC clones encompassing and flanking *PTEN* in the BAC array, and the positions of the markers utilized in allelotype analysis.

Wild-type/Pseudogene Ratio analysis (WPR)

WPR is based on the simultaneous PCR amplification, from identical primers, of an exon 9 fragment of *PTEN* that maps on chromosome 10q23 and the corresponding sequence of the highly homologous *PTEN* pseudogene on 9p. The amplified fragments contain a single nucleotide difference between *PTEN* (T) and *ψPTEN* (C), resulting in a double peak in the sequence plot at the mismatch position. Because in most prostate cancers chromosome 9p is normal [31], the T/C ratio is indicative for the *PTEN* copy number.

Fifty ng DNA was amplified using Taq polymerase (Promega, Madison, WI). Amplification settings were 35 cycles of 1 min at 95 ºC, 1 min at 50 ºC and 1 min at 72 ºC using a T1 thermocycler (Biometra, Goettingen, Germany). PTEN exon 9 primers: PTENex9F2: 5'-TAGTGACAATGAACCTGATCA–3' and PTENex9R: 5'-GGTAATCTGACACAATGTCCTA–3'. The PCR products were loaded on a 1% agarose gel, amplified 135 bp fragments were isolated from gel and purified using Qiaquick gel extraction columns according to the manufacturer's description (Qiagen, Hilden, Germany). Fragments were sequenced using the ABI Prism BigDye terminator v3.1 ready reaction cycle sequencing kit (Applied Biosystems, Foster City, CA) and primer PTENex9F2 (25 cycles of 96 °C for 10 sec, 50 °C for 5 sec, 60 °C for

4 min (ramping 1 °C/sec). Sequence reactions were run on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). To determine the *PTEN* copy number the T/C ratio at the mismatch position in the amplified fragments was corrected for differences in T and C signal intensity, based on the average signal of flanking T and C sequences. T/C< 0.4 was indicative of bi-allelic *PTEN* loss, 0.4 ≤ T/C < 0.8 as mono-allelic *PTEN* loss, and T/C ≥ 0.8 as allele retention.

Dual color fluorescent in situ hybridization

Frozen tissue sections (4 μm) were fixed on microscope slides treated with 3-aminopropyltriethoxysilane (Sigma, St. Louis, MO). The slides were air-dried at room temperature for 1 h, then submerged in methanol-acetone (1:1, v/v) at –20 °C for 20 min, washed twice in PBS containing 0.5% (v/v) Tween 20 (Sigma). Subsequently, tissue sections were treated with 0.0005% pepsin (Sigma) in 0.01 M HCl for 3 min at 37 °C, washed 5 times in water for 1 min and 5 times in PBS for 1 min. The slides were post-fixed in 1% formaldehyde in PBS for 10 min, washed 5 times in PBS for 1 min and 5 times in water for 1 min, dehydrated in an increasing ethanol series (70%, 80%, 90% and 100%, 5 min each) and air dried. The probes used were labeled by nick translation and hybridized essentially as described [32]. The chromosome 10 centromere probe pα10 RR8 was lissamin-11-dUTP (Roche, Mannheim, Germany) labeled and PAC 190P6, containing *PTEN* exons 3 to 9 inclusive (Figure 6; Genome Systems, St Louis, MO), was digoxigenin-11-dUTP labeled and visualized with FITC-conjugated sheep antidigoxigenin (Roche). To verify the results, the labels of the probes were interchanged. Labeled probes (100 ng each) and 0.5 μg Cot1 DNA were dissolved in 10 μl hybridization mixture containing 50% formamide/2xSCC/10% dextransulfate. Results were visualized using a Leica DM fluorescent microscope with a DAPI/FITC/Cy3 triple band-pass filter.

PTEN mutation analysis by single-strand conformation polymorphism and sequencing

Fragments for PCR-SSCP analysis were obtained for all *PTEN* exons using primer sets and PCR conditions as described previously [15]. Amplification reactions were in a 15 μl volume in the presence of 1 μCi [α^{32}P]dATP. The products were separated over a 6% non-denaturing polyacrylamide gel containing 5 or 10% glycerol at 7 W overnight at room temperature. Sequencing of samples containing an aberrant band was performed as described above (WPR analysis).

Immunohistochemistry

The mouse monoclonal anti-human PTEN antibody (Clone 6H2.1, Cascade Bioscience, Winchester, MA) was used for immunohistochemistry on formalin-fixed, paraffin- embedded tissue sections. The sections were pre-treated by a modified heat-induced antigen-retrieval method [33] in 10 mM citrate buffer, pH 6.0. PTEN antibody (1:200) incubation was overnight at room temperature. As a secondary antibody, biotinylated rabbit-anti-mouse was used (DAKO, Glostrup, Denmark). Signals were visualized using the avidin-biotin-Horse Radish Peroxidase complex (DAKO). PTEN staining was independently scored by 2 observers (ThHvdK, PCMSV). The cytoplasmic staining intensity was estimated as absent (0), weak (1), normal (2) or strong (3). In case of heterogeneity, the range was indicated.

References

1. Jemal, A., Murray, T., Ward, E., Samuels, A., Tiwari, R.C., Ghafoor, A., Feuer, E.J.,Thun, M.J. (2005) Cancer statistics, 2005 *CA Cancer J Clin*, **55,** 10-30.
2. Verhagen, P.C., Tilanus, M.G., de Weger, R.A., van Moorselaar, R.J., van den Tweel, J.G.,Boon, T.A. (2002) Prognostic factors in localised prostate cancer with emphasis on the application of molecular techniques *Eur Urol*, **41,** 363-371.
3. Liaw, D., Marsh, D.J., Li, J., Dahia, P.L., Wang, S.I., Zheng, Z., Bose, S., Call, K.M., Tsou, H.C., Peacocke, M., et al. (1997) Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome *Nat Genet*, **16,** 64-67.
4. Li, J., Yen, C., Liaw, D., Podsypanina, K., Bose, S., Wang, S.I., Puc, J., Miliaresis, C., Rodgers, L., McCombie, R., et al. (1997) PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer *Science*, **275,** 1943-1947.
5. Steck, P.A., Pershouse, M.A., Jasser, S.A., Yung, W.K., Lin, H., Ligon, A.H., Langford, L.A., Baumgard, M.L., Hattier, T., Davis, T., et al. (1997) Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers *Nat Genet*, **15,** 356-362.
6. Cantley, L.C. and Neel, B.G. (1999) New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway *Proc Natl Acad Sci U S A*, **96,** 4240-4245.
7. Trotman, L.C., Niki, M., Dotan, Z.A., Koutcher, J.A., Di Cristofano, A., Xiao, A., Khoo, A.S., Roy-Burman, P., Greenberg, N.M., Van Dyke, T., et al. (2003) Pten dose dictates cancer progression in the prostate *PLoS Biol*, **1,** E59.
8. Ma, X., Ziel-van der Made, A.C., Autar, B., van der Korput, H.A., Vermeij, M., van Duijn, P., Cleutjens, K.B., de Krijger, R., Krimpenfort, P., Berns, A., et al. (2005) Targeted biallelic inactivation of Pten in the mouse prostate leads to prostate cancer accompanied by increased epithelial cell proliferation but not by reduced apoptosis *Cancer Res*, **65,** 5730-5739.
9. Wang, S., Gao, J., Lei, Q., Rozengurt, N., Pritchard, C., Jiao, J., Thomas, G.V., Li, G., Roy-Burman, P., Nelson, P.S., et al. (2003) Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer *Cancer Cell*, **4,** 209-221.
10. Cairns, P., Okami, K., Halachmi, S., Halachmi, N., Esteller, M., Herman, J.G., Jen, J., Isaacs, W.B., Bova, G.S.,Sidransky, D. (1997) Frequent inactivation of PTEN/MMAC1 in primary prostate cancer *Cancer Res*, **57,** 4997-5000.
11. Pesche, S., Latil, A., Muzeau, F., Cussenot, O., Fournier, G., Longy, M., Eng, C.,Lidereau, R. (1998) PTEN/MMAC1/TEP1 involvement in primary prostate cancers *Oncogene*, **16,** 2879-2883.

12. Dong, J.T., Sipe, T.W., Hyytinen, E.R., Li, C.L., Heise, C., McClintock, D.E., Grant, C.D., Chung, L.W.,Frierson, H.F., Jr. (1998) PTEN/MMAC1 is infrequently mutated in pT2 and pT3 carcinomas of the prostate *Oncogene*, **17,** 1979-1982.
13. Wang, S.I., Parsons, R.,Ittmann, M. (1998) Homozygous deletion of the PTEN tumor suppressor gene in a subset of prostate adenocarcinomas *Clin Cancer Res*, **4,** 811-815.
14. Suzuki, H., Freije, D., Nusskern, D.R., Okami, K., Cairns, P., Sidransky, D., Isaacs, W.B.,Bova, G.S. (1998) Interfocal heterogeneity of PTEN/MMAC1 gene alterations in multiple metastatic prostate cancer tissues *Cancer Res*, **58,** 204-209.
15. Vlietstra, R.J., van Alewijk, D.C., Hermans, K.G., van Steenbrugge, G.J.,Trapman, J. (1998) Frequent inactivation of PTEN in prostate cancer cell lines and xenografts *Cancer Res*, **58,** 2720-2723.
16. Dong, J.T., Li, C.L., Sipe, T.W.,Frierson, H.F., Jr. (2001) Mutations of PTEN/MMAC1 in primary prostate cancers from Chinese patients *Clin Cancer Res*, **7,** 304-308.
17. Feilotter, H.E., Nagai, M.A., Boag, A.H., Eng, C.,Mulligan, L.M. (1998) Analysis of PTEN and the 10q23 region in primary prostate carcinomas *Oncogene*, **16,** 1743-1748.
18. McMenamin, M.E., Soung, P., Perera, S., Kaplan, I., Loda, M.,Sellers, W.R. (1999) Loss of PTEN expression in paraffin-embedded primary prostate cancer correlates with high Gleason score and advanced stage *Cancer Res*, **59,** 4291-4296.
19. Halvorsen, O.J., Haukaas, S.A.,Akslen, L.A. (2003) Combined Loss of PTEN and p27 Expression Is Associated with Tumor Cell Proliferation by Ki-67 and Increased Risk of Recurrent Disease in Localized Prostate Cancer *Clin Cancer Res*, **9,** 1474-1479.
20. Hermans, K.G., van Alewijk, D.C., Veltman, J.A., van Weerden, W., van Kessel, A.G.,Trapman, J. (2004) Loss of a small region around the PTEN locus is a major chromosome 10 alteration in prostate cancer xenografts and cell lines *Genes Chromosomes Cancer*, **39,** 171-184.
21. Gray, I.C., Stewart, L.M., Phillips, S.M., Hamilton, J.A., Gray, N.E., Watson, G.J., Spurr, N.K.,Snary, D. (1998) Mutation and expression analysis of the putative prostate tumour-suppressor gene PTEN *Br J Cancer*, **78,** 1296-1300.
22. Whang, Y.E., Wu, X., Suzuki, H., Reiter, R.E., Tran, C., Vessella, R.L., Said, J.W., Isaacs, W.B.,Sawyers, C.L. (1998) Inactivation of the tumor suppressor PTEN/MMAC1 in advanced human prostate cancer through loss of expression *Proc Natl Acad Sci U S A*, **95,** 5246-5250.
23. Konishi, N., Nakamura, M., Kishi, M., Nishimine, M., Ishida, E.,Shimada, K. (2002) Heterogeneous methylation and deletion patterns of the INK4a/ARF locus within prostate carcinomas *Am J Pathol*, **160,** 1207-1214.
24. Abate-Shen, C., Banach-Petrosky, W.A., Sun, X., Economides, K.D., Desai, N., Gregg, J.P., Borowsky, A.D., Cardiff, R.D.,Shen, M.M. (2003) Nkx3.1; Pten mutant mice develop invasive prostate adenocarcinoma and lymph node metastases *Cancer Res*, **63,** 3886-3890.

25. Kwabi-Addo, B., Giri, D., Schmidt, K., Podsypanina, K., Parsons, R., Greenberg, N.,Ittmann, M. (2001) Haploinsufficiency of the Pten tumor suppressor gene promotes prostate cancer progression *Proc Natl Acad Sci U S A*, **98,** 11563-11568.

26. You, M.J., Castrillon, D.H., Bastian, B.C., O'Hagan, R.C., Bosenberg, M.W., Parsons, R., Chin, L.,DePinho, R.A. (2002) Genetic analysis of Pten and Ink4a/Arf interactions in the suppression of tumorigenesis in mice *Proc Natl Acad Sci U S A*, **99,** 1455-1460.

27. Di Cristofano, A., De Acetis, M., Koff, A., Cordon-Cardo, C.,Pandolfi, P.P. (2001) Pten and p27KIP1 cooperate in prostate cancer tumor suppression in the mouse *Nat Genet*, **27,** 222-224.

28. Sambrook, J. and Russel, D.W., *Molecular cloning, a laboratory manual*. Third edition ed. 2001, Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

29. Fiegler, H., Carr, P., Douglas, E.J., Burford, D.C., Hunt, S., Scott, C.E., Smith, J., Vetrie, D., Gorman, P., Tomlinson, I.P., et al. (2003) DNA microarrays for comparative genomic hybridization based on DOP-PCR amplification of BAC and PAC clones *Genes Chromosomes Cancer*, **36,** 361-374.

30. Knijnenburg, J., Szuhai, K., Giltay, J., Molenaar, L., Sloos, W., Poot, M., Tanke, H.J.,Rosenberg, C. (2005) Insights from genomic microarrays into structural chromosome rearrangements *Am J Med Genet A*, **132,** 36-40.

31. van Dekken, H., Paris, P.L., Albertson, D.G., Alers, J.C., Andaya, A., Kowbel, D., van der Kwast, T.H., Pinkel, D., Schroder, F.H., Vissers, K.J., et al. (2004) Evaluation of genetic patterns in different tumor areas of intermediate-grade prostatic adenocarcinomas by high-resolution genomic array analysis *Genes Chromosomes Cancer*, **39,** 249-256.

32. Mostert, M.C., Verkerk, A.J., van de Pol, M., Heighway, J., Marynen, P., Rosenberg, C., van Kessel, A.G., van Echten, J., de Jong, B., Oosterhuis, J.W., et al. (1998) Identification of the critical region of 12p over-representation in testicular germ cell tumors of adolescents and adults *Oncogene*, **16,** 2617-2627.

33. Shi, S.R., Key, M.E.,Kalra, K.L. (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immunohistochemical staining based on microwave oven heating of tissue sections *J Histochem Cytochem*, **39,** 741-748.

Appendix I – Color figures chapter 4

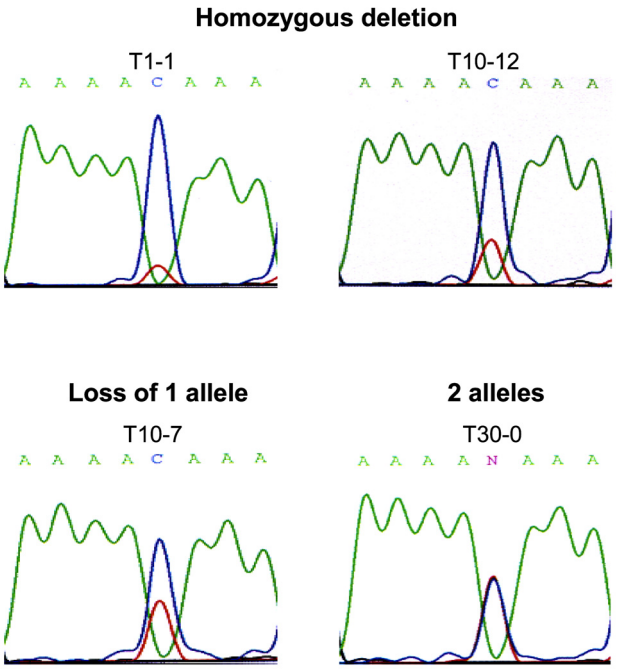


Figure 3. Examples of *PTEN* exon 9 analyzed by WPR. T10-7 contains one *PTEN* allele. T1-1 and T10-12 contain a homozygous deletion of *PTEN*. T30-0 shows the presence of two *PTEN* alleles.

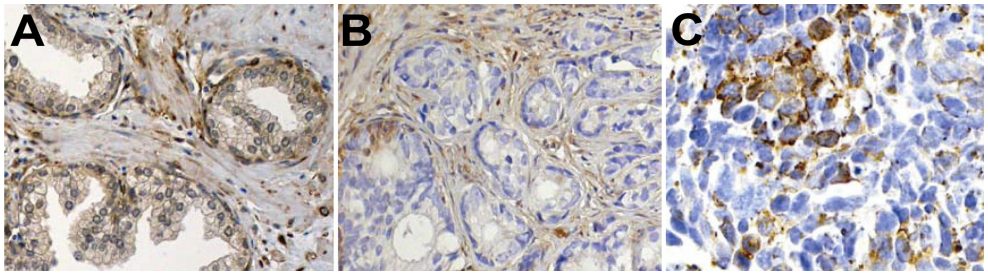


Figure 5. Immunohistochemical staining of PTEN (brown) in (A) a positive control prostate. (B) Absence of PTEN expression in the tumor cells in T10-12. Note PTEN expression in stromal cells. (C) Heterogeneous cytoplasmic staining with locally complete absence of PTEN expression in prostate tumor T1-8.

Chapter 5

Quantitative Wild-type to Pseudogene Ratio analysis (q-WPR) is an accurate method to detect gene copy number changes

Petra W. van Duijn[1], Karin G. L. Hermans[1], Wytske M. van Weerden[2] and Jan Trapman[1]

[1]Department of Pathology, and [2]Department of Urology, Josephine Nefkens Institute, Erasmus University Medical Center, Rotterdam, The Netherlands

Submitted

Abstract

The cancer genome is characterized by various structural alterations. Here, we describe a simple and accurate method, denoted quantitative Wild-type to Pseudogene Ratio (q-WPR) analysis, to detect gene copy number changes, utilizing *PTEN* in prostate cancer as a model system. Q-WPR applies a highly homologous pseudogene as an internal control, in a single step PCR based assay with one primer set, to assess the copy number of the corresponding functional gene. This omits the need for normal DNA and unrelated internal reference markers as controls. In many tumors, including prostate cancer, *PTEN* is frequently lost by small deletions. *PTEN* maps to 10q23 and its closely related pseudogene *ψPTEN* on 9p13. In q-WPR, simultaneously amplified *PTEN* and *ψPTEN* fragments are discriminated by annealing to gene specific fluorescent-tagged oligonucleotide probes. *PTEN* exon 5 and 6 specific assays were developed and subsequently validated on both normal DNAs and on DNA from previously genotyped prostate cancer cell lines and xenografts. Next, the assays were tested on clinical prostate cancer samples. Q-WPR detected mono- and bi-allelic losses of *PTEN* and was applicable on genomic DNA from frozen and formalin-fixed tumor tissues. The method will allow very accurate and simple high-throughput analysis of gene copy number changes of any gene with a highly homologous structurally related gene.

Introduction

Genetic alterations like mutations, translocations and gene copy number changes are characteristic features of various genetic disorders, including cancer. During the last decades many different technologies, varying from genome wide approaches to small scale gene-specific methods, have been developed to analyze changes of gene copy numbers [1-3]. Most recently, sophisticated array-CGH (Comparative Genomic Hybridization) and high density SNP (Single Nucleotide Polymorphism) arrays have been generated, allowing high-throughput genotyping of the whole genome [4-7].

Other techniques to determine gene copy numbers, like loss of heterozygosity [8] of polymorphic microsatellite markers are more suited for screening of specific, small chromosomal regions [9]. Recently developed Multiplex Ligation-dependent Probe Amplification (MLPA) can detect semi-quantitatively gene copy number changes simultaneously in up to 40 loci [1,10]. For the detection of amplification and losses of

specific genes in individual cells laborious interphase Fluorescent In Situ Hybridization (FISH) is the most frequently applied method [11,12].

In the present study we describe a novel, high-throughput assay, denoted quantitative Wild-type to Pseudogene Ratio analysis (q-WPR), to detect copy number changes of individual genes. Q-WPR applies a highly homologous pseudogene as an internal control to determine the copy number of the corresponding functional gene. Wild-type gene and pseudogene fragments are simultaneously amplified and discriminated by specific fluorescent-labeled oligonucleotide probes that hybridize to their target sequence during the amplification. In q-WPR reference normal control DNAs and unrelated internal controls can be omitted.

Pseudogenes are highly similar to their corresponding functional counterparts but are generally not transcribed or translated to functional proteins due to the lack of functional promoters or other regulatory elements and the presence of premature stop codons and frameshift mutations [13]. Retrotransposition and duplication of genomic DNA are the two major mechanisms giving rise to pseudogenes [13,14]. Non-processed pseudogenes often have retained the original exon-intron structures of the parental gene because they arise from genomic DNA duplication or unequal crossing over and are thus mostly located at the same chromosomal site as their functional counterpart. Retro-pseudogenes or processed pseudogenes have arisen from the reverse transcription of a mRNA transcript followed by random integration of the resulting cDNA into the genome. Mammalian genomes contain a large number of pseudogenes of which processed pseudogenes form the majority. In the human genome the number of processed pseudogenes is estimated to be 3,600 – 20,000 depending on the search methodology and stringency used [14-18]. Approximately 10% of cellular genes are thought to have at least one retrotransposed pseudogene [14,16,18]. Genes encoding ribosomal proteins, housekeeping genes like GAPDH and genes that code for metabolic enzymes frequently have multiple processed pseudogenes [13,14]. This has been ascribed to the high mRNA expression level of these genes, thereby increasing the chances of generating a processed pseudogene.

In this study we utilized the tumor suppressor *PTEN* located on chromosomal band 10q23 and its pseudogene *ψPTEN* on 9p13 to develop and validate the q-WPR assay. PTEN antagonizes the action of PI3K, in regulating the intracellular PIP3 concentration, thereby negatively regulating important biological processes

like cell growth, proliferation and survival [19]. The *PTEN* gene is frequently inactivated in various human tumors including prostate cancer [20-22]. The assessment of *PTEN* copy number changes requires sensitive methods because *PTEN* loss might involve only small genomic regions [23]. In most prostate cancers chromosome 9p copy number is unchanged, allowing the use of ψ*PTEN* as internal control [24,25]. Our data show that q-WPR is able to detect homozygous deletions and mono-allelic losses of *PTEN*. Q-WPR requires only small amounts of DNA and is suitable for fragmented DNA samples. In combination with the high-throughput format of the assay this method is very useful for the screening of large series of archival formalin-fixed paraffin-embedded patient material, allowing correlations of gene copy number changes with long-term clinical follow-up.

Results

Principle of q-WPR analysis

Q-WPR is a novel high-throughput method to detect gene copy number changes, modified from the 5'-nuclease Taqman assay [26]. The q-WPR method makes use of small structural differences between two further highly identical genes, e.g. a functional gene and a corresponding pseudogene located on a different chromosome (see Materials and Methods and Figure 1A). Importantly, amplification primers perfectly match both the normal wild-type gene and its pseudogene. Fluorescent-labeled wild-type (FAM) and pseudogene (VIC) specific probes are used to discriminate between the two simultaneously amplified gene fragments (Figure 1A). Genotypes are then assessed based on the ratio between the wild-type gene and the pseudogene fluorescence that is generated during the PCR amplification. These ratios are depicted by fluorescence clustering patterns in a bivariate plot (Figure 1A, right panel).

Q-WPR analysis can detect gene copy number changes of virtually every gene with a corresponding closely related gene and may be widely applicable in genetic screening and diagnostic studies. In cancer diagnostics q-WPR can be used to score (high level) amplifications of oncogenes and loss of tumor suppressor genes, both common features of the cancer genome. Amplification of genes causes an excess of wild-type gene fluorescence (Figure 1A, right panel). Specific clusters that represent mono- and bi-allelic loss of the wild-type gene will be obtained if loss of a tumor suppressor gene is assayed (Figure 1A, right panel).

Assay development

In this study a q-WPR assay was developed for the *PTEN* tumor suppressor gene located at 10q23 and its highly homologous processed pseudogene *ψPTEN* that maps to 9p13. In prostate cancer loss of one or two copies of *PTEN* is frequently observed allowing the use of prostate cancer cell lines, xenografts and clinical prostate cancer specimens for the testing and validation of the assay [20,23]. Due to the absence of introns in the processed pseudogene primers and probes must be designed on exon sequences. *PTEN* is composed of 9 exons and the coding sequence differs at nineteen positions from *ψPTEN*. The exons 1-4 and exon 9 of PTEN were excluded from the assay design because specific hybridization probes could not be developed for these exons. To increase reliability, q-WPR assays were developed for two exons of *PTEN*, exon 5 and exon 6. Previously, it was shown for prostate cancer xenograft DNAs that loss of *PTEN* always affected exon 5 [20]. Exon 6 was chosen as a second assay because it shows the least homology with mouse *Pten* at the position of the probe, avoiding possible false genotyping of human tumors transplanted on mice (compare mouse controls, Figure 2A). Figure 1B shows the alignment between *PTEN* exon 5 and exon 6 and *ψPTEN* sequences, and the localization of the primers and probes.

To test the specificity of the probes, we generated separate *PTEN* and *ψPTEN* exon 5 and exon 6 specific fragments by molecular cloning of PCR amplified fragments. In both the *PTEN* exon 5 and exon 6 assay the probes were found to be specific for either *PTEN* or *ψPTEN* as indicated by the green and red clusters of spots (Figure 1C, and data not shown). The yellow cluster of spots represents equally mixed *PTEN* and *ψPTEN* specific fragments, as present in normal genomic DNA (Figure 1C). In case of 1:2 (*PTEN:ψPTEN*) mixed fragments, representing mono-allelic loss of the wild-type gene, the fluorescent signal had shifted towards the *ψPTEN* control (Figure 1C). Taken together, our data showed that the designed probes were *PTEN* and *ψPTEN* specific and that separate genotype clusters could be detected.

Next, a set of 20 normal genomic DNAs was tested in duplicate to verify the specificity and reproducibility of the assay. In both exon 5 and exon 6 *PTEN* assays all normal DNAs clustered together (Figure 1D, and data not shown). In addition, the normal genomic DNAs clustered together with the equally mixed *PTEN* and *ψPTEN* controls in both assays.

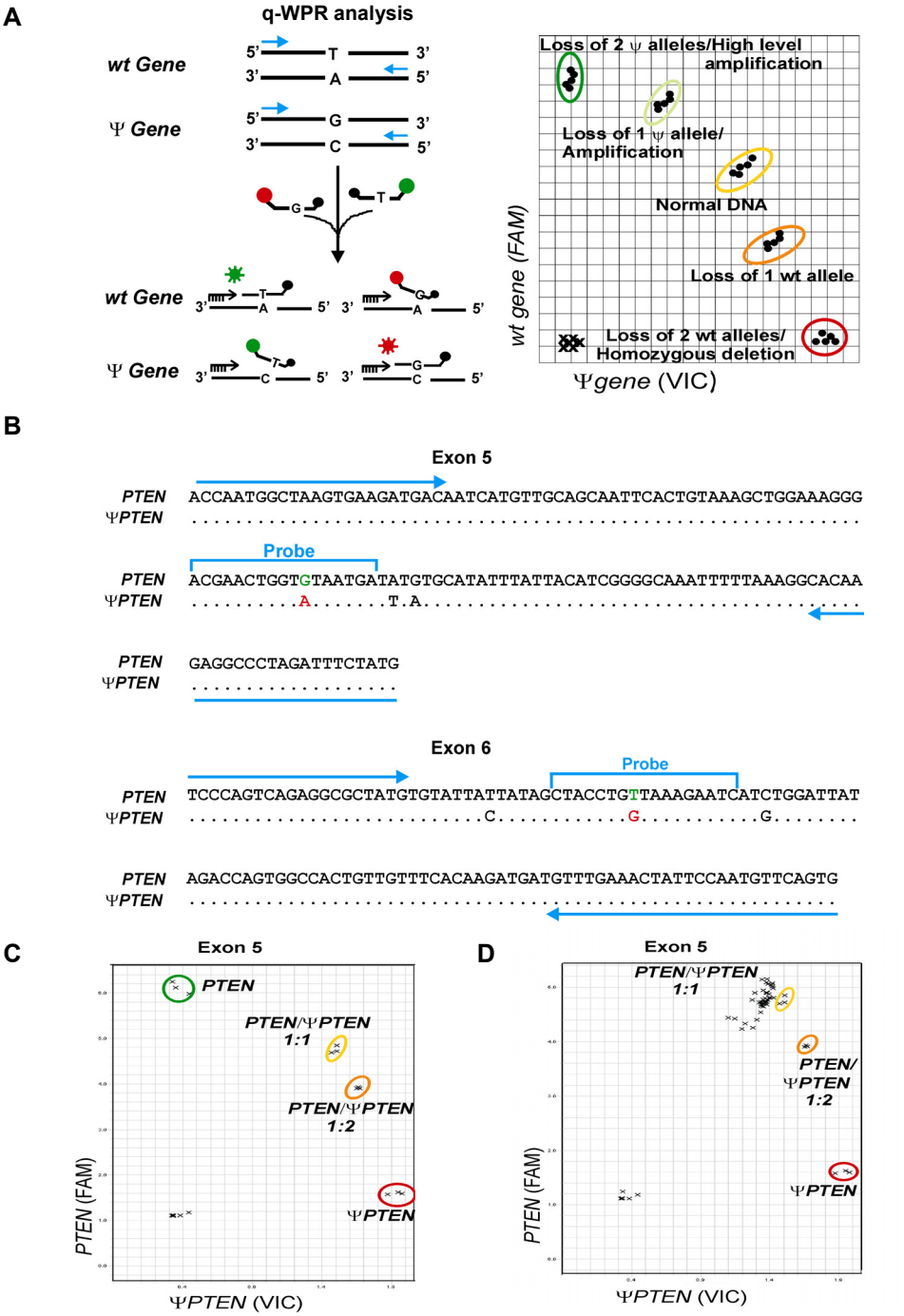


Figure 1. Principle of q-WPR analysis and PTEN specific assay development. (A) Schematic overview of q-WPR analysis. Primers are designed to perfectly match both the wild-type gene and the pseudogene sequence. Wild-type and pseudogene specific probes are respectively FAM and VIC

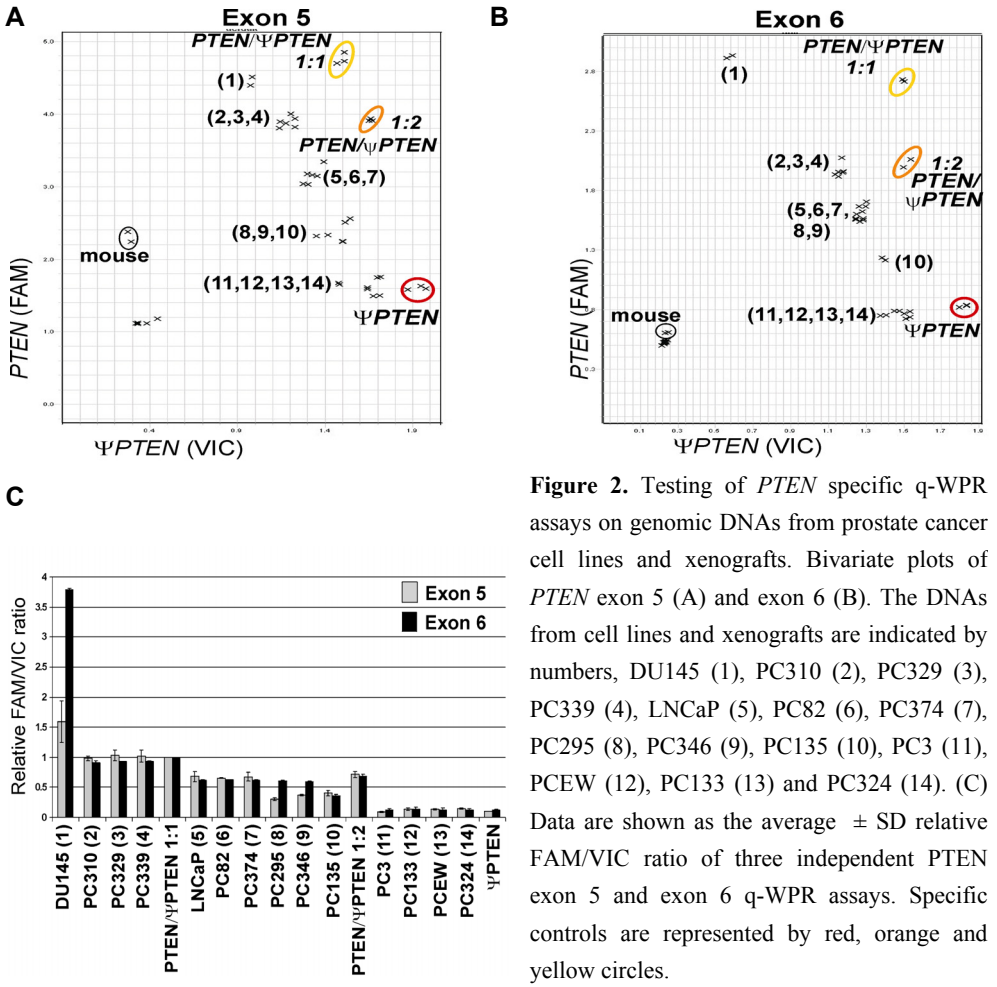


Figure 2. Testing of *PTEN* specific q-WPR assays on genomic DNAs from prostate cancer cell lines and xenografts. Bivariate plots of *PTEN* exon 5 (A) and exon 6 (B). The DNAs from cell lines and xenografts are indicated by numbers, DU145 (1), PC310 (2), PC329 (3), PC339 (4), LNCaP (5), PC82 (6), PC374 (7), PC295 (8), PC346 (9), PC135 (10), PC3 (11), PCEW (12), PC133 (13) and PC324 (14). (C) Data are shown as the average ± SD relative FAM/VIC ratio of three independent PTEN exon 5 and exon 6 q-WPR assays. Specific controls are represented by red, orange and yellow circles.

labeled. During the amplification reaction fluorescent signals are generated proportional to the copy number of the gene. Different genotypes can be identified based on the fluorescence clustering patterns in a bivariate plot (right panel). (B) Sequence alignment of *PTEN* and *ψPTEN* exon 5 and exon 6. Nucleotide differences between *PTEN* and *ψPTEN* are indicated. Arrows indicate the location of the primers, the hybridization probe sequences are also indicated. (C) *PTEN* and *ψPTEN* exon 5 specific controls were used separately and in both a 1:1 (normal DNA) and 1:2 (loss of one wild-type allele) ratio to test probe specificity and separation of the different genotype clusters. FAM and VIC fluorescence intensities are depicted on the y-axis and the x-axis, respectively. (D) For the exon 5 assay twenty normal genomic DNAs were tested in duplicate together with the fragment specific controls representing a homozygous *PTEN* deletion (*ψPTEN* fragment only), loss of one wild-type *PTEN* allele (1:2 ratio) and normal DNA (1:1 ratio). Bivariate plot of a representative experiment is shown. Water was used as non-template control.

Validation of q-WPR analysis

To test q-WPR on tumor DNAs we applied the *PTEN* exon 5 and exon 6 assays on 14 prostate cancer cell lines and xenografts that were previously genotyped for *PTEN* by allelotype analysis and array-CGH [20,23]. The xenograft PC295 is of particular interest because this xenograft contains a small homozygous deletion of *PTEN* exon 5, but loss of only one copy of exon 6. In Figure 2A,B the results of the q-WPR analysis are shown. The red, orange and yellow control clusters represent homozygous *PTEN* deletion, mono-allelic loss of *PTEN* and retention of both alleles, respectively. In both assays, most prostate cancer cell lines and xenografts clustered together with either one of the controls. Samples 2, 3 and 4 (PC310, PC329 and PC339) showed a 1:1 *PTEN/ψPTEN* ratio; 5, 6 and 7 (LNCaP, PC82 and PC374) a 1:2 *PTEN/ψPTEN* ratio; samples 11, 12, 13 and 14 (PC3, PCEW, PC133 and PC324) generated only a *ψPTEN* fluorescence signal. In Table 1 the previously by array-CGH established *PTEN* copy numbers of all investigated xenografts are summarized [23].

To better compare the exon 5 and exon 6 data the *PTEN/ψPTEN* (FAM/VIC) ratio was determined for each cell line and xenograft, and normalized to the equally mixed control. In Figure 2C the average FAM/VIC ratios of three independent experiments are shown. Both assays confirmed the previously found homozygous *PTEN* deletions for PCEW, PC133, PC324 and PC3. Also the mono-allelic loss found in PC82, PC374 and LNCaP, and the normal genotype of PC310, PC329 and PC339 corresponded with the known genotypes, as assayed by array-CGH (Table 1). In both assays the PC135 (sample 10) signal is located between the clusters of mono- and bi-allelic loss of *PTEN*. This xenograft is however, tetraploid and has lost 3 copies of *PTEN*, explaining the aberrant position of the PC135 signal. In case of the xenografts PC295 and PC346 (samples 8 and 9 respectively) the ratio obtained in the q-WPR exon 5 assay was significantly lower than the ratio found by exon 6 q-WPR analysis. As previously indicated, PC295 contains a small homozygous deletion of *PTEN* exon 5, explaining the difference between the *PTEN* exon 5 and exon 6 specific assays.

However, the FAM/VIC ratio of PC295 exon 5 is still higher compared to other samples with bi-allelic *PTEN* loss. At the wild-type probe binding site of *PTEN* exon 5 the mouse and human *PTEN* sequences are highly homologous and the relatively large contribution of mouse genomic DNA in this xenograft influences the outcome of the assay (compare FAM/VIC ratio of PC295 with mouse control,

Figure 2A). In PC346 the different results obtained in the *PTEN* exon 5 and exon 6 q-WPR assays are due to a CGA130TGA nonsense mutation present in exon 5 of *PTEN* that is located at the probe binding site, causing less efficient binding of the *PTEN* (FAM) probe. The significantly higher q-WPR ratio found in DU145 was due to amplification of *PTEN* as previously indicated by array-CGH (Table 1).

Table 1. *PTEN* copy number of prostate cancer cell lines and xenografts

Sample Number	Sample Name	PTEN copy number Array-CGH		PTEN copy number q-WPR	
		Exon 5	Exon 6	Exon 5	Exon6
1	DU145	3	3	>2	>2
2	PC310	2	2	2	2
3	PC329	2	2	2	2
4	PC339	2	2	2	2
5	LNCaP	1	1	1	1
6	PC82	1	1	1	1
7	PC374	1	1	1	1
8	PC295	1	1	<1[#]	1
9	PC346	1	1	<1[§]	1
10	PC135	1	1	<1[*]	<1
11	PC3	0	0	0	0
12	PCEW	0	0	0	0
13	PC133	0	0	0	0
14	PC324	0	0	0	0

[#] Contaminating mouse DNA in PC295 interferes with accurate copy number determination of *PTEN* exon 5

§ *PTEN* exon 5 of PC346 contains a CGA130TGA nonsense mutation at the probe binding site

* PC135 is tetraploid for chromosome 10 and has lost 3 copies of *PTEN*

Sensitivity of q-WPR analysis on DNA isolated from formalin-fixed, paraffin-embedded prostate cancer xenografts

Paraffin-embedded formalin-fixed tissue specimens stored in archives are very valuable for studying the pathogenesis of many diseases. However, DNA extracted from these tissue samples is often of poor quality, limiting the accuracy of genome

analysis. To investigate whether q-WPR is accurate on DNA from archived material, we tested six prostate cancer xenografts from which both fixed and frozen material of the same tumor was available. These xenografts were split at harvesting, one half was directly frozen, whereas the other half was formalin-fixed and embedded in paraffin. For both the *PTEN* exon 5 and exon 6 q-WPR assays comparable results were found for DNA extracted from fresh frozen samples and DNA isolated from fixed tissues (Figure 3A,B). Slightly higher FAM/VIC ratios of *PTEN* exon 5 of PC310 and PC295 were proven to be caused by the high percentage of mouse cells in these tissue samples (data not shown).

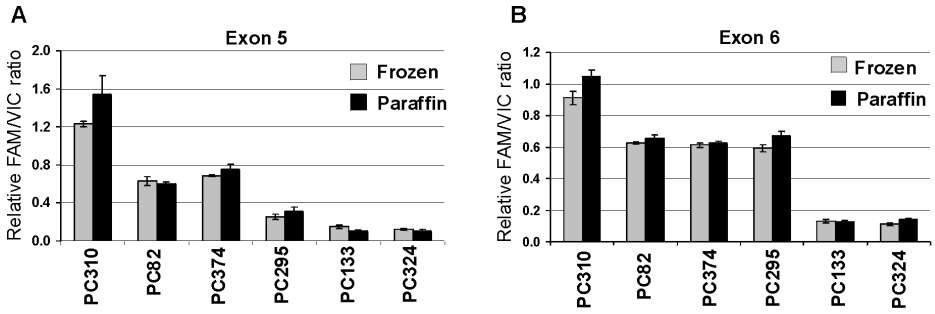


Figure 3. Application of q-WPR to DNA isolated from paraffin-embedded prostate cancer tissues. Xenograft material was split upon harvesting and either freshly frozen or formalin-fixed and paraffin-embedded. DNA was isolated in parallel from both frozen and fixed material from the same xenograft. The average relative FAM/VIC ratios ± SD of three independent PTEN exon 5 (A) and exon 6 (B) q-WPR assays are shown.

Analysis of clinical prostate cancers

Next, q-WPR analysis was applied on clinical prostate cancer samples. The bivariate plots of the PTEN exon 5 and exon 6 q-WPR assays (Figure 4A,B) indicated the presence of different genotype clusters. The relative FAM/VIC ratios suggested a homozygous PTEN deletion in T1-1 and T1-7, mono-allelic loss in T1-8, T2-9 and T8-4 and retention of both PTEN alleles in T1-4, T7-11 and T10-15 (Figure 4C). Following q-WPR analysis array-CGH was performed on these clinical samples. In T1-1 and T1-7, array-CGH detected a homozygous deletion for PTEN, thereby confirming the q-WPR results. As an example the array-CGH profile of T1-7 is shown, indicating no alterations in chromosome 9 and a clear homozygous deletion of the PTEN region at 10q (Figure 4D). Array-CGH detected

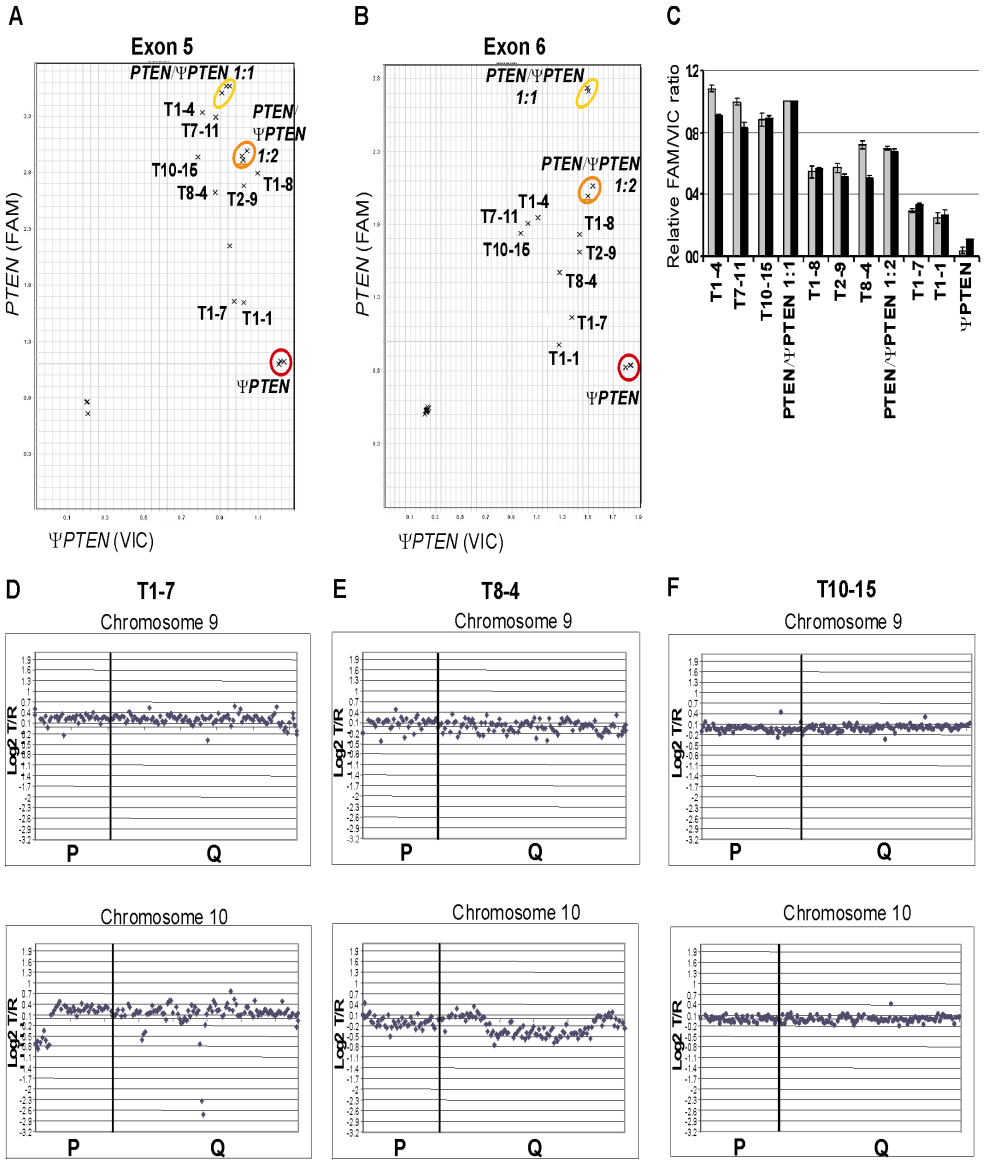


Figure 4. *PTEN* copy number analysis by q-WPR in clinical prostate cancer samples. Representative examples of a bivariate plot of the q-WPR *PTEN* exon 5 (A) and exon 6 (B) assay. Specific controls are depicted by red, orange and yellow circles indicating homozygous *PTEN* deletion, mono-allelic loss of *PTEN* and retention of both alleles, respectively. (C) The relative FAM/VIC ratio of three independent exon 5 and exon 6 q-WPR assays. Array-CGH was performed on all tumor DNAs and as examples the specific chromosome 9 and chromosome 10 profiles of T1-7 (D), T8-4 (E) and T10-15 (F) are shown, representing tumors with a homozygous *PTEN* deletion, mono-allelic loss of *PTEN* and retention of both *PTEN* alleles, respectively.

loss of one PTEN allele in T1-8, T2-9 and T8-4, as determined by q-WPR analysis. The array-CGH profile of T8-4 is shown as a representative example and indicates retention of chromosome 9 and loss of a large part of chromosome 10q, including the region where PTEN is located (Figure 4E). Q-WPR analysis suggested a normal PTEN genotype for T1-4, T7-11 and T10-15 that was confirmed by array-CGH (See T10-15, Figure 4F). Taken together q-WPR analysis is proven to be a fast, reproducible and reliable method to determine gene copy number changes even in clinical DNA samples and in DNA extracted from formalin-fixed and paraffin-embedded tissues.

Discussion

Gene deletions and amplifications are common features of the cancer genome [27]. Here we describe the development and validation of q-WPR analysis, a novel, accurate high-throughput method to determine gene copy number changes that has some unique advantages over existing genotyping techniques such as FISH, allelotype analysis [8], MLPA, array-CGH and SNP-arrays [4,5,10,11]. One major advantage is that a corresponding normal reference is not required because a pseudogene is used as internal control. Moreover, only a minimal quantity (<20 ng) of genomic DNA is required for each q-WPR assay, which is important when limited amounts of clinical materials are available. Further, q-WPR is applicable to DNA extracted from formalin-fixed paraffin-embedded tissue (Figure 3), which is often fragmented thereby affecting the efficiency of a PCR reaction [28].

Genome wide approaches to detect copy number changes like array-CGH and SNP arrays frequently use amplified genomic DNA, which involves the risk of increasing differences in DNA representation [29]. Due to the small sample size required for q-WPR analysis this amplification step can be omitted. The recently developed MLPA technique that can determine copy number changes of up to 40 loci in one PCR based assay [1,10] also requires only small amounts of DNA per assay but compared to q-WPR analysis MLPA still requires normal DNA controls, is more time consuming and less quantitative. In addition, MLPA is a multi-step assay whereas q-WPR involves only one step thereby minimizing the risk of sample cross contamination. For the detection of single gene copy number changes FISH is the most frequently used method. However, compared to q-WPR analysis FISH is much more laborious and has a much lower sample throughput. Recently, the Paralogue Ratio Test (PRT) has been described, based on the previously

published paralogous sequence quantification (PSQ) used in the diagnosis of trisomy [30,31]. Like q-WPR, PSQ and PRT are based on the simultaneous amplification of both test and reference loci that are subsequently distinguished by minor internal sequence differences [30,31]. However, q-WPR does not require further sample processing minimizing the risk of contamination and is more quantitative. Obviously, as in other genotyping methods the percentage of contaminating normal DNA in clinical tumor samples might affect the outcome of the assay.

Q-WPR analysis is not restricted to *PTEN* but can be applied to determine copy number alterations of virtually every gene with one or more corresponding and closely related genes such as processed pseudogenes, depending on their sequence and chromosomal location. The number of processed pseudogenes associated with a given gene can vary from one to multiple copies. Approximately 10% of human genes is thought to have at least one retrotransposed pseudogene indicating the applicability of q-WPR [14,16,18,32]. The tumor suppressor gene *MKK4,* located on chromosome 17p11.2 with a corresponding pseudogene on chromosome Xq13.2, is also a suitable candidate for q-WPR analysis [33]. Homozygous deletions of *MKK4* have been reported in a subset of pancreatic, biliary and breast carcinomas [34,35].

Apart from screening for loss of tumor suppressor genes q-WPR can also be used for amplification analysis, as shown by the high relative FAM/VIC ratio (>1) found in DU145 cells (Figure 2), indicating *PTEN* amplification as confirmed by array-CGH (data not shown). A candidate gene for amplification analysis is *PIK3CA* located at 3q26.3, encoding the p110α subunit of PI3K. This gene is frequently amplified in various tumor types and a *PIK3CA* pseudogene was discovered at chromosome 22 [36-40]. Other possible candidates for amplification analysis by q-WPR are *MDM2* (12q15) and *CDK4* (12q14.1) and their respective pseudogenes on chromosome 2p24.1 and 1p21.1. The *MDM2* oncogene is often amplified in human cancers [41]. In addition, several tumor types show co-amplification of *MDM2* and *CDK4* [42-44], a feature that is used to distinguish lipomas from liposarcomas because histological diagnosis of these tumors is difficult [45,46].

In conclusion, q-WPR analysis has been shown to be a rapid and reliable method for the detection of gene copy number changes. Q-WPR can be very valuable in prognostic studies due to its high sample throughput and its suitability to be used on low quality DNA.

Materials and methods

Samples

The prostate cancer cell lines LNCaP, DU145 and PC3 were cultured under standard conditions. The prostate cancer xenografts PCEW, PC82, PC133, PC135, PC295, PC310, PC324, PC329, PC339, PC346 and PC374 were propagated on male nude mice, as previously described [47-49]. Clinical prostate cancer tissues were collected by TURP (Trans Urethral Resection of the Prostate). Collection of patient samples has been performed according to national legislation concerning ethical requirements. Use of these samples has been approved by the ErasmusMC Medical Ethics Committee according to the Medical Research Involving Human Subjects Act (MEC-2004-261).

DNA preparations

Genomic DNA from cell lines, and frozen, or formalin-fixed and paraffin-embedded xenograft tissues was isolated utilizing the Puregene system (Gentra Systems, Minneapolis, MN) according to the manufacturers description. Formalin-fixed and paraffin-embedded xenograft tissue (five 20 µm tissue slides) were minced and de-paraffinized by xylene treatment prior to DNA isolation.

Genomic DNA from clinical tumor tissues was isolated from five consecutive 5 µm cryostat sections containing at least 80% tumor. The isolation procedure included overnight proteinase K incubation at 55 ˚C, phenol extraction and ethanol precipitation. DNAs were dissolved in TE buffer (10 mM Tris HCl, pH 7.8, 1 mM EDTA). DNA was further purified by RNAse treatment.

The ECACC human random control DNA panel (HRC-1) was used as normal Genomic reference DNAs (Sigma, St. Louis, MO). These DNAs were extracted from lymphoblastoid cell lines obtained by EBV transformation of peripheral blood lymphocytes from fresh blood samples of healthy donors.

Generation of PTEN fragments

Fragments of wild-type *PTEN* exon 5 and exon 6 and the corresponding fragments in *ψPTEN*, containing the q-WPR primer and probe sequences were amplified by PCR on normal human genomic DNA (Sigma) as a template. Amplification settings were 35 cycles of 1 min at 95 ºC, 1 min at 50 ºC and 1 min at 72 ºC.

Amplification primers:
PTENex5F2: 5'-CCA CAG CTA GAA CTT ATC–3'
PTENex5R2: 5'-GTC TCT GGT CCT TAC TTC-3'
PTENex6F: 5'-TCC CAG TCA GAG GCG CTA TG-3'
PTENex6R2: 5'-TTC CGC CAC TGA ACA TTG G–3'.
PCR products were purified and inserted into pGEM-Teasy (Promega, Madison, WI). *PTEN* and *ψPTEN* fragments were separated by molecular cloning. Inserts were verified by sequencing utilizing the BigDye terminator v3.1 ready reaction cycle sequencing kit and the ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA).

Quantitative Wild-type to Pseudogene Ratio analysis (q-WPR)
Q-WPR analysis is a modification of the 5'-nuclease Taqman assay [26]. In Figure 1A, the principle of q-WPR, which is based on the simultaneous amplification of a gene and its corresponding pseudogene as an accurate internal standard, is illustrated. The single primer set perfectly matches both the wild-type and the pseudogene sequence, which in the amplified region differ at one or a few nucleotides. The either FAM or VIC fluorescently labeled wild-type or pseudogene specific hybridization probes, are complementary to an internal region of the PCR product, surrounding the single nucleotide difference between the wild-type gene and the pseudogene. The ratio of the wild-type and pseudogene fluorescent signals generated during the amplification is a direct measure for the copy number of the investigated gene.

Primers and probes for the *PTEN* specific q-WPR assays were designed with Primer Express software (Applied Biosystems). Reactions were performed in a total reaction volume of 5 µl containing 2,5 µl 2 x TaqMan Universal PCR master Mix (No Amperase UNG, Applied Biosystems), each primer at a final concentration of 900 nM, 200 nM of each probe and 20 ng genomic DNA. Thermal cycling parameters for the assay were 15 min 95 ˚C followed by 35 cycles for 15 sec at 95 ˚C and 1 min at 60 ˚C. After amplification fluorescence was measured in an ABI PRISM 7900HT Sequence Detection System and analyzed using SDS software version 2.2.2 (Applied Biosystems).
Fluorescent-labeled probes (Applied Biosystems):
PTENex5-wt FAM ACG AAC TGG TGT AAT GA-MGB
PTENex5-ψ VIC ACG AAC TGG TAT AAT GA-MGB

PTENex6-wt FAM CTA CCT GTT AAA GAA TC-MGB
PTENex6-ψ VIC CTA CCT GGT AAA GAA T-MGB
Amplification primers:
PTENex5F 5'-ACC AAT GGC TAA GTG AAG ATG ACA-3'
PTENex5R 5'-CAT AGA AAT CTA GGG CCT CTT GTG-3'
PTENex6F 5'-TCC CAG TCA GAG GCG CTA TG-3'
PTENex6R 5'-CACTGAACATTGGAATAGTTTCAAACA-3'

Array-CGH

Arrays were produced from the human 3600 bacterial artificial chromosome (BAC)/P1-derived artificial chromosome genomic clone set of the Welcome Trust Sanger Institute, covering the full genome at ~ 1Mb spacing. Degenerated oligonucleotide PCR products were prepared for spotting as described [50,51]. DNA labeling and hybridization were done essentially as described [50] with minor modifications [52]. After hybridization arrays were scanned in a ScanArray Express HT (Perkin-Elmer, Freemont, CA). The resulting images were analyzed with GenePix Pro 5.0 software (Axon Instruments, Foster City, CA) and subsequently visualized with an excel macro [51].

Acknowledgements

The authors would like to thank Pascal Arp and Rowena Uttberg for technical assistance.

References

1. Schouten, J.P., McElgunn, C.J., Waaijer, R., Zwijnenburg, D., Diepvens, F.,Pals, G. (2002) Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification *Nucleic Acids Res*, **30,** e57.
2. Zhou, X., Yu, T., Cole, S.W.,Wong, D.T. (2006) Advancement in characterization of genomic alterations for improved diagnosis, treatment and prognostics in cancer *Expert Rev Mol Diagn*, **6,** 39-50.
3. Roylance, R. (2002) Methods of molecular analysis: assessing losses and gains in tumours *Mol Pathol*, **55,** 25-28.
4. Craig, D.W. and Stephan, D.A. (2005) Applications of whole-genome high-density SNP genotyping *Expert Rev Mol Diagn*, **5,** 159-170.
5. Pinkel, D. and Albertson, D.G. (2005) Array comparative genomic hybridization and its applications in cancer *Nat Genet*, **37 Suppl,** S11-17.

6. Ylstra, B., van den Ijssel, P., Carvalho, B., Brakenhoff, R.H.,Meijer, G.A. (2006) BAC to the future! or oligonucleotides: a perspective for micro array comparative genomic hybridization (array CGH) *Nucleic Acids Res*, **34,** 445-450.
7. Zhou, X., Rao, N.P., Cole, S.W., Mok, S.C., Chen, Z.,Wong, D.T. (2005) Progress in concurrent analysis of loss of heterozygosity and comparative genomic hybridization utilizing high density single nucleotide polymorphism arrays *Cancer Genet Cytogenet*, **159,** 53-57.
8. Nakayama, K., Ishida, N., Shirane, M., Inomata, A., Inoue, T., Shishido, N., Horii, I.,Loh, D.Y. (1996) Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors *Cell*, **85,** 707-720.
9. Tomlinson, I.P., Lambros, M.B.,Roylance, R.R. (2002) Loss of heterozygosity analysis: practically and conceptually flawed? *Genes Chromosomes Cancer*, **34,** 349-353.
10. Sellner, L.N. and Taylor, G.R. (2004) MLPA and MAPH: new techniques for detection of gene deletions *Hum Mutat*, **23,** 413-419.
11. Liehr, T. and Claussen, U. (2002) Current developments in human molecular cytogenetic techniques *Curr Mol Med*, **2,** 283-297.
12. Bayani, J.M. and Squire, J.A. (2002) Applications of SKY in cancer cytogenetics *Cancer Invest*, **20,** 373-386.
13. Mighell, A.J., Smith, N.R., Robinson, P.A.,Markham, A.F. (2000) Vertebrate pseudogenes *FEBS Lett*, **468,** 109-114.
14. Zhang, Z. and Gerstein, M. (2004) Large-scale analysis of pseudogenes in the human genome *Curr Opin Genet Dev*, **14,** 328-335.
15. Khelifi, A., Duret, L.,Mouchiroud, D. (2005) HOPPSIGEN: a database of human and mouse processed pseudogenes *Nucleic Acids Res*, **33,** D59-66.
16. Ohshima, K., Hattori, M., Yada, T., Gojobori, T., Sakaki, Y.,Okada, N. (2003) Whole-genome screening indicates a possible burst of formation of processed pseudogenes and Alu repeats by particular L1 subfamilies in ancestral primates *Genome Biol*, **4,** R74.
17. Torrents, D., Suyama, M., Zdobnov, E.,Bork, P. (2003) A genome-wide survey of human pseudogenes *Genome Res*, **13,** 2559-2567.
18. Zhang, Z., Harrison, P.M., Liu, Y.,Gerstein, M. (2003) Millions of years of evolution preserved: a comprehensive catalog of the processed pseudogenes in the human genome *Genome Res*, **13,** 2541-2558.
19. Vivanco, I. and Sawyers, C.L. (2002) The phosphatidylinositol 3-Kinase AKT pathway in human cancer *Nat Rev Cancer*, **2,** 489-501.
20. Vlietstra, R.J., van Alewijk, D.C., Hermans, K.G., van Steenbrugge, G.J.,Trapman, J. (1998) Frequent inactivation of PTEN in prostate cancer cell lines and xenografts *Cancer Res*, **58,** 2720-2723.

21. Bonneau, D. and Longy, M. (2000) Mutations of the human PTEN gene *Hum Mutat*, **16,** 109-122.

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

23. Hermans, K.G., van Alewijk, D.C., Veltman, J.A., van Weerden, W., van Kessel, A.G.,Trapman, J. (2004) Loss of a small region around the PTEN locus is a major chromosome 10 alteration in prostate cancer xenografts and cell lines *Genes Chromosomes Cancer*, **39,** 171-184.

24. Sun, J., Liu, W., Adams, T.S., Li, X., Turner, A.R., Chang, B., Kim, J.W., Zheng, S.L., Isaacs, W.B.,Xu, J. (2007) DNA copy number alterations in prostate cancers: a combined analysis of published CGH studies *Prostate*, **67,** 692-700.

25. van Dekken, H., Paris, P.L., Albertson, D.G., Alers, J.C., Andaya, A., Kowbel, D., van der Kwast, T.H., Pinkel, D., Schroder, F.H., Vissers, K.J., et al. (2004) Evaluation of genetic patterns in different tumor areas of intermediate-grade prostatic adenocarcinomas by high-resolution genomic array analysis *Genes Chromosomes Cancer*, **39,** 249-256.

26. Livak, K.J. (1999) Allelic discrimination using fluorogenic probes and the 5' nuclease assay *Genet Anal*, **14,** 143-149.

27. Albertson, D.G., Collins, C., McCormick, F.,Gray, J.W. (2003) Chromosome aberrations in solid tumors *Nat Genet*, **34,** 369-376.

28. Lehmann, U. and Kreipe, H. (2001) Real-time PCR analysis of DNA and RNA extracted from formalin-fixed and paraffin-embedded biopsies *Methods*, **25,** 409-418.

29. Knijnenburg, J., van der Burg, M., Nilsson, P., Ploos van Amstel, H.K., Tanke, H.,Szuhai, K. (2005) Rapid detection of genomic imbalances using micro-arrays consisting of pooled BACs covering all human chromosome arms *Nucleic Acids Res*, **33,** e159.

30. Deutsch, S., Choudhury, U., Merla, G., Howald, C., Sylvan, A.,Antonarakis, S.E. (2004) Detection of aneuploidies by paralogous sequence quantification *J Med Genet*, **41,** 908-915.

31. Armour, J.A., Palla, R., Zeeuwen, P.L., den Heijer, M., Schalkwijk, J.,Hollox, E.J. (2007) Accurate, high-throughput typing of copy number variation using paralogue ratios from dispersed repeats *Nucleic Acids Res*, **35,** e19.

32. Goncalves, I., Duret, L.,Mouchiroud, D. (2000) Nature and structure of human genes that generate retropseudogenes *Genome Res*, **10,** 672-678.

33. Chae, K.S., Ryu, B.K., Lee, M.G., Byun, D.S.,Chi, S.G. (2002) Expression and mutation analyses of MKK4, a candidate tumour suppressor gene encoded by chromosome 17p, in human gastric adenocarcinoma *Eur J Cancer*, **38,** 2048-2057.

34. Su, G.H., Hilgers, W., Shekher, M.C., Tang, D.J., Yeo, C.J., Hruban, R.H.,Kern, S.E. (1998) Alterations in pancreatic, biliary, and breast carcinomas support MKK4 as a genetically targeted tumor suppressor gene *Cancer Res*, **58,** 2339-2342.

35. Teng, D.H., Perry, W.L., 3rd, Hogan, J.K., Baumgard, M., Bell, R., Berry, S., Davis, T., Frank, D., Frye, C., Hattier, T., et al. (1997) Human mitogen-activated protein kinase kinase 4 as a candidate tumor suppressor *Cancer Res*, **57,** 4177-4182.

36. Bertelsen, B.I., Steine, S.J., Sandvei, R., Molven, A.,Laerum, O.D. (2006) Molecular analysis of the PI3K-AKT pathway in uterine cervical neoplasia: frequent PIK3CA amplification and AKT phosphorylation *Int J Cancer*, **118,** 1877-1883.

37. Muller, C.I., Miller, C.W., Hofmann, W.K., Gross, M.E., Walsh, C.S., Kawamata, N., Luong, Q.T.,Koeffler, H.P. (2007) Rare mutations of the PIK3CA gene in malignancies of the hematopoietic system as well as endometrium, ovary, prostate and osteosarcomas, and discovery of a PIK3CA pseudogene *Leuk Res*, **31,** 27-32.

38. Wu, G., Mambo, E., Guo, Z., Hu, S., Huang, X., Gollin, S.M., Trink, B., Ladenson, P.W., Sidransky, D.,Xing, M. (2005) Uncommon mutation, but common amplifications, of the PIK3CA gene in thyroid tumors *J Clin Endocrinol Metab*, **90,** 4688-4693.

39. Ma, Y.Y., Wei, S.J., Lin, Y.C., Lung, J.C., Chang, T.C., Whang-Peng, J., Liu, J.M., Yang, D.M., Yang, W.K.,Shen, C.Y. (2000) PIK3CA as an oncogene in cervical cancer *Oncogene*, **19,** 2739-2744.

40. Miyake, T., Yoshino, K., Enomoto, T., Takata, T., Ugaki, H., Kim, A., Fujiwara, K., Miyatake, T., Fujita, M.,Kimura, T. (2008) PIK3CA gene mutations and amplifications in uterine cancers, identified by methods that avoid confounding by PIK3CA pseudogene sequences *Cancer Lett*, **261,** 120-126.

41. Momand, J., Jung, D., Wilczynski, S.,Niland, J. (1998) The MDM2 gene amplification database *Nucleic Acids Res*, **26,** 3453-3459.

42. Muthusamy, V., Hobbs, C., Nogueira, C., Cordon-Cardo, C., McKee, P.H., Chin, L.,Bosenberg, M.W. (2006) Amplification of CDK4 and MDM2 in malignant melanoma *Genes Chromosomes Cancer*, **45,** 447-454.

43. Hernandez, L., Bea, S., Pinyol, M., Ott, G., Katzenberger, T., Rosenwald, A., Bosch, F., Lopez-Guillermo, A., Delabie, J., Colomer, D., et al. (2005) CDK4 and MDM2 gene alterations mainly occur in highly proliferative and aggressive mantle cell lymphomas with wild-type INK4a/ARF locus *Cancer Res*, **65,** 2199-2206.

44. Wikman, H., Nymark, P., Vayrynen, A., Jarmalaite, S., Kallioniemi, A., Salmenkivi, K., Vainio-Siukola, K., Husgafvel-Pursiainen, K., Knuutila, S., Wolf, M., et al. (2005) CDK4 is a probable target gene in a novel amplicon at 12q13.3-q14.1 in lung cancer *Genes Chromosomes Cancer*, **42,** 193-199.

45. Hostein, I., Pelmus, M., Aurias, A., Pedeutour, F., Mathoulin-Pelissier, S.,Coindre, J.M. (2004) Evaluation of MDM2 and CDK4 amplification by real-time PCR on paraffin wax-embedded material: a potential tool for the diagnosis of atypical lipomatous tumours/well-differentiated liposarcomas *J Pathol*, **202,** 95-102.

46. Sirvent, N., Coindre, J.M., Maire, G., Hostein, I., Keslair, F., Guillou, L., Ranchere-Vince, D., Terrier, P.,Pedeutour, F. (2007) Detection of MDM2-CDK4 amplification by fluorescence in situ hybridization in 200 paraffin-embedded tumor samples: utility in diagnosing adipocytic lesions and comparison with immunohistochemistry and real-time PCR *Am J Surg Pathol*, **31,** 1476-1489.

47. Hoehn, W., Schroeder, F.H., Reimann, J.F., Joebsis, A.C.,Hermanek, P. (1980) Human prostatic adenocarcinoma: some characteristics of a serially transplantable line in nude mice (PC 82) *Prostate*, **1,** 95-104.

48. Hoehn, W., Wagner, M., Riemann, J.F., Hermanek, P., Williams, E., Walther, R.,Schrueffer, R. (1984) Prostatic adenocarcinoma PC EW, a new human tumor line transplantable in nude mice *Prostate*, **5,** 445-452.

49. van Weerden, W.M., de Ridder, C.M., Verdaasdonk, C.L., Romijn, J.C., van der Kwast, T.H., Schroder, F.H.,van Steenbrugge, G.J. (1996) Development of seven new human prostate tumor xenograft models and their histopathological characterization *Am J Pathol*, **149,** 1055-1062.

50. Fiegler, H., Carr, P., Douglas, E.J., Burford, D.C., Hunt, S., Scott, C.E., Smith, J., Vetrie, D., Gorman, P., Tomlinson, I.P., et al. (2003) DNA microarrays for comparative genomic hybridization based on DOP-PCR amplification of BAC and PAC clones *Genes Chromosomes Cancer*, **36,** 361-374.

51. Knijnenburg, J., Szuhai, K., Giltay, J., Molenaar, L., Sloos, W., Poot, M., Tanke, H.J.,Rosenberg, C. (2005) Insights from genomic microarrays into structural chromosome rearrangements *Am J Med Genet A*, **132,** 36-40.

52. Verhagen, P.C., van Duijn, P.W., Hermans, K.G., Looijenga, L.H., van Gurp, R.J., Stoop, H., van der Kwast, T.H.,Trapman, J. (2006) The PTEN gene in locally progressive prostate cancer is preferentially inactivated by bi-allelic gene deletion *J Pathol*, **208,** 699-707.

Chapter 6

General discussion and future directions

General discussion and future directions

As the second cause of male cancer deaths in the Western world, prostate cancer is a serious health problem, however, treatment options of advanced disease are still limited. The tumor suppressor *PTEN* is the most frequently inactivated gene in prostate cancer and a good understanding of its molecular functions in prostate cancer is therefore of utmost importance for the development of novel, targeted therapies. In addition, it may reveal whether PTEN, like in other types of cancer, can serve as a novel prognostic marker for prostate cancer progression or recurrence.

Activation of PTEN signaling by genetic alterations

Genetic and epigenetic changes of members of the PTEN/PI3K pathway can result in constitutive activation of this signaling cascade, a feature that is frequently observed in a variety of sporadic human tumor types. Interestingly, deregulation of PTEN/PI3K signaling appears to occur in a tissue-specific manner. In addition each tumor type might have a predominant mechanism of (in)activation of *PTEN* and *PIK3CA*, encoding the p110α catalytic subunit of PI3K.

PI3KCA was mapped to chromosome 3q26, an area frequently amplified in various human cancers including ovarian, thyroid and cervical cancers [1-3]. Instead of *PIK3CA* copy number changes several other tumor types such as liver, colon, gastric, brain and breast cancers display high rates of *PIK3CA* point mutations, which are predominantly clustered at two mutational hot spots located in the exons 9 and 20 [4].

Somatic *PTEN* abnormalities have been found in many tumors but the highest frequencies of *PTEN* inactivation are found in glioblastomas, endometrial carcinomas and prostate cancers [5]. An important mechanism of *PTEN* inactivation is homozygous deletion. Unfortunately homozygous deletions are difficult to detect in clinical tumor samples due to the presence of contaminating normal DNA in these samples. Chapter 4 of this thesis describes a comprehensive analysis of the *PTEN* status in locally progressive prostate cancers, which revealed that in this tumor type the *PTEN* gene is preferentially inactivated by bi-allelic loss. From the 12, out of the 40 investigated tumor specimens, in which complete *PTEN* inactivation was found, 9 displayed bi-allelic loss of the gene. The other three samples contained an inactivating *PTEN* mutation in either exon 7 or exon 8 of the gene. Although point mutations in *PTEN* have been detected in prostate cancer

their frequency is far less compared to glioblastomas and endometrial carcinomas (Table 3 of the general introduction). Most of the point mutations in the prostate cancer samples are scattered over the exons 5-9.

As previously mentioned, *PTEN* inactivation in glioblastomas and endometrial carcinomas predominantly occurs by point mutations [6]. A large number of mutations in both tumor types are found in exon 5, which encodes the catalytic core motif of PTEN. In glioblastomas also a high proportion of the mutations was found in exon 6, whereas endometrial carcinomas showed a high mutation frequency of the exons 7 and 8 [6]. Exon 6 encodes a part of the so-called TI loop of PTEN, which is responsible for the larger width of the active site pocket of PTEN compared to other protein phosphatases [7]. Therefore, the exon 6 mutations observed in glioblastomas may also affect the phosphatase activity of PTEN. The exons 7 and 8 encode elements that are involved in the membrane binding of PTEN, thus an altered membrane binding due to mutation of these elements could also affect the function of PTEN [7]. The point mutations found in the exons 7 and 8 are located in repetitive motifs. In addition, the endometrial carcinomas in which *PTEN* mutations were found displayed a high frequency of microsatellite instability. It is therefore thought that the defect of mismatch repair in these tumors could be responsible for the high mutation rates of the exons 7 and 8 [6].

In many cancers simultaneous alteration of *PIK3CA* and *PTEN* has been thought to be mutually exclusive [8-10]. However, a recent study reports coexistent mutations of *PIK3CA* and *PTEN* in endometrial carcinoma. In a small subset of breast cancers *PTEN* loss was found to be associated with *PIK3CA* mutations [11,12]. These findings are unexpected because loss of PTEN function and activation of PI3K are thought to have similar effects on the PIP3 pool, and thereby the activation of the downstream signaling pathway. It might however be that more than one event is required for the complete activation of this pathway. In favor of this hypothesis is the report of Velasco et al. (2006) showing that *PIK3CA* mutations predominantly co-exist with mono-allelic inactivation of *PTEN* thereby supporting the hypothesis that *PIK3CA* mutations may have an additive effect on *PTEN* alterations in certain tumor types [13].

Various molecular mechanisms are thus involved in activating the PTEN/PI3K driven tumorigenesis. A helpful tool in determining the underlying inactivation mechanism of certain genes is the q-WPR assay as described in chapter 5 of this thesis. This is a modification of the wild-type to pseudogene ratio assessment

(WPR) assay used in chapter 4 to determine the mechanism of *PTEN* inactivation in primary prostate cancers. Whereas WPR makes use of a sequencing reaction to obtain a semi-quantitative estimate of DNA copy number, the q-WPR assay as described in chapter 5 is based on the previously described 5'-nuclease Taqman assay [14]. Compared to the initial WPR assay, q-WPR analysis is even faster, applicable in a high-throughput format, does not require further sample processing minimizing the risk of contamination and is much more quantitative.

The enhanced sensitivity of q-WPR analysis compared to the previous described WPR technique is further demonstrated by the observation that for one of the tumor specimens investigated in chapter 4 (T8-4) q-WPR analysis predicted loss of one *PTEN* allele whereas the former WPR technique and even the FISH and LOH studies predicted complete loss of *PTEN* in this tumor. Array-CGH, which is a powerful tool to detect copy number changes at high resolution, then showed that in T8-4 there is indeed loss of one *PTEN* allele. Moreover, also in case of T10-15 q-WPR was proven to be more sensitive than the initial WPR method that predicted loss of one allele of *PTEN*. However, q-WPR analysis indicated a normal *PTEN* copy number for this tumor, which was later confirmed by array-CGH.

The q-WPR assay cannot only be used for the detection of gene deletions but is also suitable for amplification analysis of any gene with a highly homologous structurally related gene. A recent study described the discovery of a *PIK3CA* pseudogene spanning exons 9-13 on chromosome 22 [15], which opens perspectives for the application of q-WPR in studies investigating the mechanism underlying activation of the PTEN/PI3K pathway.

PTEN dosage

A phenomenon closely related with the *PTEN* copy number is haploinsufficiency. In mice a study investigating the effect of *Pten* dose on cancer progression using a so-called hypomorphic mouse mutant series, in which the *Pten* dose was decreased even below heterozygous levels, revealed that *Pten* plays a crucial dose-dependent role in prostate cancer progression [16]. Whereas the prostates of the *Pten* heterozygous mice were not enlarged and displayed only moderate/low grade PIN foci further reduction of the *Pten* dose in resulted in markedly enlarged prostates and moreover 25% of these mice showed histological signs of local invasion at 6 months of age. In human prostate cancer specimens representing various stages of the disease it was recently showed that *PTEN* is more frequently lost in prostate

cancer samples compared to precursor lesions implicating a role for PTEN haploinsufficiency in the transition from preneoplastic PIN to prostate cancer [17]. Thus although there are indications that reduced PTEN function is important in the progression of prostate cancer further research is needed to elucidate the question whether PTEN haploinsufficiency is indeed critical in prostate tumor progression. Further insight into the role of *PTEN* dosage in human prostate cancer development may be obtained by a gradually knock down of PTEN expression in normal prostate cells using a tightly controlled, inducible short hairpin RNA (vector based siRNA) expression system.

Model systems to study PTEN function

An important issue in unraveling the cell biological and molecular functions of PTEN in prostate cancer is the choice of the model system. Prostate-specific *Pten* knock-out mice are a major component of the current prostate cancer research. However, the onset and progression of tumor formation upon *Pten* inactivation in mice seem to occur earlier than in humans. Whereas in human prostate tumors complete loss of PTEN function is associated with the later stages of the disease, in the *PB-Cre;Pten-loxP/loxP* mice invasive carcinomas had already developed at 9 weeks of age [18]. The process of prostate cancer development in *PSA-Cre;Pten-loxP/loxP* mice was slower, massive hyperplasia and focal PIN were first observed at 4-5 months of age while carcinomas were detected at 10-14 months [19]. Recently, a mouse model was described in which prostate carcinogenesis is initiated after puberty which may more closely resemble the course of human prostate cancer development [20]. Moreover, it must be kept in mind that PTEN might also display species specific functions. Therefore caution must be taken in interpreting the observed functions of Pten in mouse model systems with respect to the significance of these functions in human cancer.

In the studies described in chapters 2 and 3 of this thesis tumor derived cell lines were used to study PTEN function. Since cell lines can be easily propagated large amounts of material are available. In addition, in cell lines manipulation of signaling pathways is relatively easy to perform and in contrast to mouse models results can be obtained within a few days. However, it must be noted that tumor derived cell lines display multiple genetic alterations, which adds another level of complexity in the identification of PTEN regulated processes. Normal prostate cell lines might therefore be the most preferred model system to investigate the role of

PTEN in prostate cancer. Only a few reports describe the use of non tumorigenic PNT1a or PNT2 prostate cells with respect to PTEN research [21,22]. However, most times small molecules or PTEN over-expression was used to manipulate PTEN/PI3K signaling, which would not be the preferred method of choice in cell lines with a wild-type PTEN background. In one study PTEN expression was also knocked down by siRNA, which resulted in high steady state levels of active Akt but thus far no functional PTEN studies were performed in these cell lines [21].

Recently, the fusion of the androgen regulated gene *TMPRSS2* to genes encoding members of the ETS transcription factor family, such as *ERG*, has been identified as a frequently occurring rearrangement in prostate cancer [23-25]. Overexpression of the *TMPRSS2:ERG* gene fusion product in normal prostate cells promoted invasion, and expression in transgenic mice initiated the development of PIN lesions [26,27]. These models provide valuable information concerning the significance of the *TMPRSS2:ERG* rearrangement in prostate cancer tumorigenesis. Together with the inactivation of *PTEN* it is the most common genetic alteration in prostate cancer. Since complete loss of PTEN function is correlated with later stages of the disease it would be interesting to investigate the effect of additional *PTEN* loss in the further progression of prostate cancer in the *TMPRSS2:ERG* model systems.

Modulation of PTEN signaling

The PTEN/PI3K signal transduction cascade is very complex and modulation of this signaling pathway by over-expression of PTEN, inducible systems or small molecules that inhibit PI3K function has been proven to be very useful to increase the insight in the cell biological functions of PTEN and its downstream targets. However, drawbacks of these methods are the non-specific side effects of small molecules and the non-physiological expression of the protein of interest in over-expression studies, thereby complicating the identification of normal cell biological functions and direct downstream signaling targets. The last few years RNA interfering as a method for specific post-transcriptional gene silencing has been proven to be a new potent tool to analyze signal transduction cascades. By systematically targeting a specific member of a certain signal transduction cascade much can be learned about its role in that particular pathway. For example, the specific knockdown of the three Akt isoforms in various tumor cell lines revealed that the relative importance of the different isoforms is cell line specific [28]. In

chapter 2 of this thesis siRNA technology was used to investigate the role of PTEN/Akt signaling in the regulation of $p27^{kip1}$ and in chapter 3 Akt specific siRNA was used to discriminate between Akt dependent and independent mechanisms of PTEN mediated transcription regulation.

Thus, RNAi technology facilitates complex pathway analysis by dissecting signaling cascades in a systematic way. The functional pathway analysis can even be improved by the combinatorial use of several siRNAs within one cell [29]. Also the development of inducible short hairpin RNA (vector based siRNA) expression systems especially in combination with lenti-viral delivery systems for an efficient and stable expression of the siRNA, will enhance the research studying complex biological processes [30].

Next to the exogenously applied siRNAs also the recently discovered miRNAs could have an important function in gene silencing and introduce a new level and mechanism of regulation [31]. MiRNAs are an abundant class of endogenously expressed noncoding RNAs of approximately 22 nucleotides in length that can function as negative regulators of gene expression by inducing degradation or inhibiting translation of select mRNAs [32]. As previously described in the general introduction of this thesis, to date miR-21, miR-214, and the miR17-92 cluster are reported to function in the regulation of PTEN [33-35]. In addition, the miR17-92 cluster was also found to suppress the expression of the pro-apoptotic factor BIM, a putative target of FOXO transcription factors [35,36].

There are only a limited number of studies that have investigated the role of miRNAs in prostate cancer but interestingly the expression of miR-125b was frequently altered [37-39]. Down-regulation of miR-125b in tumor samples was associated with an increased expression of the Akt/mTOR pathway target 4E-BP1 [38]. Furthermore, in prostate cancer cell lines the miRNAs miR-221 and miR-222 were implicated in the regulation of $p27^{kip1}$ [40]. In the investigated cell lines miR-221 and miR-222 expression seemed to correlate with the growth rate of the cell line; whereas they were highly detectable in PC3 cells they were almost absent in the slow growing LNCaP and 22Rv-1 cell lines [40]. Moreover, their expression inversely correlated with the $p27^{kip1}$ expression level. As described previously in chapter 2 of this thesis, in our panel of prostate cancer cell lines the $p27^{kip1}$ expression level more strongly correlated with the growth rate than with the PTEN/Akt status of a particular cell line. Thus maybe, apart from the described

role of Skp2 in $p27^{kip1}$ regulation, miR-221 and miR-222 have also a role in the regulation of $p27^{kip1}$ in prostate cancer cell lines.

Cross-talk of PTEN/PI3K signaling with other signal transduction cascades

PTEN might display at least part of its function through cross-talk with other signaling pathways. Since both the AR and activation of PTEN/PI3K signaling have a major function in prostate cancer growth many efforts have been put in the investigation of a functional link between these two signal transduction cascades. Although *in vitro* Akt has been found to phosphorylate the AR, *in vivo* the effect of Akt dependent phosphorylation in transactivation of the AR is still a matter of debate [41-44].

Since both *PTEN* inactivation and fusion of *TMPRSS2* to *ETS* genes are frequently occurring alterations in prostate cancer it might be of interest to study cross-talk between PTEN and ETS pathways. ETS transcription factors are involved in the regulation of various biological processes [45]. A study by Iljin et al., (2006) aiming to identify ETS regulated processes in prostate cancer revealed that the WNT and PITX2 cascades were the most highly enriched pathways in ERG positive tumors [46]. In addition, these ERG positive tumors also displayed over-expression of HDAC1 [46]. Parallel knockdown of several targets using RNAi technology will be a valuable tool to study cross-talk between PTEN/AR and PTEN/ETS pathways in a systematic way and help to elucidate their role in the development and progression of prostate cancer.

PTEN in cell cycle regulation

Progression through the cell cycle is a highly complex and tightly coordinated process. The role of PTEN therein is observed at several levels of regulation [47]. At the protein level direct phosphorylation by Akt alters the cellular distribution and thereby the activity of $p21^{Cip1/Waf}$ and $p27^{kip1}$ [48,49]. In addition, Akt dependent phosphorylation of these cell cycle inhibitors can also target them for degradation by the ubiquitin-proteasome system. Moreover, cyclin D1 is targeted for degradation by the ubiquitin-proteasome system upon phosphorylation by the Akt target GSK3 [50].

Protein degradation by the ubiquitin-proteasome system is an important aspect in cell cycle regulation. One of the major E3 ubiquitin ligases involved in the proteolysis of many cell cycle proteins is Skp2 [51]. In chapter 2 we reported that in

PC3 and DU145 cells Skp2 is the main determinant of the Akt dependent $p27^{kip1}$ regulation. By that time the underlying mechanisms of Akt dependent Skp2 regulation remained to be elucidated. Although many aspects are still unclear, roles for GSK3, mTOR and E2F transcription factors were suggested in the PTEN/PI3K dependent regulation Skp2 expression [52-54]. Although E2F transcription factors were found to up-regulate Skp2 mRNA in pancreatic ductal adenocarcinoma cells[53], we could not detect regulation of Skp2 expression at the transcriptional level in the prostate cancer cells PC3 and DU145. Since many proteins are regulated at various different levels it might very well be that cell-type specific regulatory mechanisms exist. This hypothesis is further strengthened by the observation that in intestinal cells over-expression of active GSK3 resulted in a reduced Skp2 expression and that inhibition of mTOR activity by rapamycin in breast cancer cells not only affected Skp2 mRNA expression but also enhanced the Skp2 protein degradation rate [52,54].

Next to PTEN/Akt mediated modulations at the protein level PTEN dependent transcription regulation is also important in cell cycle regulation as described in chapter 3 of this thesis. PTEN is known to modulate transcription by affecting the activity of the FOXO family of transcription factors and in several cell types a contribution of FOXO to G_1/S cell-cycle arrest by regulating the expression of negative cell-cycle regulators, such as $p27^{kip1}$ and RBL2, has been described [55-58]. However, we found that in prostate cancer cells Akt/FOXO signaling is not a major regulator of $p27^{kip1}$ mRNA expression and also the observed PTEN dependent transcription regulation of RBL2 was independent of Akt/FOXO signaling (see chapters 2 and 3). Although FOXO transcription factors are probably implicated in the regulation of cyclin G2 the transcription factors responsible for the altered mRNA expression of the other genes remained to be identified. Screening a siRNA library targeting various transcription factors would be a useful tool to reveal which transcription factors are involved and shed a new light on PTEN dependent transcription regulation.

Nuclear PTEN functions

A critical part of the tumor suppressive function of PTEN is due to its lipid phosphatase activity, a process occurring at the cellular membrane [59]. Despite the extensively studied role of PTEN in signaling at the cellular membrane the presence of PTEN in the nucleus of many different cell types suggested that also its

nuclear localization may contribute to its tumor suppressor activity [60,61]. Many components of the PI3K signaling pathway are also found in the nucleus but a recent study indicated that nuclear PTEN does not dephosphorylate the nuclear PIP3 pool [62,63]. The exact role of nuclear PTEN in its tumor suppressive function thus remains to be elucidated although nuclear PTEN has been proposed to act as a pro-apoptotic factor and a regulator of chromosomal integrity [64,65]. Intriguingly, higher levels of nuclear PTEN were associated with G_0-G_1 phase, and lower nuclear PTEN levels were associated with S phase progression in MCF7 cells [66]. Further research indicated that nuclear PTEN is required for PTEN mediated cell cycle arrest whereas cytoplasmic PTEN mainly regulates apoptosis [67]. In chapter 3 we reported that G_0/G_1 cell cycle arrest was one of the most prominent processes regulated by PTEN in LNCaP/PTEN cells. However, in this study no discrimination was made between nuclear and cytoplasmic PTEN functions. In these cells PTEN dependent transcription regulation, which is executed in the nucleus, was of particular importance in the regulation of the G_0/G_1 cell cycle arrest. Therefore, it may be interesting to investigate the role of nuclear PTEN in cell cycle regulation of prostate cancer cells.

PTEN and DNA replication

In the search for PTEN regulated processes altered expression of several genes involved in DNA replication was observed, such as CDC6, CDT1 and MCM10 (chapter 3). Although the process of DNA replication is closely linked to cell cycle control as indicated by the function of CDKs both in cell cycle regulation and as initiator proteins of DNA replication, this seemed a more indirect regulated process since the expression of these genes first started to decrease at 24 h after PTEN induction [68]. Thus far, less is known about the function of PTEN in DNA replication but interestingly, Skp2 was recently implicated in the regulation of CDT1 [69,70]. Since we and others have detected a PTEN mediated regulation of Skp2 (71 and chapter 2 of this thesis) it may be interesting to further explore the role of PTEN and Skp2 in the regulation of DNA replication in prostate cancer. It might be that although at the transcriptional level PTEN dependent regulation of DNA replication is a more indirect effect, PTEN is more directly involved in the regulation of DNA replication at the protein level via regulation of Skp2 expression.

Concluding remarks

The research described in this thesis increased the insight in the genetic and cell biological aspects of PTEN in prostate cancer. However, many issues remain to be elucidated, such as the question whether PTEN haploinsufficiency is indeed critical in prostate cancer tumor progression. At the cell biological level experiments in normal prostate cell lines will largely contribute to an increased understanding of normal PTEN function in the prostate. Inducible shRNA expression systems will be an essential tool to dissect the PTEN/PI3K signaling pathway and its possible interactions with other signal transduction cascades. Special interest may be paid to the role of nuclear PTEN in cell cycle regulation and also the question whether and by which regulatory mechanism PTEN might be involved in the regulation of DNA replication provides an interesting opportunity for future research. Thus although PTEN signaling pathway has been extensively studied and much progress has been made in defining the role of PTEN in tumor suppression continuing research will be essential in unraveling additional functions of PTEN. Further understanding of the possible tissue specific regulation of the PTEN pathway in prostate cancer may provide opportunities for the development of novel therapeutic options.

References

1. Wu, G., Mambo, E., Guo, Z., Hu, S., Huang, X., Gollin, S.M., Trink, B., Ladenson, P.W., Sidransky, D.,Xing, M. (2005) Uncommon mutation, but common amplifications, of the PIK3CA gene in thyroid tumors *J Clin Endocrinol Metab*, **90,** 4688-4693.
2. Ma, Y.Y., Wei, S.J., Lin, Y.C., Lung, J.C., Chang, T.C., Whang-Peng, J., Liu, J.M., Yang, D.M., Yang, W.K.,Shen, C.Y. (2000) PIK3CA as an oncogene in cervical cancer *Oncogene*, **19,** 2739-2744.
3. Shayesteh, L., Lu, Y., Kuo, W.L., Baldocchi, R., Godfrey, T., Collins, C., Pinkel, D., Powell, B., Mills, G.B.,Gray, J.W. (1999) PIK3CA is implicated as an oncogene in ovarian cancer *Nat Genet*, **21,** 99-102.
4. Samuels, Y. and Velculescu, V.E. (2004) Oncogenic mutations of PIK3CA in human cancers *Cell Cycle*, **3,** 1221-1224.
5. Dahia, P.L. (2000) PTEN, a unique tumor suppressor gene *Endocr Relat Cancer*, **7,** 115-129.
6. Bonneau, D. and Longy, M. (2000) Mutations of the human PTEN gene *Hum Mutat*, **16,** 109-122.
7. Lee, J.O., Yang, H., Georgescu, M.M., Di Cristofano, A., Maehama, T., Shi, Y., Dixon, J.E., Pandolfi, P.,Pavletich, N.P. (1999) Crystal structure of the PTEN tumor suppressor:

implications for its phosphoinositide phosphatase activity and membrane association *Cell*, **99,** 323-334.

8. Saal, L.H., Holm, K., Maurer, M., Memeo, L., Su, T., Wang, X., Yu, J.S., Malmstrom, P.O., Mansukhani, M., Enoksson, J., et al. (2005) PIK3CA mutations correlate with hormone receptors, node metastasis, and ERBB2, and are mutually exclusive with PTEN loss in human breast carcinoma *Cancer Res*, **65,** 2554-2559.
9. Abubaker, J., Bavi, P.P., Al-Harbi, S., Siraj, A.K., Al-Dayel, F., Uddin, S.,Al-Kuraya, K. (2007) PIK3CA mutations are mutually exclusive with PTEN loss in diffuse large B-cell lymphoma *Leukemia*, **21,** 2368-2370.
10. Byun, D.S., Cho, K., Ryu, B.K., Lee, M.G., Park, J.I., Chae, K.S., Kim, H.J.,Chi, S.G. (2003) Frequent monoallelic deletion of PTEN and its reciprocal associatioin with PIK3CA amplification in gastric carcinoma *Int J Cancer*, **104,** 318-327.
11. Perez-Tenorio, G., Alkhori, L., Olsson, B., Waltersson, M.A., Nordenskjold, B., Rutqvist, L.E., Skoog, L.,Stal, O. (2007) PIK3CA mutations and PTEN loss correlate with similar prognostic factors and are not mutually exclusive in breast cancer *Clin Cancer Res*, **13,** 3577-3584.
12. Oda, K., Stokoe, D., Taketani, Y.,McCormick, F. (2005) High frequency of coexistent mutations of PIK3CA and PTEN genes in endometrial carcinoma *Cancer Res*, **65,** 10669-10673.
13. Velasco, A., Bussaglia, E., Pallares, J., Dolcet, X., Llobet, D., Encinas, M., Llecha, N., Palacios, J., Prat, J.,Matias-Guiu, X. (2006) PIK3CA gene mutations in endometrial carcinoma: correlation with PTEN and K-RAS alterations *Hum Pathol*, **37,** 1465-1472.
14. Livak, K.J. (1999) Allelic discrimination using fluorogenic probes and the 5' nuclease assay *Genet Anal*, **14,** 143-149.
15. Muller, C.I., Miller, C.W., Hofmann, W.K., Gross, M.E., Walsh, C.S., Kawamata, N., Luong, Q.T.,Koeffler, H.P. (2007) Rare mutations of the PIK3CA gene in malignancies of the hematopoietic system as well as endometrium, ovary, prostate and osteosarcomas, and discovery of a PIK3CA pseudogene *Leuk Res*, **31,** 27-32.
16. Trotman, L.C., Niki, M., Dotan, Z.A., Koutcher, J.A., Di Cristofano, A., Xiao, A., Khoo, A.S., Roy-Burman, P., Greenberg, N.M., Van Dyke, T., et al. (2003) Pten dose dictates cancer progression in the prostate *PLoS Biol*, **1,** E59.
17. Yoshimoto, M., Cutz, J.C., Nuin, P.A., Joshua, A.M., Bayani, J., Evans, A.J., Zielenska, M.,Squire, J.A. (2006) Interphase FISH analysis of PTEN in histologic sections shows genomic deletions in 68% of primary prostate cancer and 23% of high-grade prostatic intra-epithelial neoplasias *Cancer Genet Cytogenet*, **169,** 128-137.

18. Wang, S., Gao, J., Lei, Q., Rozengurt, N., Pritchard, C., Jiao, J., Thomas, G.V., Li, G., Roy-Burman, P., Nelson, P.S., et al. (2003) Prostate-specific deletion of the murine Pten tumor suppressor gene leads to metastatic prostate cancer *Cancer Cell*, **4,** 209-221.
19. Ma, X., Ziel-van der Made, A.C., Autar, B., van der Korput, H.A., Vermeij, M., van Duijn, P., Cleutjens, K.B., de Krijger, R., Krimpenfort, P., Berns, A., et al. (2005) Targeted biallelic inactivation of Pten in the mouse prostate leads to prostate cancer accompanied by increased epithelial cell proliferation but not by reduced apoptosis *Cancer Res*, **65,** 5730-5739.
20. Ratnacaram, C.K., Teletin, M., Jiang, M., Meng, X., Chambon, P.,Metzger, D. (2008) Temporally controlled ablation of PTEN in adult mouse prostate epithelium generates a model of invasive prostatic adenocarcinoma *Proc Natl Acad Sci U S A*, **105,** 2521-2526.
21. Sharrard, R.M. and Maitland, N.J. (2007) Regulation of protein kinase B activity by PTEN and SHIP2 in human prostate-derived cell lines *Cell Signal*, **19,** 129-138.
22. Sharrard, R.M. and Maitland, N.J. (2000) Phenotypic effects of overexpression of the MMAC1 gene in prostate epithelial cells *Br J Cancer*, **83,** 1102-1109.
23. Mehra, R., Tomlins, S.A., Shen, R., Nadeem, O., Wang, L., Wei, J.T., Pienta, K.J., Ghosh, D., Rubin, M.A., Chinnaiyan, A.M., et al. (2007) Comprehensive assessment of TMPRSS2 and ETS family gene aberrations in clinically localized prostate cancer *Mod Pathol*, **20,** 538-544.
24. Hermans, K.G., van Marion, R., van Dekken, H., Jenster, G., van Weerden, W.M.,Trapman, J. (2006) TMPRSS2:ERG fusion by translocation or interstitial deletion is highly relevant in androgen-dependent prostate cancer, but is bypassed in late-stage androgen receptor-negative prostate cancer *Cancer Res*, **66,** 10658-10663.
25. Tomlins, S.A., Rhodes, D.R., Perner, S., Dhanasekaran, S.M., Mehra, R., Sun, X.W., Varambally, S., Cao, X., Tchinda, J., Kuefer, R., et al. (2005) Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer *Science*, **310,** 644-648.
26. Klezovitch, O., Risk, M., Coleman, I., Lucas, J.M., Null, M., True, L.D., Nelson, P.S.,Vasioukhin, V. (2008) A causal role for ERG in neoplastic transformation of prostate epithelium *Proc Natl Acad Sci U S A*, **105,** 2105-2110.
27. Tomlins, S.A., Laxman, B., Varambally, S., Cao, X., Yu, J., Helgeson, B.E., Cao, Q., Prensner, J.R., Rubin, M.A., Shah, R.B., et al. (2008) Role of the TMPRSS2-ERG gene fusion in prostate cancer *Neoplasia*, **10,** 177-188.
28. Koseoglu, S., Lu, Z., Kumar, C., Kirschmeier, P.,Zou, J. (2007) AKT1, AKT2 and AKT3-Dependent Cell Survival is Cell Line-specific and Knockdown of All Three Isoforms Selectively Induces Apoptosis in 20 Human Tumor Cell Lines *Cancer Biol Ther*, **6**.

29. Sahin, O., Lobke, C., Korf, U., Appelhans, H., Sultmann, H., Poustka, A., Wiemann, S.,Arlt, D. (2007) Combinatorial RNAi for quantitative protein network analysis *Proc Natl Acad Sci U S A*, **104,** 6579-6584.

30. Sumimoto, H. and Kawakami, Y. (2007) Lentiviral vector-mediated RNAi and its use for cancer research *Future Oncol*, **3,** 655-664.

31. Hagan, J.P. and Croce, C.M. (2007) MicroRNAs in carcinogenesis *Cytogenet Genome Res*, **118,** 252-259.

32. Bartel, D.P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function *Cell*, **116,** 281-297.

33. Meng, F., Henson, R., Wehbe-Janek, H., Ghoshal, K., Jacob, S.T.,Patel, T. (2007) MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer *Gastroenterology*, **133,** 647-658.

34. Yang, H., Kong, W., He, L., Zhao, J.J., O'Donnell, J.D., Wang, J., Wenham, R.M., Coppola, D., Kruk, P.A., Nicosia, S.V., et al. (2008) MicroRNA expression profiling in human ovarian cancer: miR-214 induces cell survival and cisplatin resistance by targeting PTEN *Cancer Res*, **68,** 425-433.

35. Xiao, C., Srinivasan, L., Calado, D.P., Patterson, H.C., Zhang, B., Wang, J., Henderson, J.M., Kutok, J.L.,Rajewsky, K. (2008) Lymphoproliferative disease and autoimmunity in mice with increased miR-17-92 expression in lymphocytes *Nat Immunol*, **9,** 405-414.

36. Urbich, C., Knau, A., Fichtlscherer, S., Walter, D.H., Bruhl, T., Potente, M., Hofmann, W.K., de Vos, S., Zeiher, A.M.,Dimmeler, S. (2005) FOXO-dependent expression of the proapoptotic protein Bim: pivotal role for apoptosis signaling in endothelial progenitor cells *Faseb J*, **19,** 974-976.

37. Porkka, K.P., Pfeiffer, M.J., Waltering, K.K., Vessella, R.L., Tammela, T.L.,Visakorpi, T. (2007) MicroRNA expression profiling in prostate cancer *Cancer Res*, **67,** 6130-6135.

38. Ozen, M., Creighton, C.J., Ozdemir, M.,Ittmann, M. (2008) Widespread deregulation of microRNA expression in human prostate cancer *Oncogene*, **27,** 1788-1793.

39. Shi, X.B., Tepper, C.G.,White, R.W. (2008) Cancerous miRNAs and their regulation *Cell Cycle*, **7**.

40. Galardi, S., Mercatelli, N., Giorda, E., Massalini, S., Frajese, G.V., Ciafre, S.A.,Farace, M.G. (2007) miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27Kip1 *J Biol Chem*, **282,** 23716-23724.

41. Li, P., Nicosia, S.V.,Bai, W. (2001) Antagonism between PTEN/MMAC1/TEP-1 and androgen receptor in growth and apoptosis of prostatic cancer cells *J Biol Chem*, **276,** 20444-20450.

42. Lin, H.K., Yeh, S., Kang, H.Y.,Chang, C. (2001) Akt suppresses androgen-induced apoptosis by phosphorylating and inhibiting androgen receptor *Proc Natl Acad Sci U S A*, **98,** 7200-7205.

43. Gioeli, D., Ficarro, S.B., Kwiek, J.J., Aaronson, D., Hancock, M., Catling, A.D., White, F.M., Christian, R.E., Settlage, R.E., Shabanowitz, J., et al. (2002) Androgen receptor phosphorylation. Regulation and identification of the phosphorylation sites *J Biol Chem*, **277,** 29304-29314.

44. Wen, Y., Hu, M.C., Makino, K., Spohn, B., Bartholomeusz, G., Yan, D.H.,Hung, M.C. (2000) HER-2/neu promotes androgen-independent survival and growth of prostate cancer cells through the Akt pathway *Cancer Res*, **60,** 6841-6845.

45. Seth, A. and Watson, D.K. (2005) ETS transcription factors and their emerging roles in human cancer *Eur J Cancer*, **41,** 2462-2478.

46. Iljin, K., Wolf, M., Edgren, H., Gupta, S., Kilpinen, S., Skotheim, R.I., Peltola, M., Smit, F., Verhaegh, G., Schalken, J., et al. (2006) TMPRSS2 fusions with oncogenic ETS factors in prostate cancer involve unbalanced genomic rearrangements and are associated with HDAC1 and epigenetic reprogramming *Cancer Res*, **66,** 10242-10246.

47. Liang, J. and Slingerland, J.M. (2003) Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression *Cell Cycle*, **2,** 339-345.

48. Liang, J., Zubovitz, J., Petrocelli, T., Kotchetkov, R., Connor, M.K., Han, K., Lee, J.H., Ciarallo, S., Catzavelos, C., Beniston, R., et al. (2002) PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest *Nat Med*, **8,** 1153-1160.

49. Li, Y., Dowbenko, D.,Lasky, L.A. (2002) AKT/PKB phosphorylation of p21Cip/WAF1 enhances protein stability of p21Cip/WAF1 and promotes cell survival *J Biol Chem*, **277,** 11352-11361.

50. Diehl, J.A., Cheng, M., Roussel, M.F.,Sherr, C.J. (1998) Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization *Genes Dev*, **12,** 3499-3511.

51. Nakayama, K.I. and Nakayama, K. (2005) Regulation of the cell cycle by SCF-type ubiquitin ligases *Semin Cell Dev Biol*, **16,** 323-333.

52. Shapira, M., Kakiashvili, E., Rosenberg, T.,Hershko, D.D. (2006) The mTOR inhibitor rapamycin down-regulates the expression of the ubiquitin ligase subunit SKP2 in breast cancer cells *Breast Cancer Res*, **8,** R46.

53. Reichert, M., Saur, D., Hamacher, R., Schmid, R.M.,Schneider, G. (2007) Phosphoinositide-3-kinase signaling controls S-phase kinase-associated protein 2 transcription via E2F1 in pancreatic ductal adenocarcinoma cells *Cancer Res*, **67,** 4149-4156.

54. Wang, Q., Zhou, Y., Wang, X.,Evers, B.M. (2008) p27 Kip1 nuclear localization and cyclin-dependent kinase inhibitory activity are regulated by glycogen synthase kinase-3 in human colon cancer cells *Cell Death Differ*, **15,** 908-919.

55. Kops, G.J., Medema, R.H., Glassford, J., Essers, M.A., Dijkers, P.F., Coffer, P.J., Lam, E.W.,Burgering, B.M. (2002) Control of cell cycle exit and entry by protein kinase B-regulated forkhead transcription factors *Mol Cell Biol*, **22,** 2025-2036.

56. Collado, M., Medema, R.H., Garcia-Cao, I., Dubuisson, M.L., Barradas, M., Glassford, J., Rivas, C., Burgering, B.M., Serrano, M.,Lam, E.W. (2000) Inhibition of the phosphoinositide 3-kinase pathway induces a senescence-like arrest mediated by p27Kip1 *J Biol Chem*, **275,** 21960-21968.

57. Medema, R.H., Kops, G.J., Bos, J.L.,Burgering, B.M. (2000) AFX-like Forkhead transcription factors mediate cell-cycle regulation by Ras and PKB through p27kip1 *Nature*, **404,** 782-787.

58. Nakamura, N., Ramaswamy, S., Vazquez, F., Signoretti, S., Loda, M.,Sellers, W.R. (2000) Forkhead transcription factors are critical effectors of cell death and cell cycle arrest downstream of PTEN *Mol Cell Biol*, **20,** 8969-8982.

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

60. Lachyankar, M.B., Sultana, N., Schonhoff, C.M., Mitra, P., Poluha, W., Lambert, S., Quesenberry, P.J., Litofsky, N.S., Recht, L.D., Nabi, R., et al. (2000) A role for nuclear PTEN in neuronal differentiation *J Neurosci*, **20,** 1404-1413.

61. Gimm, O., Perren, A., Weng, L.P., Marsh, D.J., Yeh, J.J., Ziebold, U., Gil, E., Hinze, R., Delbridge, L., Lees, J.A., et al. (2000) Differential nuclear and cytoplasmic expression of PTEN in normal thyroid tissue, and benign and malignant epithelial thyroid tumors *Am J Pathol*, **156,** 1693-1700.

62. Deleris, P., Gayral, S.,Breton-Douillon, M. (2006) Nuclear Ptdlns(3,4,5)P3 signaling: an ongoing story *J Cell Biochem*, **98,** 469-485.

63. Lindsay, Y., McCoull, D., Davidson, L., Leslie, N.R., Fairservice, A., Gray, A., Lucocq, J.,Downes, C.P. (2006) Localization of agonist-sensitive PtdIns(3,4,5)P3 reveals a nuclear pool that is insensitive to PTEN expression *J Cell Sci*, **119,** 5160-5168.

64. Gil, A., Andres-Pons, A., Fernandez, E., Valiente, M., Torres, J., Cervera, J.,Pulido, R. (2006) Nuclear localization of PTEN by a Ran-dependent mechanism enhances apoptosis: Involvement of an N-terminal nuclear localization domain and multiple nuclear exclusion motifs *Mol Biol Cell*, **17,** 4002-4013.

65. Shen, W.H., Balajee, A.S., Wang, J., Wu, H., Eng, C., Pandolfi, P.P.,Yin, Y. (2007) Essential role for nuclear PTEN in maintaining chromosomal integrity *Cell*, **128,** 157-170.

66. Ginn-Pease, M.E. and Eng, C. (2003) Increased nuclear phosphatase and tensin homologue deleted on chromosome 10 is associated with G0-G1 in MCF-7 cells *Cancer Res*, **63,** 282-286.

67. Chung, J.H. and Eng, C. (2005) Nuclear-cytoplasmic partitioning of phosphatase and tensin homologue deleted on chromosome 10 (PTEN) differentially regulates the cell cycle and apoptosis *Cancer Res*, **65,** 8096-8100.

68. Nishitani, H. and Lygerou, Z. (2002) Control of DNA replication licensing in a cell cycle *Genes Cells*, **7,** 523-534.

69. Li, X., Zhao, Q., Liao, R., Sun, P.,Wu, X. (2003) The SCF(SKP2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation *J Biol Chem*, **278,** 30854-30858.

70. Nishitani, H., Sugimoto, N., Roukos, V., Nakanishi, Y., Saijo, M., Obuse, C., Tsurimoto, T., Nakayama, K.I., Nakayama, K., Fujita, M., et al. (2006) Two E3 ubiquitin ligases, SCF-SKP2 and DDB1-Cul4, target human Cdt1 for proteolysis *Embo J*, **25,** 1126-1136.

71. Mamillapalli, R., Gavrilova, N., Mihaylova, V.T., Tsvetkov, L.M., Wu, H., Zhang, H.,Sun, H. (2001) PTEN regulates the ubiquitin-dependent degradation of the CDK inhibitor p27(KIP1) through the ubiquitin E3 ligase SCF(SKP2) *Curr Biol*, **11,** 263-267.

Chapter 7

Summary

Summary

Uncontrolled proliferation, enhanced migration and decreased apoptosis are important hallmarks of tumor cells. A major regulator of these biological processes is the PI3K/Akt signaling pathway, of which the tumor suppressor PTEN is a key player. Due to its ability to dephosphorylate PIP3, a lipid second messenger produced by PI3K, PTEN counteracts PI3K and functionally antagonizes signaling pathways that rely on the activity of PI3K. Thereby PTEN has a major influence on the regulation of various cell biological processes. In prostate cancer *PTEN* is the most frequently inactivated gene. Complete *PTEN* inactivation is correlated with the later stages of the disease. The aim of the research described in this thesis was to gain further insight into the role of PTEN in the development and progression of prostate cancer. This will augment our understanding of prostate tumorigenesis and may result in novel treatment options for advanced disease.

In **chapter 1** an overview of the cell biological and molecular properties of PTEN is presented. The chapter starts with a general description of its structure, cell biological and molecular functions. In the second part of this chapter the role of PTEN in prostate cancer is specifically emphasized. It discusses the rate and underlying mechanism of *PTEN* inactivation and addresses the presumed role of *PTEN* haploinsufficiency in the progression of prostate cancer. Further, it describes the cell biological processes that are affected by PTEN in prostate cancer, its role in androgen independence, and the cross talk between PTEN and AR signal transduction cascades. Moreover, several prostate cancer mouse models and the prognostic value of PTEN inactivation in prostate cancer will be discussed.

Chapter 2 is focused on the role of PTEN in the regulation of the cell cycle inhibitor $p27^{kip1}$. In prostate cancer low expression of $p27^{kip1}$ is a marker of poor prognosis. The expression level of $p27^{kip1}$ is controlled by the integration of several signaling pathways, including the PTEN/Akt pathway. We described a differential effect of PTEN/Akt signaling on the expression of $p27^{kip1}$ in the three PTEN negative prostate cancer cell lines LNCaP, PC3 and PC346, and the PTEN positive cell line DU145. PTEN/Akt signaling affects the $p27^{kip1}$ expression in PC3 and DU145 cells but is not a major factor in the regulation of $p27^{kip1}$ in LNCaP and PC346. In PC3 and DU145 cells PTEN mediated its effect on $p27^{kip1}$ expression by PTEN/Akt dependent regulation of the E3 ubiquitin ligase Skp2.

Chapter 3 reported on the generation and characterization of the novel cell line LNCaP/PTEN, which stably expresses PTEN under inducible control. PTEN

expression strongly influenced proliferation by arresting the cells in the G_0/G_1 phase of the cell cycle. Throughout the years, the role of the PTEN/Akt pathway in the regulation of cell cycle proteins at the protein level by altered distribution, or phosphorylation has been extensively studied. In this chapter we showed that PTEN dependent transcriptional regulation of specific cell cycle proteins might be involved in the PTEN mediated cell cycle arrest. Micro-array analysis and q-PCR showed that E2F2, Cdc25a, RBL2 and cyclin G2 are important transcriptional targets of PTEN signaling. This PTEN regulated transcription was mediated by both Akt dependent and independent mechanisms.

In this thesis we also focused on the mechanism of *PTEN* inactivation in prostate cancer. **Chapter 4** presents a comprehensive study to detect *PTEN* inactivation in 40 locally progressive prostate cancers obtained by TURP. To assess *PTEN* inactivation in these tumor samples we utilized a variety of complementary technical approaches including allelotype analysis, dual color FISH, array-CGH, IHC and the novel WPR (wild-type/pseudogene ratio analysis) technique. Overall, the various methods showed consistent results and complete inactivation of *PTEN* was found in 12 out of 40 tumor specimens, with bi-allelic deletion being the major mechanism of PTEN inactivation.

In **chapter 5** the development and validation of quantitative wild-type/pseudogene ratio analysis (q-WPR), a novel and accurate method to detect gene copy number changes, utilizing *PTEN* in prostate cancer as a model system is described. Sensitive methods are required to assess the *PTEN* copy number in tumor samples, since loss of *PTEN* frequently encompasses a small genomic region. Q-WPR is based on the 5'-nuclease taqman assay and applies a highly homologous pseudogene as internal control, which is a major advantage over already existing genotyping methods. The method will allow high-throughput analysis of gene copy number changes of any gene with a highly homologous structurally related gene. Q-WPR was very accurate in detection of mono- and bi-allelic loss of *PTEN* and was applicable on genomic DNA from frozen and from formalin-fixed, paraffin-embedded tumor tissues.

In conclusion, the research described in this thesis has increased our knowledge about the genetic aspects, and the cell biological and molecular functions of PTEN in prostate cancer. A solid understanding of the prostate specific functions and regulation of PTEN signaling may provide opportunities for the development of novel therapeutic options.

Chapter 8

Samenvatting

Samenvatting

Vergeleken met normale cellen worden kankercellen o.a. gekenmerkt door migratie, ongecontroleerde groei en een verminderde mate van geprogrammeerde celdood. De PI3K/Akt signaaltransductieroute speelt een grote rol in de regulatie van deze celbiologische processen. Het tumorsupressor eiwit PTEN is een essentieel onderdeel van deze signaaltransductieroute. PTEN is een phosphatase met als belangrijkste substraat het phospholipide PIP3, een second messenger die geproduceerd wordt door PI3K. Door zijn vermogen om PIP3 te defosforyleren, en daarmee het effect van PI3K op de activatie van diverse signaaltransductieroutes teniet te doen, is PTEN direct betrokken bij de regulatie van verschillende celbiologische processen. Het *PTEN* gen is het meest geïnactiveerde gen in prostaatkanker. Volledige inactivatie van PTEN functie wordt vooral gevonden in de latere stadia van de ziekte. De in dit proefschrift beschreven studies naar de rol van PTEN in prostaatkanker dragen bij aan het vergroten van de kennis omtrent de moleculaire en celbiologische mechanismen die betrokken zijn bij de groei van prostaatkanker. Een beter begrip van deze mechanismen kan leiden tot de ontwikkeling van nieuwe en betere behandelingen van prostaatkanker.

Hoofdstuk 1 geeft een algemene beschrijving van de structuur en de celbiologische en moleculaire functies van PTEN. De door PTEN gereguleerde processen kunnen echter per celtype verschillen en in het tweede deel van hoofdstuk 1 wordt dan ook extra nadruk gelegd op de rol van PTEN in prostaatkanker. Belangrijke aspecten die besproken zullen worden zijn de frequentie en het mechanisme van *PTEN* inactivering en de veronderstelde rol van PTEN haplo-insufficiëntie in de progressie van prostaatkanker. Verder is er aandacht voor de door PTEN gereguleerde celbiologische processen in de prostaat, de rol van PTEN in het ontstaan van androgeen onafhankelijkheid, en de mogelijke overlap van de door PTEN, en de door de androgeenreceptor, gereguleerde signaaltransductieroutes. Tenslotte wordt de ontwikkeling van een aantal prostaatspecifieke muismodellen besproken en wordt de voorspellende waarde van een afwijking in PTEN op de progressie van prostaatkanker bediscussieerd.

In **hoofdstuk 2** wordt de rol van PTEN in de regulatie van $p27^{kip1}$ onderzocht. $P27^{kip1}$ is een eiwit dat bijdraagt aan de remming van de celcyclus en daarmee ongecontroleerde celgroei, en dus tumorvorming tegen gaat. Bij prostaatkanker is een lage expressie van $p27^{kip1}$ een indicatie voor een slechte prognose. Er zijn verschillende signaaltransductieroutes die een effect kunnen hebben op het

expressieniveau van $p27^{kip1}$, waaronder de PI3K/Akt signaaltransductieroute. Het effect van deze route op de expressie van $p27^{kip1}$ bleek nogal wisselend te zijn in de onderzochte prostaatkankercellijnen. In de PTEN negatieve prostaatkankercellijnen PC346 en LNCaP was de PI3K/Akt signaaltransductieroute geen belangrijke factor in de regulatie van $p27^{kip1}$. Dit in tegenstelling tot het effect in de derde PTEN negatieve prostaatkankercellijn PC3 en de PTEN positieve lijn DU145, waarbij activatie van de PI3K/Akt signaaltransductieroute een verminderde expressie van $p27^{kip1}$ tot gevolg had. Dit effect werd voornamelijk veroorzaakt door de PTEN/Akt afhankelijke regulatie van het E3 ubiquitine ligase Skp2, wat zorgt voor een verhoogde afbraak van $p27^{kip1}$ eiwit in het proteasoom.

In **hoofdstuk 3** wordt de ontwikkeling en de karakterisatie van de nieuwe cellijn LNCaP/PTEN beschreven. Deze cellen kunnen PTEN geïnduceerd tot expressie brengen. Expressie van PTEN in deze cellen bleek een sterk remmend effect te hebben op de celgroei, wat veroorzaakt werd door een remming van de cellen in de G_0/G_1 fase van de celcyclus. De afgelopen jaren is er veel onderzoek gedaan naar de rol van PTEN in de regulatie van de celcyclus op eiwitniveau, bijvoorbeeld door een veranderde fosforylatie of localisatie van bepaalde celcycluseiwitten. In dit hoofdstuk wordt aangetoond dat PTEN ook de transcriptie van een aantal belangrijke celcycluseiwitten kan reguleren. Micro-array analyse en q-PCR toonden aan dat de celcycluseiwitten E2F2, Cdc25a, RBL2 en cycline G2 op transcriptieniveau door PTEN gereguleerd kunnen worden. Verder onderzoek wees uit dat er zowel Akt afhankelijke als Akt onafhankelijke mechanismen betrokken zijn bij de PTEN afhankelijke transcriptieregulatie van deze genen.

Naast het bestuderen van de moleculaire functie van PTEN in prostaatkanker is er ook onderzoek gedaan naar de genetische aspecten van PTEN in prostaatkanker. En dan met name naar de frequentie en het mechanisme van *PTEN* inactivatie in klinische prostaattumoren. In de studie die beschreven wordt in **hoofdstuk 4** is onderzoek gedaan naar de inactivatie van het *PTEN* gen in 40 locaal progressieve tumoren (TURP). Er werden verschillende, elkaar aanvullende, methoden gebruikt om de inactivatie van *PTEN* te kunnen detecteren waaronder FISH, array-CGH, immunohistochemie, en de nieuw ontwikkelde methode WPR (wild-type/pseudogen ratio analyse). Over het algemeen gaven de verschillende technieken een vergelijkbaar beeld. In 12 van de 40 onderzochte tumoren werd een inactivatie van *PTEN* gevonden. De belangrijkste oorzaak van deze inactivatie bleek het verlies van beide kopieën van het *PTEN* gen te zijn.

Hoofdstuk 5 beschrijft tenslotte de ontwikkeling van q-WPR (quantitatieve-Wild-type/Pseudogen Ratio analyse), een nieuwe, gevoelige methode om het aantal kopieën van een gen te bepalen. Omdat in veel tumoren, waaronder prostaatkanker, verlies van één of twee kopieën van *PTEN* word gevonden, wordt het *PTEN* gen in prostaatkanker gebruikt als modelsysteem om de methode te valideren. Q-WPR is gebaseerd op de eerder beschreven 5'-nuclease taqman assay. Een groot voordeel van q-WPR ten opzichte van al bestaande methoden is het gebruik van een sterk homoloog pseudogen als interne controle. Q-WPR bleek zeer nauwkeurig in het bepalen van het verlies van één of twee kopieën van *PTEN*. Verder is de methode goed toepasbaar op genomisch DNA verkregen uit gefixeerd en in paraffine ingebed weefsel, wat vaak van mindere kwaliteit is. Tenslotte is q-WPR niet alleen toepasbaar op *PTEN* maar op vrijwel elk gen met een bijbehorend structureel verwant pseudogen.

Samenvattend kan gezegd worden dat de in dit proefschrift beschreven studies een belangrijke bijdrage hebben geleverd aan het vergroten van het inzicht in de rol van PTEN in prostaatkanker. Een goed inzicht in de prostaat specifieke functies van PTEN kan op termijn resulteren in de ontwikkeling van nieuwe en verbeterde therapiën voor prostaatkanker.

List of abbreviations

AR	Androgen receptor
BPH	Benign prostatic hyperplasia
BZS	Bannayan-zonana syndrome
CaP	Carcinoma of the prostate
CD	Cowden disease
CDC6	Cell division cycle 6
Cdc25a	Cell division cycle 25 homolog A
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
cDNA	Copy DNA
CDT1	Chromatin licensing and DNA replication factor 1
CGH	Comparative genomic hybridization
E2F2	E2F transcription factor 2
ERG	ETS related gene
ES cells	Embryonic stem cells
ETS	E26 transforming sequence
FACS	Fluorescent activated cell sorting
FISH	Fluorescent in situ hybridization
FOXO	Forkhead box O
GSK3	Glycogen synthase kinase-3
HD	Homozygous deletion
IGF	Insulin growth factor
IHC	Immunohistochemistry
IPA	Ingenuity pathway analysis
IRS2	Insulin receptor substrate 2
LDD	Lhermitte-duclos disease
LOH	Loss of heterozygosity
MCM10	Minichromosome maintenance 10
MDM2	Mouse double-minute 2
MEF	Mouse embryonic fibroblast
miRNA	Micro RNA
MLPA	Multiplex ligation-dependent probe amplification
MPAKT	Murine prostate restricted Akt kinase model
mRNA	Messenger RNA

mTOR	Mammalian target of rapamycin
NLS	Nuclear localization signal
PB	Probasin
PDK1	3-Phosphoinositide-dependent protein kinase-1
PDZ domain	Acronym of PSD95, DlgA, Zo-1 proteins, also known as GLGF domain (Glycine Leucine Glycine Phenylalanine)
PEST motif	Proline-Glutamic acid-Serine-Threonine motif
PHTS	PTEN hamartoma tumor syndrome
PI3K	Phosphatidyl inositol- 3 kinase
PIN	Prostatic intraepithelial neoplasia
PSA	Prostate specific antigen
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PTP	Protein tyrosine phosphatases
Q-PCR	Quantitative polymerase chain reaction
Q-WPR	Quantitative wild-type to pseudogene ratio
RBL2	Retino blastoma-like 2
RP	Radical prostatectomy
RT-PCR	Reverse transcription polymerase chain reaction
siRNA	Small interfering RNA
Skp2	S-phase kinase associated protein 2
SNP	Single nucleotide polymorphism
SSCP	Single strand conformation polymorphism
TMPRSS2	Transmembrane Protease, Serine 2
TRAMP	Transgenic adenocarcinoma mouse prostate
TURP	Trans urethral resection of the prostate
UTR	Untranslated region
WPR	Wild-type to pseudogene ratio

Curriculum Vitae

Petra Wilhelmina van Duijn werd geboren op 9 februari 1976 te Utrecht. In 1994 behaalde zij haar VWO-diploma aan het Dr. F.H. de Bruijne Lyceum te Utrecht, waarna zij startte met een studie Biologie aan de Universiteit Utrecht. Tijdens deze studie verrichtte zij een stage op de afdeling Celbiologie van de faculteit Geneeskunde van de Universiteit Utrecht in de groep van Dr. P. van der Sluijs. Haar hoofdvakstage volgde zij op de afdeling Moleculaire Celbiologie van de faculteit Biologie onder leiding van Prof. dr. S. Smeekens. In augustus 1999 werd het doctoraalexamen behaald. In oktober 1999 begon zij haar promotieonderzoek aan de Erasmus Universiteit Rotterdam op de afdeling Pathologie in de groep van Prof. dr. J. Trapman, waarvan de resultaten staan beschreven in dit proefschrift. Van 2006 tot 2008 werkte zij als postdoc bij DeltaCell b.v. te Leiden. Vanaf oktober 2008 zal zij werkzaam zijn als postdoc bij het Hubrecht Instituut in de groep van Prof. dr. J. den Hertog.

List of publications

Van Duijn, P.W. and Trapman, J. PI3K/Akt signaling regulates $p27^{kip1}$ expression via Skp2 in PC3 and DU145 prostate cancer cells, but is not a major factor in $p27^{kip1}$ regulation in LNCaP and PC346 cells. *The Prostate* 66(7): 749-760 (2006).

Verhagen, P.C.M.S., **Van Duijn, P.W.,** Hermans, K.G.L., Looijenga, L.H.J., Van Gurp, R.J.H.L.M., Stoop, H., Van der Kwast, T.H. and Trapman, J. The pten gene in locally progressive prostate cancer is preferentially inactivated by bi-allelic gene deletion. *Journal of Pathology* 208(5): 699-707 (2006).

van Duijn, **P.W**., van der Korput, J.A.G.M., Ziel-van der Made, A.C.J., and Trapman, J. PTEN mediated G1 growth arrest in LNCaP prostate cancer cells is associated with transcriptional regulation of cell cycle regulators.
Submitted

van Duijn, P.W., Hermans, K.G.L., and Trapman, J. Quantitative Wildtype to Pseudogen (q-WPR) Ratio analysis: a novel method to detect gene copy number changes.
Submitted

Ma, X., Van der Made, A.C., Autar, B., Van der Korput, H.A., Vermeij, M., **Van Duijn, P.,** Cleutjens, K.B., de Krijger, R., Krimpenfort, P., Berns, A., Van der Kwast, T. H. and Trapman, J. Targeted biallelic inactivation of Pten in the mouse prostate leads to prostate cancer accompanied by increased epithelial cell proliferation but not by reduced apoptosis. *Cancer Research* 65: 5730-5739 (2005)

Dankwoord

Na een aantal jaren hard werken is het dan toch zover, mijn boekje is *bijna* af!
Bijna, omdat ik tenslotte nog iedereen wil bedanken die heeft bijgedragen aan het tot stand komen van dit proefschrift. Zonder iemand te kort te willen doen, wil ik echter een aantal mensen in het bijzonder bedanken.

In de eerste plaats mijn promotor Jan Trapman. Jan, ik wil je bedanken voor de mogelijkheid die je me hebt gegeven om mijn promotie tot een goed einde te brengen. Als directe begeleider was je nauw betrokken bij mijn onderzoek. Van je kritische kijk op mijn werk heb ik veel geleerd. Bedankt hiervoor.

Natuurlijk wil ik ook al mijn labgenoten van Be320 bedanken voor de gezelligheid, hun steun en interesse van de afgelopen jaren. Hetty, je hebt me wegwijs gemaakt op het lab en ik heb veel van je geleerd. Later was je ook als kamergenoot erg gezellig. Angelique ook jij bedankt voor je gezelligheid en vooral ook voor alle Q-PCRs die je nog voor me gedaan hebt toen ik allang weg was. Zonder jou en Hetty had hoofdstuk 3 er heel anders uitgezien. Erik-jan, behalve een fijne collega ook een stadsgenoot. We hebben dan ook heel wat gesprekken gevoerd tijdens de treinreis of meestal nog gedurende de fietstocht van het station naar het lab. Ik heb veel gehad aan je vaak nuttige adviezen. Karin, bedankt voor alle array-CGH plaatjes en heel veel succes in Toronto.

Speciale dank ook voor mijn kamergenoten, Karine, Pascal, Hanneke, Martin en Hetty. Bedankt voor jullie gezelschap, advies en hulp bij computercrissisen. Martin, nog drie weken! Ik kijk er naar uit. Hanneke, ik heb de afgelopen jaren veel plezier beleefd aan onze gesprekken over van alles en nog wat. Ook na mijn vertrek hield je me op de hoogte van de gebeurtenissen en de laatste nieuwtjes van het lab. Leuk dat je mijn paranimf wilt zijn!

Verder wil ik ook alle andere collega's van de afdelingen Pathologie en Urologie bedanken voor hun hulp en interesse. Ook mijn ex-collega's van DeltaCell, bedankt voor het meeleven met de laatste loodjes.

En tenslotte natuurlijk papa, mama, Es en Peter, bedankt voor het vertrouwen en jullie onvoorwaardelijke steun, ik moet toegeven jullie hadden gelijk, het is goed gekomen. Es, onze shop-sessies en andere uitstapjes waren een welkome afleiding in soms drukke tijden. Ik ben heel blij met jou als zus en natuurlijk ook als paranimf (met ervaring én nu zelfs dubbele support!!! zit het helemaal goed). En pap, mam, nogmaals bedankt voor alles!!