Targeted Biallelic Inactivation of Pten in the Mouse Prostate Leads to Prostate Cancer Accompanied by Increased Epithelial Cell Proliferation but not by Reduced Apoptosis

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Abstract

The PTEN tumor suppressor gene is frequently inactivated in human tumors, including prostate cancer. Based on the Cre/loxP system, we generated a novel mouse prostate cancer model by targeted inactivation of the Pten gene. In this model, Cre recombinase was expressed under the control of the prostate-specific antigen (PSA) promoter. Conditional biallelic and monoallelic Pten knock-out mice were viable and Pten recombination was prostate-specific. Mouse cohorts were systematically characterized at 4 to 5, 7 to 9, and 10 to 14 months. A slightly increased proliferation rate of epithelial cells was observed in all prostate lobes of monoallelic Pten knock-out mice (PSA-Cre:Pten-loxP/loxP), but minimal pathologic changes were detected. All homozygous knock-out mice (PSA-Cre:Pten-loxP/loxP) showed an increased size of the luminal epithelial cells, large areas of hyperplasia, focal prostate intraepithelial neoplasia lesions and an increased prostate weight at 4 to 5 months. More extensive prostate intraepithelial neoplasia and focal microinvasion occurred at 7 to 9 months; invasive prostate carcinoma was detected in all male PSA-Cre:Pten-loxP/loxP mice at 10 to 14 months. At 15 to 16 months, a rare lymph node metastasis was found. In hyperplastic cells and in tumor cells, the expression of phospho-AKT was up-regulated. In hyperplastic and tumor cells, expression of luminal epithelial cell cytokeratins was up-regulated; tumor cells were negative for basal epithelial cell cytokeratins. Androgen receptor expression remained detectable at all stages of tumor development. The up-regulation of phospho-AKT correlated with an increased proliferation rate of the epithelial cells, but not with a reduced apoptosis. (Cancer Res 2005; 65(13): 5730-9)

Introduction

The PTEN tumor suppressor gene, also known as MMAC1 or TEP1, is one of the most frequently inactivated genes in human cancer (1–4). The main molecular function of PTEN is counteracting phosphoinositide-3-kinase signaling by removing the D3 phosphate from the inositol ring of phosphatidylinositol (3,4,5)-phosphate. The kinase AKT/PKB is the major downstream target of phosphatidylinositol (3,4,5)-phosphate (5, 6). Functional loss of PTEN results in the accumulation of phosphatidylinositol (3,4,5)-phosphate and up-regulation of phospho-AKT, consequently modulating the activities of its downstream effectors. These molecular changes can affect many biological functions of cells such as proliferation, apoptosis, cell size, cell adhesion, and migration (7–12).

Germ line mutations of PTEN are the cause of Cowden’s disease, Bannayan-Zonana syndrome and Lhermitte-Duclos disease, which are characterized by the development of hamartomas and an increased risk of cancer (13–15). Somatic mutations of PTEN have been detected at high frequency in many sporadic cancers, including glioblastoma, endometrial cancer and prostate cancer (4). Complete PTEN inactivation has been found in ~15% of primary prostate tumors, and in up to 60% of prostate cancer metastases and prostate cancer xenografts (16–19). Immunohistochemical studies showed the absence of PTEN in 20% of primary prostate tumors (20).

Pten is essential in mouse development (21). Conventional Pten−/− mice show developmental defects and die at embryonic days 6.5 to 9.5 (22–24). Pten+/− mice are viable and display a variety of hyperplastic and dysplastic changes in different tissues. Female heterozygous mice mainly develop endometrial and breast tumors (25), most male Pten+/− mice develop lymphoma (23–25), and at lower frequency, hyperplasia and dysplasia of several other tissues, including the prostate. Tumor development might be associated with loss of the wild-type Pten allele (23).

Biallelic conditional knock-out mice completely lacking Pten function might develop tumors in specific tissues (26). Recently, it has been shown that prostate-targeted Pten knock-out mice develop hyperplasia, and at older age, metastatic tumors in the prostate (27–29). In these studies, Cre expression was regulated by the mouse mammary tumor virus (MMTV) promoter or a modified probasin promoter. We generated a novel targeted Pten mouse prostate cancer model, applying the well-characterized prostate-specific antigen (PSA) promoter (30, 31) to drive prostate-specific Cre expression. The Pten gene was inactivated by PSA-Cre targeted deletion of exon 5, resulting in the expression of a truncated protein lacking the phosphatase domain. The same deletion has previously been found in a human prostate cancer xenograft (18). Cohorts of male PSA-Cre:Pten-loxP/loxP and PSA-Cre:Pten-loxP/+/ mice, and control littermates were systematically characterized at different ages. All PSA-Cre:Pten-loxP/loxP mice showed the gradual development of prostate hyperplasia, followed by prostate intraepithelial neoplasia (PIN) lesions and prostate cancer. Hyperplasia, PIN, and cancer were accompanied by increased cell size and epithelial cell proliferation, but not by reduced apoptosis. Prostate epithelial cells in all stages of tumor development showed increased expression of cytokeratins. The effect of monoallelic...
prostate-specific deletion of Pten was limited. At old age, focal
dysplasia was observed in some mice, but none of the PSA-
Cre;Pten-loxP/+ mice developed a prostate tumor.

Materials and Methods

Generation of prostate-targeted Pten knock-out mice. The PSA-Cre
construct was generated by linkage of the 6 kb HindIII/HindIII fragment of
the human PSA promoter to Cre cDNA. The PSA promoter has been proven
to target transgene expression to the luminal epithelial cells of the prostate
(30). The mouse prostate gene provided an intron and the 3’- untranslated
region, including the polyadenylation signal (Fig. 1A). Transgenic PSA-Cre
founders were generated by injection of a 7.5 kb PSA-Cre-Prot fragment
into the pronuclei of fertilized oocytes of FVB mice. Prostate-specific
expression of the transgene was assessed by crossing with CAG-loxP-CAT-
loxP-LacZ indicator mice (ref. 32; data not shown). The founder line PSA-
CreD4 showing high, prostate-specific Cre activity was used in the
experiments described in this study.

The generation of mice (strain 129Ola) carrying the Pten-loxP allele has
been described elsewhere (33). In these mice, Pten was targeted by
introduction of a loxP recognition sequence and a flanking diagnostic
EcoRI site in intron 5, and a neomycin (neo) cassette flanked by loxP sites
in intron 4 (Fig. 1A). A 9 kb fragment, encompassing the targeted Pten exon 5,
was electroporated into the embryonic stem cell line E14 subclone IB10 (34).
By crossbreeding PSA-Cre transgenic mice with Pten-loxP/loxP mice and
subsequent breeding of PSA-Cre;Pten-loxP/+ F1 offspring with Pten-loxP/
loxP mice, biallelic and monoallelic prostate-targeted Pten knock-out mice
were generated. Cre negative Pten-loxP/loxP littermates were kept as
controls. All experiments were done in a 129Ola/FVB background. Mice
were housed according to institutional guidelines, and procedures
were carried out in compliance with the standards for use of laboratory animals.

PCR for genotyping and detection of specific recombination.

Genomic DNA was isolated from tail tips by standard procedures. PSA-Cre
mice were genotyped by standard PCR, using two sets of primers: 5’-
CCTCTGGAGATGGGATTAG3’ (Cre-F) and 5’-GGACTTGCTC-
TATTCTGTGCATCTAG3’ (Pro-R) for amplification of Cre cDNA and
the prostate 3’-untranslated region, and 5’-CTTTGATGGTGACCAGAGCAG3’
(PSA-F) and 5’-CGACGGGATCTCTGCAAAGATG3’ (PSA-R) to
amplify the PSA promoter.

The primers used for genotyping of Pten-loxP mice by standard PCR were
5’-GCTTCACTTAGTAAAGCAG3’ (In5F2) and 5’-GCGAAAGATC-
TCTTGGTTAC3’ (In5R3). For detection of Pten recombination in each prostate
in genomic DNA, we used the primers 5’-TGCTGAATGAGAAGATG3’
(In5F2) and In5R3. Primers for amplification of the control genomic Gapdh
fragment: 5’-TCCGCCCCCTCCGTGATGCC-3’ (Gapdh1) and 5’-
TAGTGCGCCCTGCGGCGCCGTG-3’ (Gapdh2). For long-range PCR, we
used the PCR core kit of Qiagen (Hilden, Germany).

Southern blotting. The Genta DNA isolation kit (BIOzym, Landgraffa,
the Netherlands) was used for isolation of genomic DNA. BglII-digested
DNA was loaded on a 1% agarose gel in TAE buffer. Next, the DNA was
blotted on Hybond-N+ membrane (Amersham Biosciences, Little Chalfont,
England), and hybridized with a 32P-labeled Pten probe. Following washing
under high stringent conditions, the filter was exposed to X-ray
film. The 320 bp hybridization probe was generated by PCR on
Pten intron 5 in vector DNA (Fig. 1A). PCR primers were 5’-CTGATATGAGCATGGACCTTG3’
(Pten-SA-F) and 5’-GGCGCTTAAGAGTCTTCCACA3’ (Pten-SA-R).

Histology and immunohistochemistry. Tissues for histology were fixed
in freshly prepared buffered 4% formalin for 16 hours at 4°C, dehydrated
and embedded in paraffin. Five micrometer sections were attached to AAS-
coated slides and H&E-stained. The antibodies used for immunostaining
were directed against basal epithelial cell cytokeratins (mouse monoclonal
antibody 34E12, diluted 1:1000; DAKO, Glostrup, Denmark), phospho-AKT
(rabbit polyclonal antisemur, 1:200; Cell Signaling Technology, Beverly, MA),
androgen receptor (rabbit polyclonal antisemur SP197, direct against the NH2
terminus (1:500)), and luminal epithelial cytokeratin (mouse monoclonal
antibody, 1:50; BD, San Diego, CA). Microwave pretreatment for antigen
unmasking was applied by boiling in 10 mmol/L sodium citrate for 15
minutes and cooling at room temperature for 1 hour. Primary antibodies
were incubated overnight at 4°C, and biotin-labeled secondary antibodies
(1:400, DAKO) were incubated at room temperature for 1 hour. Immune-
activity was visualized by the streptavidin-peroxidase (1:50, BioGenex, San
Ramon, CA) or streptavidin-alkaline phosphatase (1:50, BioGenex) systems
(for phospho-AKT).

Western blotting. Frozen prostate lobes were pulverized under liquid
nitrogen and tissue powder was transferred into 1× Laemmli sample buffer
(10 μl sample buffer per microgram of tissue powder). The samples were
sonicated for 2×20 seconds, boiled for 5 minutes, and centrifuged
(5 minutes at 14,000 rpm, Eppendorf centrifuge). Equal amounts of protein
were subjected to 10% SDS-PAGE. After electrophoresis, proteins were
transferred to protran membrane (Schleicher & Schuell, Dassel, Germany)
and immunoblotted. Primary antibodies used were rabbit polyclonal
antibodies to AKT, phospho-AKT (Ser473, Cell Signaling Technology),
and Pten (Neomarkers, Fremont, CA; all at 1:1000) and mouse monoclonal
α-actin antibody (Sigma, St. Louis, MO; 1:10,000).

Analysis of cell proliferation and apoptosis. For assessing in vivo cell
proliferation, mice were subjected to i.p. injection of bromodeoxyuridine
(BrdUrd, Sigma; 40 mg/kg body weight) at 30 minutes prior to sacrifice.
Formalin-fixed, paraflin-embedded tissue slides from prostate lobes were
treated with 1% protease (Sigma) for 10 minutes at 37°C, rinsed with cold
PBS and incubated in 2 mol/L HCl for 30 minutes at 37°C. Primary mouse
monoclonal BrdUrd antibody IB5 (1:200, donated by Dr. Frans Ramaekers,
Department of Pathology, University of Maastricht, Maastricht, the Nether-
lands) was applied for overnight incubation at 4°C. Secondary antibody
incubation and visualization were as described above. The proliferation
index was calculated as number of BrdUrd positive cells per 1000 adjacent
luminal epithelial cells from three independent counts. The Student’s t test
was applied for statistical analyses.

For assessment of apoptosis, tissue sections of prostate lobes were
immunostained for active caspase-3 positive cells (R&D System, Minneap-
olis, MN; 1:500; ref. 35). The apoptotic index was calculated as the number
of active caspase-3-positive cells per 1,000 luminal epithelial cells. Tissue
pretreatment, visualization of immunoreactivity, calculations, and statistics
were as described above. The frequently applied terminal nucleotidyl
transferase-mediated nick end labeling (TUNEL) assay gave less reproduc-
ible results, in accordance with previously published findings (36).

Results

Pten recombination occurs in the prostates of conditional
knock-out mice and gives rise to high-level phospho-AKT in
bimalleolar knock-out mice. At 4 to 5 months, genomic DNA was
isolated from 19 different tissues of Cre-positive mice and PCR was
done to determine Pten recombination. As visualized by the
presence of a 330 bp band, Cre-specific recombination of Pten
exon 5 was detected in the anterior, dorsolateral, and ventral prostate
lobes of PSA-Cre;Pten-loxP/loxP and PSA-Cre;Pten-loxP/+ mice
(Fig. 1B, lanes 1-3). Pten recombination efficiency was somewhat
variable in individual mice, however, all Cre-positive mice displayed
Pten recombination in each prostate lobe. None of the other
investigated tissues contained the recombined band (Fig. 1B, lanes
4-19), except for occasional recombination in adrenal glands (data
not shown). We were unable to detect a recombinant band in the
first weeks after birth (data not shown). To obtain insight into the
Pten recombination efficiency, long-range PCR over exon 5 was
carried out. Primers detected both the recombinated and the
nonrecombined allele (Fig. 1C). In prostate DNA of both PSA-Cre;Pten-
loxP/+ and PSA-Cre;Pten-loxP/loxP mice, the recombinated band
was clearly visible (Fig. 1C, lanes 4 and 6). Southern blotting of prostate
DNA from mice of each genotype confirmed the PCR data (Fig. 1D).

In PSA-Cre;Pten-loxP/loxP mice, >50% of Pten was recombinated in the
prostate (Fig. 1D, lane 3); as expected in PSA-Cre;Pten-loxP/+ mice, the recombinated 5.7 kb band was less pronounced (Fig. 1D,

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We systematically examined the secretion rate of prostate epithelial cells, but is not sufficient for or two functional prostates; it was low in mice carrying one PSA-Cre;Pten-loxP/loxP prostate. Phospho-AKT expression was substantially increased in prostate lobes, lowest expression was observed in the anterior mice, Pten expression seemed to be different in the distinct Pten expression level in PSA-Cre;Pten-loxP/+ completely absent in PSA-Cre;Pten-loxP/loxP mice was most likely derived from cells lacking Cre expression, e.g., stromal cells.

As shown by immunoblotting, Pten expression was almost completely absent in PSA-Cre;Pten-loxP/loxP prostate lobes; the Pten expression level in PSA-Cre;Pten-loxP/+ prostates was not obviously different from control prostates (Fig. 1E). In control mice, Pten expression seemed to be different in the distinct prostate lobes, lowest expression was observed in the anterior prostate. Phospho-AKT expression was substantially increased in PSA-Cre;Pten-loxP/+ prostates; it was low in mice carrying one or two functional Pten alleles.

Monoallelic inactivation of Pten slightly affects the proliferation rate of prostate epithelial cells, but is not sufficient for prostate cancer development. We systematically examined the prostate lobes of three different genotypes of mice. There was no obvious down-regulation of PTEN or up-regulation of phospho-AKT in the prostate lobes of PSA-Cre;Pten-loxP/loxP mice (lanes 3 and 4), and PSA-Cre;Pten-loxP/+ mice (lanes 5 and 6). Lanes 1, 3, and 5, DNA from spleen; lanes 2, 4 and 6, DNA from the anterior prostate lobe. The 2 kbp band indicates no recombination. The 2.3 kbp fragment represents the 2.3 kbp fragment of the PSA-Cre;Pten-loxP/+ mouse. Recombination-specific fragments were detected in the prostate lobes, not in the remaining tissues. Gapdh was used as a loading control: 1, anterior prostate; 2, ventral prostate; 3, dorsolateral prostate; 4, epididymis; 5, vas deferens; 6, testis; 7, seminal vesicle; 8, kidney; 9, bladder; 10, liver; 11, spleen; 12, lung; 13, heart; 14, thymus; 15, salivary gland; 16, brain; 17, thymus; 18, pituitary gland; 19, adrenal.

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from at least 10 PSA-Cre;Pten-loxP/loxP mice at three time points (see Fig. 3). The PSA-Cre;Pten-loxP/loxP mice showed progressive tumor development, including hyperplasia and focal PIN lesions at 4 to 5 months, more widespread PIN and focal microinvasion at 7 to 9 months, and invasive tumors at 10 to 14 months (see Table 1). All prostate lobes were affected, however, the pathologic changes in dorsolateral and anterior lobes were more prominent than in ventral lobes.

At 4 to 5 months, most glands of the different prostate lobes of PSA-Cre;Pten-loxP/loxP mice displayed hyperplasia manifested by an accumulation of luminal cells within the glandular lumen (Fig. 3A-D). This was associated with a distension of the individual preexisting glands, whereas the borders of the glands remained well-circumscribed. Compared with the regularly layered columnar epithelial lining of prostate tubules in the control littermates, a lining by disorganized multilayered ductal epithelial cells with tufting and/or cribriform growth pattern was observed. The epithelial cells in the prostates of knock-out mice were generally enlarged. Some foci showed distinguished epithelial dysplasia characterized by a number of distinct atypical epithelial cells possessing dense cytoplasm, enlarged and somewhat vesicular nuclei with more conspicuous nucleoli (Fig. 3A-D). These lesions shared the histologic features of human PIN that is widely accepted as a precursor lesion of prostate cancer (37).

At 7 to 9 months, the process of hyperplasia and distension of preexisting glands continued, whereas dysplasia became more diffuse (about 40-50% luminal epithelial cells involved) with more extensive cytonuclear atypia (Fig. 3E and F). In some cases, the border of the hyperplastic glands was more irregular as a consequence of outpouchings of glandular structures, displaying some dysplasia. The latter phenomenon might be considered as microinvasive carcinoma. This was also found in association with hyperplastic glands.

In all 10- to 14-month-old PSA-Cre;Pten-loxP/loxP mice, carcinoma was observed in common with more extensive involvement of the prostate lobes (Table 1). All tumors were of epithelial origin; histopathologic features classified to varied stages of adenocarcinoma like relatively low and moderately grade-differentiated (Fig. 3G-I), and poorly differentiated adenocarcinoma (Fig. 3K and L). In general, a rapid transition was seen from glandular formations of cancer cells characteristic of low-grade adenocarcinoma to solid fields of carcinoma lacking features of glandular differentiation, suggestive of a rapid process of dedifferentiation.

To further characterize the prostates of PSA-Cre;Pten-loxP/loxP knock-out mice, several immunohistochemical stainings were done on formalin-fixed tissue sections. Phospho-AKT expression

![Figure 2](image-url)

**Figure 2.** The effect of monoallelic inactivation of Pten on the mouse prostate. A, comparison of prostate weight between PSA-Cre;Pten-loxP/+ and control mice. One of each prostate lobe was measured; 9, 6, and 17 mice were analyzed for control mice ages 4 to 5, 7 to 9, and 10 to 14 months, respectively; and 12, 7, and 13 mice for PSA-Cre;Pten-loxP/+ mice ages 4 to 5, 7 to 9, and 10 to 14 months, respectively; bars, SE. B, proliferation rate of prostate epithelial cells. BrdUrd-positive cells per 1,000 epithelial cells were counted. There were 9, 5, and 8 mice for PSA-Cre;Pten-loxP/+ mice ages 4 to 5, 7 to 9, and 10 to 14 months, respectively; and 10, 6, 9 mice for the control group ages 4 to 5, 7 to 9, and 10 to 14 months, respectively; bars, SE. C-F, comparison of histologic features of prostates from PSA-Cre;Pten-loxP/+ mice and control littermates at 10 to 14 months. C and E, prostate section from control mice, representing anterior and dorsolateral lobes, respectively. D and F, prostate sections from PSA-Cre;Pten-loxP/+ mice, representing anterior and dorsolateral lobes, respectively. There are no obvious pathologic changes in most PSA-Cre;Pten-loxP/+ prostates (D). Arrows, focal epithelial dysplasia (F). Magnification, ×200 (C-F).

![Table 1](image-url)

**Table 1.** Morphologic alterations in the prostates of targeted Pten knock-out mice

<table>
<thead>
<tr>
<th>Mouse genotype</th>
<th>4 to 5 months</th>
<th>7 to 9 months</th>
<th>10 to 14 months</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>10 of 10 normal</td>
<td>10 of 10 normal</td>
<td>8 of 8 normal</td>
</tr>
<tr>
<td>PSA-Cre; Pten-loxP/+</td>
<td>12 of 12 normal</td>
<td>10 of 10 normal</td>
<td>2 of 10 focal</td>
</tr>
<tr>
<td>PSA-Cre; Pten-loxP/loxP</td>
<td>12 of 12 hyperplasia,</td>
<td>11 of 11 hyperplasia/PIN,</td>
<td>17 of 17 carcinoma</td>
</tr>
<tr>
<td></td>
<td>7 of 12 focal PIN</td>
<td>5 of 11 focal microinvasion</td>
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</tbody>
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was up-regulated in hyperplasia/PIN lesions and prostate cancer (Fig. 4A–C), confirming the immunoblot results shown in Fig. 1E and indicating that inactivation of Pten efficiently triggered direct downstream targets. Basal cell cytokeratin staining showed that the continuous basal cell layers in the prostate of control mice became fragmented in PIN lesions at 4 to 5 months. Basal cells almost disappeared in prostate cancer, although they were clearly detectable in adjacent benign glands (Fig. 4D–F). As expected, the luminal epithelial cells in normal prostate were androgen receptor–positive. We also observed ~90% positive staining of epithelial cells both in hyperplasia/PIN and cancer (Fig. 4G–I), indicating that the tumors might be sensitive to hormone deprivation treatment. The expression level and pattern of luminal epithelial cytokeratins were changed in PSA-Cre;Pten-loxP/loxP prostates, showing dominant membrane staining in hyperplasia/PIN and prostate adenocarcinoma as compared with the local staining at the epithelial cell surface in the prostate acini of normal control mice (Fig. 4J–L).
Prostate hyperplasia and cancer are associated with increased proliferation rate but do not correlate with a decreased apoptotic index in biallelic Pten knock-out mice. Macroscopic examination showed an obvious enlargement of each prostate lobe in PSA-Cre;Pten-loxP/loxP mice at the age of 4 to 5 months. Compared with the control mice, the average weight of PSA-Cre;Pten-loxP/loxP prostates was more than 2-fold increased at 4 to 5 months and almost 3-fold increased at 7 to 9 months. In prostate cancer at 10 to 14 months, the average weight had rapidly increased (Fig. 5A).

BrdUrd and activated caspase-3 immunostaining were done to examine whether the increased prostate weight was associated with stimulation of proliferation and inhibition of apoptosis. The number of BrdUrd-positive cells in prostates of PSA-Cre;Pten-loxP/loxP mice was significantly increased as compared with the controls at all three time points, the highest number of BrdUrd-positive cells was detected in the 10- to 14-month-old group (Fig. 5B, D-F). The proliferation rate correlated with the increase of prostate weight. Interestingly, we detected more apoptotic cells in the prostates of PSA-Cre;Pten-loxP/loxP mice as well. The average number of activated caspase-3-positive cells was significantly higher in the prostates of PSA-Cre;Pten-loxP/loxP mice than in control mice at all three time points (Fig. 5C, G-I). The apoptotic index did not vary much at different ages, indicating that enhanced apoptosis already occurred at early stages of tumor development.

Complete inactivation of Pten in the prostate leads to rare metastatic cancer at old age. In order to identify any metastases in multiple organs, including lymphoid nodes, liver and lung, nine PSA-Cre;Pten-loxP/loxP mice (10-16 months old) were systematically screened. Serial sections of each organ were collected and H&E stained. We found one case of metastatic adenocarcinoma seeding at the subcapsular sinusoidal region of a caudal para-aortic lymph node (Fig. 6A and B). Phospho-AKT and androgen receptor staining showed a moderate expression level of both proteins (Fig. 6C and D). The metastatic locus was tiny, hence, we might have missed even smaller metastases in other mice.

Discussion

PTEN inactivation is the most frequent genetic alteration in human prostate cancer (16, 18, 19, 38, 39). The main molecular function of PTEN is to counteract phosphoinositide-3-kinase in regulating the phosphatidylinositol (3,4,5)-phosphate level in the cell. In vitro and in vivo studies have shown that PTEN affects many biological functions of the cell, including proliferation, differentiation, apoptosis, polarity, and size (21, 40). Because of the

![Figure 4](https://www.aacrjournals.org/CancerRes/065_13_5735_Fig4a.png)
importance of PTEN in tumorigenesis and the complexity of its biological function, much effort has been put in the characterization of \textit{Pten} knock-out mice. Initially, several groups reported the properties of conventional \textit{Pten} knock-out mice (9, 22–24, 41). In all studies, \textit{Pten} \textit{-/-} conferred embryonic lethality whereas \textit{Pten} \textit{+/-} mice developed hyperplasia, and to a variable extent dysplasia and tumors in a variety of organs. \textit{Pten} \textit{+/-} mice most frequently developed lymphoma and intestinal polyps, and in female mice, breast cancer and endometrial cancer, indicating the tissue-dependent role of \textit{Pten} inactivation in tumorigenesis.

The reported pathologic changes in the prostate of \textit{Pten} \textit{+/-} mice were variable, and included various stages of hyperplasia and dysplasia. Prostate cancer was rare, possibly because mice died earlier from more rapidly developing malignancies, e.g., lymphoma, hampering more detailed analyses of prostate cancer development. Importantly, hypomorphic \textit{Pten} (\textit{Pten hy/-}) mutant mice showing an even lower level of \textit{Pten} expression than \textit{Pten} \textit{+/-} mice more rapidly developed prostate hyperplasia that in a proportion of cases progressed to focal invasive carcinoma at older age, indicating \textit{Pten} dose-dependent development of prostate lesions (27). Obviously, a drawback of conventional knock-out mice is that all cell types in all tissues are affected.

Embryonic lethality of \textit{Pten} \textit{-/-} mice and pleiotropic effects in \textit{Pten} \textit{+/-} mice have been overcome by tissue-specific \textit{Pten} inactivation. Tissue-specific biallelic \textit{Pten} inactivation has been reported for thymus, mammary gland, testis, liver, spleen, and brain (33, 42–47). In some tissues, \textit{Pten} inactivation induced tumors, including T cell lymphoma, germ cell tumors, mammary

Figure 5. Increased prostate weight is associated with an increased proliferation rate and an increased apoptotic index in PSA-Cre;\textit{Pten-loxP/loxP} mice. A, the average weight of prostates from normal adult mice and biallelic \textit{Pten} knock-out mice. There were 9, 7, and 17 mice analyzed for control mice ages 4 to 5, 7 to 9, and 10 to 14 months; and 9, 12, and 18 mice analyzed for PSA-Cre;\textit{Pten-loxP/loxP} mice ages 4 to 5, 7 to 9, and 10 and 14 months, respectively; bars, SE. B, the average number of BrdUrd-positive epithelial cells. There were 9, 5, and 9 animals analyzed for control mice ages 4 to 5, 7 to 9, and 10 to 14 months, respectively; and 10, 10, and 9 animals analyzed for PSA-Cre;\textit{Pten-loxP/loxP} mice ages 4 to 5, 7 to 9, and 10 to 14 months, respectively. Numbers indicate BrdUrd-positive cells per 1,000 epithelial cells; bars, SE. C, the average number of positive cells for active caspase-3 staining. There were 10, 6, and 6 mice for control littermates ages 4 to 5, 7 to 9, and 10 to 14 months, respectively; and there were 10, 10, and 8 mice for PSA-Cre;\textit{Pten-loxP/loxP} mice ages 4 to 5, 7 to 9, and 10 to 14 months. The number of caspase-3-positive cells is indicated as positive cells per 1,000 epithelial cells; bars, SE. D-F, examples of BrdUrd staining in a control mouse prostate (\textit{D}), a PSA-Cre;\textit{Pten-loxP/loxP} mouse prostate at 4 to 5 months (\textit{E}) and a prostate from PSA-Cre;\textit{Pten-loxP/loxP} mouse at 10 to 14 months (\textit{F}). Compared with the number of positive epithelial cells in the prostate of normal 4- to 5-month-old littermates (\textit{D}), an increased number of positively stained epithelial cells was observed in hyperplasia/PIN lesions from 4- to 5-month-old PSA-Cre;\textit{Pten-loxP/loxP} mice (\textit{E}). An even further increased number of positive staining epithelial cells was observed in adenocarcinoma from 10- to 14-month-old PSA-Cre;\textit{Pten-loxP/loxP} mice (\textit{F}). G-I, examples of active caspase-3-staining cells in the prostates of control mice (\textit{G}) and prostates of PSA-Cre;\textit{Pten-loxP/loxP} mice at 4 to 5 months (\textit{H}) and at 10 to 14 months (\textit{I}). There are hardly any positive cells in normal adult prostate (\textit{G}), the number of staining cells increased during tumorigenesis (\textit{H} and \textit{I}). Magnifications, (\textit{D-I}) ×400.
Prostate-Targeted Pten Mutant Mice

tumors, and hepatocellular carcinoma. Interestingly, in other tissues, like spleen and brain, complete Pten inactivation did not give rise to tumor formation, although inducing a variety of morphologic abnormalities and molecular alterations (33, 44). Taken together, it seems that tumorigenesis or developmental abnormalities caused by Pten inactivation are rather dependent on tempo-spatial inactivation in certain tissue/cell types.

Complementary to the prostate cancer model presented here, two models have recently been described, based on prostate-specific inactivation of Pten in the Cre-loxP system, one using the MMTV promoter and a second using a (modified) rat probasin promoter for Cre expression, respectively (27–29). Although there are parallels, the models differ in various aspects. Differences might be explained by differences in activity and cell specificity of the promoters driving Cre expression, and different genetic backgrounds. MMTV-Cre:Pten-loxP/loxP mice have a C57BL6/129 background, PB-Cre4:Pten-loxP/loxP mice have a complex C57BL/6xDBA2/129/BalbC background, and PSA-Cre:Pten-loxP/loxP mice are 129Ola/FVB. MMTV-Cre-based knock-out mice showed limited tissue specificity, resulting in Pten recombination not only in the prostate but also in several other tissues, including skin, thymus, and mammary gland (29). In MMTV-Cre:Pten-loxP/loxP mice, hyperplastic prostates were detected a few days after birth; PIN lesions were present as early as 2 weeks postnatally. Focal invasion occurred at 6 weeks and invasive adenocarcinoma at 9 weeks of age. Metastases were detected in half of the older mice. In our study, detectable Pten recombination appeared at ~4 weeks (data not shown). PSA-Cre:Pten-loxP/loxP mice showed massive hyperplasia and focal PIN at 16 to 20 weeks. At older age, more extensive PIN developed, followed by invasive carcinoma (Table 1); one lymph node metastasis was found. We propose that in addition to the different genetic backgrounds, the differences in activity and cell specificity of the probasin and PSA promoters are the main underlying mechanisms responsible for the phenotypic differences between our PSA-Cre-based model and PB-Cre-based model. It can be presumed that the probasin promoter is active in more primitive progenitor cells of mature epithelial cells than the PSA promoter. Although the slower tumor development in the PSA-Cre:Pten-loxP/loxP model has its practical disadvantage, it has the important advantage above the PB-Cre4-based model that the consecutive stages of prostate cancer development can be identified more easily. Recently, the PSA promoter has also been used for prostate-specific inactivation of Nkx3.1 (48).

Although there was a slight increase in proliferation rate in prostate epithelial cells in PSA-Cre-Pten-loxP/+ mice, we did not find any apparent pathologic changes in adult prostates until old age, when low-penetrant focal dysplasia was detected, indicating that monoallelic inactivation of Pten in prostate epithelial cells is insufficient for prostate cancer development. However, it is quite well possible that monoallelic Pten loss plus alterations in other signaling routes lead to tumor development (see below). The more pronounced prostate phenotypes observed in conventional Pten+/− knock-out mice (9, 22) could be explained by the germ line inactivation of one Pten allele, affecting prenatal and postnatal prostate development. In addition, the absence of one active allele in adult prostate stromal cells and in other tissues might indirectly influence the properties of prostate epithelial cells.

In our prostate cancer model, as well as in the other models, biallelic Pten inactivation caused an increased proliferation rate of prostate epithelial cells, indicating an important contribution to tumorigenesis (28, 29). Thus far, little is known about the effect of Pten inactivation on apoptosis in the prostate. We did not observe, as might have been expected, decreased apoptosis, but in contrast, increased apoptosis in parallel with the increased proliferation rate in all stages of the tumor model, coupling the apoptotic process to the proliferation program in PSA-Cre-Pten-loxP/loxP mice. This finding apparently contradicts previous in vitro and in vivo observations, which correlate Pten inactivation with decreased apoptosis (42, 49). However, in a few other in vivo studies, Pten inactivation did not lead to reducing apoptosis in agreement with our findings. Podsypanina and colleagues (24) showed absence of reduced apoptosis in some subsets of thymus and spleen lymphocytes, although there was significantly reduced apoptosis in a variety of bone marrow cell lineages in the same Pten knock-out mouse model. Backman et al. (50) reported the absence of differences between control and Pten-deficient brain cells in TUNEL staining pattern, and Groszer et al. (51) showed a significant increase in the number of TUNEL-positive cells in the telencephalon of Pten knock-out mice. Most importantly, similar to our prostate cancer model, in a testis-targeted Pten knock-out mouse model, increased proliferation of primordial germ cells was associated with increased apoptosis (43). Several hypotheses might explain these observations. Increased apoptosis might be a secondary event following stimulated cell proliferation or changed cell polarity in the Pten-deficient cells. Alternative, Pten-regulated

Figure 6. Complete inactivation of Pten in the prostate leads to rare metastatic disease. A-D, metastatic prostate adenocarcinoma in a caudal para-aortic lymph node. A and B, H&E staining showing two foci of cribriform malignant prostate cells at the subcapsular sinusoidal region of the lymphoid node. C, phospho-AKT staining shows intermediate positive malignant epithelial cells. D, androgen receptor staining shows positive nuclei of the malignant epithelial cells. Magnifications, ×100 (A); ×400 (B-D).
apoptosis might be a cell or tissue type–specific event. Clearly, the role of Pten in apoptosis in vivo seems more complex than originally envisaged.

Tumor initiation and progression generally need multiple genetic and epigenetic events. In the Pten model, we can discriminate several clearly defined morphologic stages leading to prostate cancer, starting with hyperplasia, followed by PIN lesions, local invasion, invasive growth in surrounding tissues, and finally metastasis. We speculate that hyperplasia might solely result from Pten inactivation. Later stages of tumor development might include secondary Pten-dependent or independent genetic or epigenetic alterations. The PSA-Cre:Pten-loxP/loxP model is very well suited to study these different biological and molecular stages of prostate tumorigenesis.

Although monoallelic Pten inactivation in our model does not lead to dramatic pathologic changes in prostate, PSA-Cre:Pten-loxP/+ mice are important for experiments aimed at investigating the functional synergism between Pten and other tumor suppressor genes and oncogenes in prostate cancer. Recently, conventional Nkx3.1 knock-out mice as well as TRAMP mice have been crossed with Pten+/− knock-out mice. Pten+/−:Nkx3.1+−/− and Pten+/−:Nkx3.1−−/− compound mice displayed an increased incidence of PIN lesions as compared with Nkx3.1+/− and Nkx3.1−−/− mice. In Pten+/−:TRAMP compound mice the progression of prostate cancer was accelerated (53). Hence, both models indicate that monoallelic loss of Pten promotes the progressive growth of prostate cancer under specific conditions. Studies with Pten+/−:Ink4a/Arf−−/− and Pten+/−:Cdkn1b−−/− compound mouse models reach similar conclusions (54, 55).

The tissue-targeted Pten prostate tumor model described in the present study permits careful definition of the synergism between Pten loss and other genetic changes at well-defined phases of tumor development. This can be achieved by crossing our model with other mutant mice as well as by careful monitoring of additional genetic changes occurring during the distinct phases of prostate cancer development we have described here.

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**References**


Targeted Biallelic Inactivation of \textit{Pten} in the Mouse Prostate Leads to Prostate Cancer Accompanied by Increased Epithelial Cell Proliferation but not by Reduced Apoptosis

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