Title: SOX4 is essential for prostate tumorigenesis initiated by PTEN ablation

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Abstract

Understanding remains incomplete of the mechanisms underlying initiation and progression of prostate cancer, the most commonly diagnosed cancer in American men. The transcription factor SOX4 is overexpressed in many human cancers, including prostate cancer, suggesting it may participate in prostate tumorigenesis. In this study, we investigated this possibility by genetically deleting Sox4 in a mouse model of prostate cancer initiated by loss of the tumor suppressor Pten. We found that specific homozygous deletion of Sox4 in the adult prostate epithelium strongly inhibited tumor progression initiated by homozygous loss of Pten. Mechanistically, Sox4 ablation reduced activation of AKT and β-catenin, leading to an attenuated invasive phenotype. Furthermore, SOX4 expression was induced by Pten loss as a result of the activation of PI3K-AKT-mTOR signaling, suggesting a positive feedback loop between SOX4 and PI3K-AKT-mTOR activity. Collectively, our findings establish that SOX4 is a critical component of the PTEN-PI3K-AKT pathway in prostate cancer, with potential implications for combination targeted therapies against both primary and advanced prostate cancers.

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Introduction

Prostate cancer is the most commonly diagnosed malignancy and the second leading cause of cancer death among men in the United States. It is estimated that approximately 220,800 new cases of prostate cancer and 27,540 deaths from prostate cancer will occur in the U.S. in 2015 (1). Prostate cancer develops through a series of clinical states, including prostatic intraepithelial neoplasia (PIN), adenocarcinoma and metastasis (2). Most of the tumors in prostate cancer patients are indolent, but a small fraction of the tumors behave aggressively resulting in mortality (3). Androgen deprivation therapy (ADT) is the first-line treatment for metastatic prostate cancer (4). Although 70-80% of the cases respond initially to ADT, most tumors relapse and become hormone-refractory; this recurrent phenotype is termed castration-resistant prostate cancer (CRPC). While it is known that both the PI3K-AKT pathway and the Wnt signaling pathway likely play important roles in CRPC, it is not well understood how these pathways cross-talk to promote sustained cancer cell survival and proliferation. Because there is currently no curative treatment for CRPC, there is an urgent need for better understanding of the molecular basis of the initiation and progression of the disease, which can ultimately lead to the development of novel therapeutic targets.

Large-scale gene expression studies have identified increased expression levels of the *SOX4* gene in a variety of human cancers, including prostate (5), breast (6), bladder (7), lung (8) and colorectal cancer (9), and *SOX4* has been recognized as one of the 64 'cancer signature' genes, suggesting that SOX4 plays a fundamental role in tumorigenesis and tumor progression (10). *SOX4* belongs to a family of 20 genes in mammals that encode transcription factors containing a highly conserved high-mobility group (HMG) DNA-binding domain that is

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structurally related to TCF/LEF family of transcription factors that regulate the expression of

Wnt target genes (11). SOX4 expression is widespread during embryonic development, with the

highest expression levels in neural and mesenchymal progenitor cells, suggesting that SOX4

transcription factor plays important roles in early embryogenesis and cell fate decisions (12).

Previous studies have shown that SOX4 expression is significantly upregulated at the mRNA and

protein levels in prostate cancer patients, and the increased levels of SOX4 expression correlate

with more aggressive tumors and poor prognosis (5). Moreover, SOX4 overexpression in non-

transformed prostate cells can induce anchorage-independent growth, suggesting that SOX4 can

act as an oncogene in prostate cancer (5). Genome-wide localization analysis of direct

transcriptional targets of SOX4 in human prostate cancer cells identified a set of important

cancer-related genes regulating miRNA processing, embryogenesis, growth factor signaling, and

tumor metastasis (13). These findings provide strong evidence that SOX4 may play a crucial role

in the development and progression of prostate cancer. However, the molecular mechanisms by

which SOX4 overexpression contributes to progression to CRPC are not well understood, nor is

it understood how SOX4 is upregulated in so many diverse types of cancers.

Deletion or mutation of the phosphatase and tensin homolog deleted on chromosome 10

(*PTEN*) gene is one of the most frequent genetic alterations in many of the sporadic human

cancers, including prostate cancer (14). Somatic *PTEN* mutations have been detected in 30% of

primary prostate tumors and in about 63% of metastatic prostate cancers (15). The tumor

suppressor *PTEN* gene encodes a dual lipid and protein phosphatase that catalyzes the

dephosphorylation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3), resulting in

downregulation of phosphatidylinositol-3-kinase-AKT-mammalian target of rapamycin (PI3K-

AKT-mTOR) signaling pathway (16). Loss-of-function mutations of *PTEN* leads to the

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accumulation of PIP3 that activates AKT, which is a key mediator in modulation of a variety of

downstream effectors involved in cell proliferation, apoptosis, cell growth, cell adhesion and

migration (17). Studies using mouse models of PTEN loss highlighted the critical importance of

the level of PTEN expression in prostate carcinogenesis. Heterozygous *Pten* knockout (*Pten*^{+/-})

mice develop neoplasia in a number of organs including endometrium and thyroid as well as

prostatic intraepithelial neoplasia (PIN) in the prostate but no progression to invasive

adenocarcinoma, indicating that loss of one allele of *Pten* is not sufficient to produce invasive,

metastatic prostate cancer (18). Mouse models with conditional homozygous *Pten* deletion in the

prostate epithelium recapitulate the disease progression in human prostate cancer from PIN to

invasive carcinoma that metastasizes to lymph nodes and lung (19).

To understand whether SOX4 is essential for prostate cancer initiation and progression

and to determine whether SOX4 might be a potential drug target in prostate cancer, we have

developed a novel mouse model in which conditional homozygous deletion of both *Sox4* and

Pten is specifically generated in the mouse prostate epithelium using Cre-*loxP* site-specific

recombination. Tamoxifen-inducible prostate-specific expression of Cre-ER[T2] recombinase is

driven under the control of the human prostate-specific antigen (PSA) promoter in this system

(20). We show in this study that biallelic deletion of *Sox4* in the adult prostate epithelium

strongly inhibits tumor development initiated by homozygous loss of *Pten*, demonstrating the

key role of SOX4 in the development of prostate cancer. Additionally, we show that SOX4

expression is regulated by the PI3K-AKT-mTOR signaling pathway and that SOX4 is necessary

for maintenance of AKT activation. We also show that SOX4 is critical for maintenance of

active β-catenin *in vivo*, suggesting that SOX4 plays a key role in the cross-talk between PI3K-

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AKT and Wnt signaling pathways. Our findings suggest that targeting SOX4 directly or

indirectly may be of benefit to prostate cancer patients.

Materials and Methods

Ethics Statement

All animal protocols in this study were approved by the Institutional Animal Care and Use Committee of the Emory University, which is accredited by the American Association for the Accreditation of Laboratory Animal Care (AALAC).

Generation of Prostate Epithelium-Specific *Sox4* and *Pten* Conditional Double-Knockout Mice

Mice carrying the *PSA-Cre-ER^{T2}* transgene and *Pten^{flox/flox}* allele have been described (20). Mice with conditional (*floxed*) alleles of *Sox4* have also been described (21). To drive prostate epithelium-specific deletion of both *Sox4* and *Pten*, the detailed breeding schemes used to obtain the control and mutant animals that were studied are illustrated in Supplemental Figure S1. Genotypes of interest in this study were (i) *PSA-Cre-ER^{T2}/Pten^{flox/flox}* (*Pten^{-/-}*), (ii) *PSA-Cre-ER^{T2}/Sox4^{flox/flox}* (*Sox4^{-/-}*) and (iii) *PSA-Cre-ER^{T2}/Pten^{flox/flox}/Sox4^{flox/flox}* (*Pten^{-/-}/Sox4^{-/-}*). Littermate controls lacking the *PSA-Cre-ER^{T2}* transgene (wild-type) were included in all experiments. All mouse lines were maintained on a pure C57BL/6 strain background.

Tamoxifen Administration

Tamoxifen (Sigma) was resuspended in sunflower oil at a concentration of 10 mg/ml by sonication. To induce *Pten* and/or *Sox4* deletion, mice at eight weeks of age were injected with tamoxifen (1 mg/kg body weight) intraperitoneally daily for five consecutive days. At 24 weeks after tamoxifen administration, animals were euthanized, and prostate, liver, kidney, pancreas

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and spleen were collected. Half of the prostate sample was snap-frozen in liquid nitrogen for RNA and protein analyses, and the other half was formalin-fixed for histological analysis.

Genotyping of Mice

Genomic DNA was extracted from 8-day-old mouse toes by incubating in lysis buffer (100 mM Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl) containing 0.5 µg/mL proteinase K (Invitrogen) at 56°C overnight. Genomic DNA from dorsolateral prostate lobe was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). Mice carrying *PSA-Cre-ER^{T2}*, *Pten$^{flox/flox}$* or *Sox4$^{flox/flox}$* were genotyped using PCR as described previously (20, 21). PCR was carried out using a denaturation step at 95°C for 10 minutes, followed by 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 1 minute, and an extension step for 5 minutes at 72°C.

Histopathology

Prostate tissues, which were fixed in 10% neutral-buffered formalin overnight, were processed by standard procedures and embedded in paraffin. The paraffin-embedded tissues were sectioned (5 µm), deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E), and Ki-67 by Winship Cancer Institute Pathology Core Laboratory. Histological analyses of the H&E- and Ki67-stained prostate tissues were performed by a board certified pathologist. Images were captured at 100X magnification, and cell proliferation index was calculated as the percentage of Ki67-positive nuclei to the total number of nuclei.

Immunofluorescence

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Fixed prostate sections were deparaffinized in xylene and antigen retrieval was performed by in 10 mM sodium citrate, 0.05% Tween 20 (pH 6.0) for 20 minutes in a microwave oven. Sections were blocked in 10% normal goat serum with 1% BSA in PBS for 2 hours and incubated with primary antibodies overnight at 4°C. Primary antibodies used were SOX4 (# 86809; 1:200; Abcam, Cambridge, MA) and Cytokeratin 8 (TROMA-I; 1:6; Developmental Studies Hybridoma Bank, Iowa City, IA). Secondary antibodies were Alexa Fluor® 546-conjugated goat anti-rabbit (A11010; 1:1,000: Molecular Probes, Eugene, OR) and Alexa Fluor® 488-conjugated goat anti-rat (A11006; 1:1,000: Molecular Probes, Eugene, OR), incubated for 1 hour at room temperature in the dark. Sections were counterstained with Hoechst dye, mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL), visualized using Nikon Eclipse 80i (Tokyo, Japan), and images were captured at 200X total magnification using SPOT Advanced software (Sterling Heights, MI).

Immunohistochemistry

Sections were deparaffinized, antigens retrieved, and blocked as described above. Primary antibodies used were phospho-AKT (Ser 473) (AF887; 1:200; R&D Systems, Minneapolis, MN) and active β-catenin (clone 8E7, #05-665; 1:300; Millipore, Billerica, MA). After washing, sections were incubated in 3% H_2O_2 in PBS for 15 minutes to block endogenous peroxidase activity. Sections were then incubated with secondary antibodies for 1 hour at room temperature. Secondary antibodies were Horseradish Peroxidase (HRP)-conjugated goat anti-rabbit (ab6721; 1:1,000; Abcam, Cambridge, MA) and (HRP)-conjugated goat anti-mouse (#7076; 1:1,000; Cell Signaling Technology, Danvers, MA). Protein was visualized using, 3,3' Diaminobenzidine (DAB) substrate kit according to manufacturer's instructions (Abcam).

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Sections were counterstained with Hematoxylin, dehydrated, and mounted with Cytoseal60 (Thermo Scientific). Cells were visualized using Nikon Eclipse E400 (Tokyo, Japan), and images were captured at 40X or 100X total magnification using QCapture software (Surrey, BC, Canada).

Protein Extraction from Dorsolateral Prostate Tissues and Western Blot Analysis

Whole cell lysates were prepared in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Promega, San Luis Obispo, CA). Thirty µg total protein was separated on 10% SDS-polyacrylamide gel, and transferred to nitrocellulose membrane. Primary antibodies used were phospho-AKT (Ser473) (# 9271; 1:1,000; Cell Signaling), AKT1 (sc-5298; 1:1,000; Santa Cruz Biotechnology, Dallas, TX) and active β-catenin (clone 8E7, #05-665; 1:300; Millipore, Billerica, MA). β-actin (# 3700; 1:5000; Cell Signaling) was used as normalization control. For all proteins other than β-actin, signals were visualized using enhanced chemiluminescence (Pierce, Rockford, IL). To detect β-actin protein, membrane was washed three times in PBS with 0.2% Tween 20 and incubated with IRDye 800CW goat anti-mouse secondary antibody (# 926-32210; 1:5,000; LI-COR Biosciences, Lincoln, NE) for 1 hour at room temperature, and signals were visualized using the Odyssey infrared imaging system (LI-COR).

RNA Isolation from Dorsolateral Prostate Tissues and Quantitative Real-time RT-PCR Analysis

Total RNA was extracted from frozen dorsolateral prostate tissues using the *mir*Vana miRNA Isolation Kit (Life Tecchnologies, Grand Islands, NY), followed by RNA clean-up using

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the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was quantitated using NanoDrop 1000

(NanoDrop, Wilmington, DE), and reverse transcribed into cDNA using iScript cDNA Synthesis

Kit (Bio-Rad Laboratories, Hercules, CA). Quantitative real-time PCR was performed using iQ

SYBR Green Supermix (Bio-Rad Laboratories) on a Bio-Rad CFX Connect instrument.

Sequences of the primers for mouse *Sox4* were 5'-TCCCACTTCGAATTCCCGGACTAT-3'

(forward) and 5'-AAGACCAGGTTAGAGATGCTGGAC-3' (reverse). Sequences of the

primers for exon 5 of mouse *Pten* were 5'-AGACCATAACCCACCACAGC-3' (forward) and

5'-TACACCAGTCCGTCCCTTTC-3' (reverse). Sequences of the primers for mouse *18S* were

5'-TCAACTTTCGATGGTAGTCGCCGT-3' (forward) and 5'-

TCCTTGGATGTGGTAGCCGTTTCT-3' (reverse). The relative changes in gene expression

data were analyzed by the $2^{-\Delta\Delta CT}$ method. *18S* was used as an internal control. Triplicates were

run for each sample in three independent experiments.

Cell Culture

LNCaP cell line (Lot #58590545) was obtained from American Type Culture Collection

(Manassas, VA) in October 2010. Certificate of analysis was last updated by ATCC 11/29/2012.

Cells have not been genetically authenticated since they were received, but do have the

morphology of LNCaP cells, express PSA and androgen receptor, and are responsive to

MVD3100 as shown in Figure 4E. Cells were maintained in RPMI-1640 medium (Gibco, Grand

Island, NY) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals,

Lawrenceville, GA), 2 mM L-Glutamine (Gibco, Grand Island, NY) and 50 U/ml Penicillin-50

µg/ml Streptomycin antibiotics (Gibco, Grand Island, NY). Cells were cultured in a 37°C

incubator with humidified atmosphere of 5% CO_2.

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Inhibition of PI3K, AR and MAPK/ERK Signaling Pathways and Western Blot Analysis

Cells were serum-starved for 24 hours prior to the treatment with 50 µM of LY294002 (# 440202; EMD Millipore, Billerica, MA), 2 µM of MK-2206 2HCl (S1078; Selleckchem, Houston, TX) or 10 nM of Rapamycin (S1039; Selleckchem) for 24 hours, or treatment with 20 µM of MG-132 (# 474790, EMD Millipore) for 4 hours. To antagonize AR signaling, cells were treated with 1 µM or 10 µM of MDV3100 (S1250; Selleckchem, Houston, TX). For the inhibition of MAPK/MEK signaling, cells were treated with 1 µM or 10 µM of Selumetinib (S-4490; LC Laboratories, Woburn, MA) prior to treatment with 5 ng/ml of recombinant human EGF (PHG0314; Life Technologies, Grand Islands, NY). Following the treatments, whole cell lysates were prepared in lysis buffer containing 137 mM NaCl, 20 mM Tris-HCl(pH 8.0), 10% glycerol, 1% NP-40, and protease inhibitor cocktail (Promega, San Luis Obispo, CA). 50 µg total protein was separated on 10% SDS-polyacrylamide gel, and transferred to nitrocellulose membrane. Primary antibodies were SOX4 (sc-20090; 1:1,000; Santa Cruz), phosho-AKT (Ser473) (# 9271; 1:1,000; Cell Signaling), phospho-p70 S6 kinase (Thr389) (# 9234; 1:1,000; Cell Signaling), ubiquitin (MMS-257P; 1:1,000; Covance, Emeryville, CA), PSA (# 5365; 1:1,000; Cell Signaling), and phosho-ERK1/2 (Thr202/Tyr204) (# 4370; 1:1,000; Cell Signaling). Immunoblots were repeated with lysates from three independent experiments.

Statistical Analysis

Student's *t*-test (two-tailed, equal variance) was used to determine significant differences between two groups of data. *p* values of <0.05 were considered as statistically significant, and indicated by asterisk (*).

PARIS Analysis of Achilles and CCLE Datasets

The version 2.4.3 release of Project Achilles data (http://www.broadinstitute.org/achilles) with

ATARiS gene level scores for 216 cell lines passing quality control (p=0.05) were analyzed

using the PARIS (Probability Analysis by Ranked Information Score) module on the Broad

Institute GenePattern server. Cancer Cell Line Encyclopedia (CCLE) mRNA expression data

(http://www.broadinstitute.org/ccle/home) from Affymetrix U133+2 arrays was downloaded and

integrated with Achilles Data in the PARIS analysis. Analysis was performed with default

parameters examining negative RNMI values to identify shRNA's from Achilles that are selected

against in cells with high SOX4 expression levels.

Analysis of Prostate Adenocarcinoma TCGA datasets

Normalized RNAseqV2 data was downloaded from the Broad Institute genome data analysis

center (http://gdac.broadinstitute.org/runs/analyses__latest/). Copy number and mutational data

for PTEN, PIK3CA, and PIK3CB were obtained from the cBioPortal website using the TCGA

PRAD provisional dataset for 419 cases with expression, copy number, and mutational data

available (http://www.cbioportal.org/index.do). Patients with homozygous loss of PTEN,

hemizygous loss of PTEN plus a mutation of the retained allele, or mutations in either PIK3CA

or PIK3CB were designated as having the pathway mutated. Other patients were considered as

not mutated. SOX4 level 3 gene expression RNAseq data was used to determine if SOX4 levels

was significantly different between groups. A two-sided student's T-test was used to determine

statistical significance.

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Results

Generation of Prostate Epithelium-Specific *Sox4* and *Pten* Conditional Double-Knockout Mice

Previous studies have shown that SOX4 expression is correlated with prostate cancer progression (5), promotes the epithelial to mesenchymal transition (EMT) (22), and is critical for tumor metastasis (6). Thus, we hypothesized that tissue-specific deletion of *Sox4* in the mouse prostate might interfere with prostate cancer progression. Some of the most useful mouse models of human prostate cancer include tissue-specific deletion of *Pten* (20, 23). To study the role of SOX4 in the prostate tumorigenesis, we crossed *Sox4$^{flox/flox}$* mice (21) with *PSA-Cre-ER^{T2}/Pten$^{flox/flox}$* mice (20) to generate conditional homozygous deletion of both *Sox4* and *Pten* in the prostate epithelium. The breeding scheme for the generation of various genotypes is summarized in Supplemental Figure S1. We selected this model for *Pten* deletion because we observed that Probasin-Cre induced Cre activity in the developing pancreas (Supplemental Figure S2), and wanted to avoid confounding effects of *Sox4* deletion on the prostate and pancreas during normal development. In the *PSA-Cre-ER^{T2}/Pten$^{flox/flox}$* mouse model, expression of tamoxifen-dependent Cre-ER^{T2} recombinase is driven by the human PSA promoter, resulting in prostate epithelial cell-specific expression. To induce deletion of *Pten* and *Sox4* alleles, mice were injected intraperitoneally with tamoxifen at eight weeks of age after reaching sexual maturity (Figure 1A). Twenty-four weeks after tamoxifen administration, animals were sacrificed, and prostate tissue samples were subjected to histological and molecular analyses. All mice with different genotypes were injected and sacrificed on the same schedule. Genotypes

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were verified by PCR analysis of genomic DNA isolated from prostate glands and toes of the

animals (Figure 1B). To further confirm loss of SOX4 expression in the mouse prostate, we

performed immunofluorescence staining. Representative images from the dorsolateral lobe,

which has the highest efficiency of *PSA-Cre-ER^{T2}* recombinase (20), are shown in Figure 1C.

The wild-type mouse exhibits strong nuclear staining of SOX4 (red) in the prostate whereas no

SOX4 staining is observed in the nucleus of *Sox4^{-/-}* mouse.

Prostate-Specific Homozygous Deletion of *Sox4* Inhibits Prostate Tumorigenesis

To test whether *Pten*-null prostate tumors require functional SOX4, we explored whether

deletion of *Sox4* ameliorates tumorigenesis induced by PTEN loss in the mouse prostate. Seventy

per cent of the *Pten^{-/-}* mice (7/10) developed tumors in the prostate, as expected from previous

studies (20). These tumors were solid masses, and the gross morphology exhibited denser glands

compared with those of wild-type mice (Figure 2A and Supplemental Figure S3). Strikingly,

only 11 per cent of the double knockout *Pten^{-/-}/Sox4^{-/-}* mice (2/18) had cancerous lesions in the

prostate. At necroscopy, prostate of the *Pten^{-/-}/Sox4^{-/-}* mice generally appeared benign.

Representative images of histological analysis are shown in Figure 2B. While *Pten^{-/-}* mice

developed invasive adenocarcinoma in the dorsolateral prostate, *Pten^{-/-}/Sox4^{-/-}* mice progressed

primarily to high-grade PIN (HGPIN) but not invasive cancer. Histological analysis was

summarized graphically in Figure 2D. The difference in the incidence of prostate cancer between

the conditional *Pten*-null and *Pten/Sox4* double-knockout mice was statistically significant (p =

0.0028). To gain insights into the molecular events associated with *Sox4* deletion, resulting in the

inhibition of prostate tumorigenesis induced by PTEN loss, we assessed the cell proliferation in

the prostates of *Pten*-null and *Pten/Sox4* double-knockout mice (Figure 2C). Analysis of Ki-67

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staining showed a significant increase in $Pten^{-/-}$ mice compared with the control mice (Figure 2E). Notably, *Sox4* deletion significantly reduced the percentage of Ki-67 positive cells in the prostate to only 16% relative to that of *Pten*-knockout mice (28%). Taken together, our findings demonstrate that SOX4 cooperates with PTEN loss to drive prostate cancer progression and cancer cell proliferation.

Loss of SOX4 Inhibits Activation of AKT and β-Catenin

It is well-established that loss of PTEN results in activated PI3K-AKT signaling. To elucidate the molecular mechanism by which *Sox4* deletion inhibits PTEN loss-driven tumorigenesis, we examined the phosphorylation levels of AKT in *Pten*-null and *Pten/Sox4* double-knockout mice. Immunohistochemical staining of dorsolateral prostate lobes indicated that deletion of *Sox4* reduced the PTEN loss-induced phosphorylation of AKT at serine 473 (Figure 3A). It is known that AKT can phosphorylate and inhibit GSK3β, resulting in increased activity of the Wnt pathway mediator β-catenin (24-26). Strikingly, a significant decrease of active β-catenin was also observed by immunohistochemical analysis of the dorsolateral prostate lobes of *Pten/Sox4* double-knockout mice compared to *Pten*-null prostates (Figure 3A). These findings were also confirmed by Western blotting analysis of dorsolateral prostate lobes of wild-type, $Sox4^{-/-}$, $Pten^{-/-}$ and $Pten^{-/-}/Sox4^{-/-}$ mice (Figure 3B). Furthermore, we observed that homozygous deletion of *Sox4* reduced the expression of AKT1 in the mouse prostate, suggesting that SOX4 may have an important role in the regulation of AKT1 (Figure 3B).

SOX4 is Regulated by the PI3K-AKT-mTOR Signaling Pathway

Interestingly, quantitative real-time RT-PCR analysis of SOX4 in *Pten*-null mice

revealed that SOX4 expression is strongly induced by loss of PTEN, suggesting that SOX4

expression is regulated by the PI3K signaling pathway (Figure 3C). To investigate whether

SOX4 expression is increased in prostate cancer patients with activation of the PI3K pathway,

we analyzed TCGA RNAseqV2 gene level data, GISTIC copy number data, and gene mutational

data from a set of 419 PRAD patients with complete data that is publicly available from the

Broad Institute TCGA Data Center. We identified the set of 172 patients with either

homozygous deletion of PTEN, hemizygous loss of PTEN with mutation on the other allele, or

mutations in either the PIK3CA or PIK3CB genes. We classified these 172 patients as the

PTEN-PI3K-AKT active set of patients, and the remaining 247 patients as those with the PTEN-

PI3K-AKT pathway as inactive. Comparison of the SOX4 mRNA between these two sets of

patients indicated that those with the PTEN-PI3K-AKT pathway active have 1.3 fold higher

SOX4 mRNA (p = 1.1E-9) than those with the pathway inactive (Figure 3D).

To further examine the role of PI3K signaling in the regulation of SOX4 in human

prostate cancer, human LNCaP prostate cancer cells were treated with the inhibitors of PI3K,

AKT or mTOR. Inhibition of PI3K signaling at each level of the PI3K-AKT-mTOR pathway

significantly reduced SOX4 expression, confirming the regulation of SOX4 via the PI3K

pathway (Figure 4A-C). Notably, concomitant treatment of LNCaP cells with inhibitors of AKT

and the proteasome restored SOX4 expression, suggesting that PI3K signaling is important for

the stabilization of SOX4 protein (Figure 4D). To show that the effect was pathway specific, we

tested the effect of inhibition of non-PI3K pathways including AR and MAPK/ERK signaling

cascades, and observed that inhibitors of these pathways have no effect on SOX4 expression

(Figure 4E-F).

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Cancer Cells with High SOX4 are Sensitive to AKT1 shRNA Knockdown

To test whether the relationship between SOX4 and AKT is generalizable to other types of cancer, we mined the publicly available datasets from Project Achilles (27) and the Cancer Cell Line Encyclopedia (CCLE) (28). We applied the PARIS (Probability Analysis by Ranked Information Score) module from GenePattern (29), which uses the rescaled normalized mutual information (RNMI) metric to identify statistically significant gene interactions in large datasets. The Achilles 2.4.3 dataset includes shRNA sensitivity data on 5711 genes in 216 cancer cell lines derived from approximately 20 tissue types, including three prostate cancer cell lines (22RV1, NCIHCC60, and VCAP), while the CCLE dataset includes gene expression data from 1037 cell lines, including 215 of the Achilles cell lines. PARIS analysis of the overlapping data from the Achilles and CCLE datasets to identify shRNA targets that are associated with high expression of SOX4 identified six genes with a false discovery rate (FDR) < 0.05. Interestingly, cell lines with high SOX4 gene expression were highly sensitive to shRNAs that target the *AKT1* gene (p = 1.75e-5, FDR = 0) (Figure 4G), suggesting that combined targeting of SOX4 and AKT1 could have synergistic effects.

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Discussion

The SOX4 transcription factor is highly overexpressed in many types of human cancers, including leukemias, melanomas, glioblastomas, medulloblastomas, and cancers of the bladder, lung, prostate, and breast, suggesting that it plays a fundamental role in many of these malignancies (reviewed in (30)). A lingering unanswered question for many years, has been, why is SOX4 overexpressed in so many cancer types? The data from this study indicate that SOX4 expression is strongly induced by the PI3K-AKT pathway, which is activated in most cancer types, providing a potential answer to this question. Although our data demonstrate a link between PI3K-AKT signals and SOX4 expression, it also is important to emphasize that there are likely multiple mechanisms that affect SOX4 levels, and PI3K-AKT signals represent only one of those pathways.

A second question relates to whether expression of SOX4 is required for cancer progression, or whether it is simply a 'passenger' effect of perturbed developmental pathways. Our data demonstrate that deletion of *Sox4* in the mouse prostate arrests PTEN loss-driven prostate cancer progression, thus indicating that SOX4 expression is indeed necessary for development of invasive prostate adenocarcinoma. A limitation of our current study is that mice were sacrificed at a single timepoint, and thus is it possible that SOX4 deletion may delay, rather than completely inhibit, cancer progression. Consistent with the essential role of SOX4 in stimulation of cell proliferation, our results indicate that loss of SOX4 significantly reduces prostate cancer cell proliferation *in vivo*. Our observation that *Sox4* deletion reduces active phospho-AKT suggests that SOX4 is necessary for maintenance of PTEN-PI3K-AKT activity as part of a positive feedback loop between AKT activation and SOX4 activity. This feedback loop raises the questions of whether AKT directly phosphorylates SOX4 or whether SOX4 directly

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activates transcription of AKT genes, questions that are currently under investigation (Figure 5).

Previous studies by our group (13) have found that *PIK3R1*, *PIK3R4,* and *FZD5* may be

regulated by SOX4 in prostate cancer cells, while other studies in acute lymphoblastic leukemia

(31) have identified *Pik3r2, Pik3r3,* and *Mtor* as *Sox4* targets in transgenic mice. Thus, there are

potentially several mechanisms that could mediate positive feedback stabilization of PTEN-

PI3K-AKT signaling through SOX4 activity (Figure 5). Moreover, our finding that inhibition of

the proteasome partially restores SOX4 expression are consistent with a model in which AKT

phosphorylation of the transactivation domain of SOX4, which also controls proteasomal

degradation of SOX4 (32), enhances protein stability by inhibiting ubiquitin-mediated

proteolysis. Our analysis of Achilles and CCLE datasets across 215 cell lines from 20 tissue

types supports the hypothesis that SOX4 interactions with AKT1 are important in many types of

cancer, and also suggests that drug combinations that target both SOX4 and PI3K-AKT might

have synergistic effects.

Another important question relates to the role of the PTEN-PI3K-AKT pathway, SOX4,

and tumor metastasis. Recently, several studies have identified *PTEN* as not only a critical

tumor suppressor gene, but also as a metastasis-suppressor gene (33, 34).   In each of these

studies *PTEN* deletion seemed to cooperate with *KRAS* activation, *RB1* loss, or *CDKN2A*

deletion to promote tumor metastasis. SOX4 has also been shown to play an important role in

metastasis, since shRNA knockdown of SOX4 inhibited lung metastases of breast cancer

xenografts (6) and SOX4 acts as a master-regulator of EMT in breast cancer cells (22). Thus,

our data suggest that SOX4 may mediate the metastatic effects of the PTEN-PI3K-AKT

pathway, although additional studies with more metastatic models of prostate cancer will be

necessary to adequately address this question.

There is growing evidence that Wnt signaling is important for the progression of CPRC (35-37), with one study demonstrating nuclear β-catenin in 37% of bone metastases (37). SOX4 is important for progenitor cell differentiation and Wnt signaling (38, 39), and SOX4 interacts directly with β-catenin (13, 39). Here we have shown that deletion of *Sox4* reduces levels of active β-catenin *in vivo*, but the mechanisms of how SOX4 loss inhibits β-catenin activation remains to be determined. It is well established that there is cross-talk between the PI3K-AKT and APC-β-catenin pathways via AKT phosphorylation and inhibition of GSK3β (25). Moreover, PI3K-AKT and β-catenin can cooperate to stimulate AR signaling in CRPC (40), and β-catenin can interact directly with AR (41). Thus, it is conceivable that SOX4 stimulates β-catenin activity indirectly via maintenance of active AKT, and that it modulates AR-β-catenin activity directly via protein-protein interactions. These hypotheses raise the possibility that SOX4 could be an attractive target for treatment of CRPC, since it may function at the intersection of PI3K-AKT, Wnt-β-catenin, and AR signaling pathways. Definitive answers to these questions will require additional studies.

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Figure Legends

Figure 1. Generation of prostate epithelium-specific *Sox4* and *Pten* conditional double-knockout mice. (*A*) Schematic representation of the experimental setup for tamoxifen treatment. Mice at eight weeks of age were injected with tamoxifen (1 mg/kg) or vehicle i.p. daily for five days. At 24 weeks after tamoxifen administration, animals were sacrificed. (*B*) PCR analysis of genomic DNA isolated from wild-type, heterozygous and homozygous mice for *Pten*, *Sox4* and *Cre* alleles. (*C*) Immunofluorescence staining of SOX4 in the dorsolateral prostate gland of wild-type and *Sox4*$^{-/-}$ mice. SOX4 staining (red) is strong in the nucleus of wild-type mouse but is absent in *Sox4*$^{-/-}$ mouse.

Figure 2. Loss of SOX4 significantly inhibits *PTEN* deletion-induced prostate tumorigenesis. (*A*) Representative images of prostate glands from wild-type, *Pten*$^{-/-}$ and *Pten*$^{-/-}$/*Sox4*$^{-/-}$ mice are shown. Homozygous deletion of *Sox4* significantly reduces tumor incidence in *Pten*$^{-/-}$/*Sox4*$^{-/-}$ mice. (*B* and *C*) Representative H&E- and Ki-67-stained paraffin-embedded tissue sections of the prostate glands from wild-type, *Pten*$^{-/-}$ and *Pten*$^{-/-}$/*Sox4*$^{-/-}$ mice are shown in (*B*) and (*C*), respectively. (*D*) Quantitation of histopathological analysis shows that while 70% of *Pten*$^{-/-}$ (n = 10) mice developed cancer by 32 weeks, only 11% of double-knockout *Pten*$^{-/-}$/*Sox4*$^{-/-}$ (n = 18) mice developed cancer. (*E*) Quantitative analysis of proliferation shows that the percentage of Ki-67(+) cells was 28% in *Pten*$^{-/-}$ mice but only 16% Ki-67(+) cells in *Pten*$^{-/-}$/*Sox4*$^{-/-}$ mice. Error bars represent standard error.

Figure 3. Loss of SOX4 inhibits activation of AKT and β-catenin. (*A*) Representative images of immunohistochemical staining of phospho-AKT (Ser473) and active β-catenin in the dorsolateral prostate lobes of wild-type, $Sox4^{-/-}$, $Pten^{-/-}$ and $Pten^{-/-}/Sox4^{-/-}$ mice are shown. (*B*) Whole cell lysates were prepared from wild-type, $Sox4^{-/-}$, $Pten^{-/-}$ and $Pten^{-/-}/Sox4^{-/-}$ mice, and analyzed by western blot for phospho-AKT, AKT1 and active β-catenin. Loss of SOX4 reduces phospho-AKT, AKT1 and active β-catenin levels. (*C*) Total RNA was assessed for *Sox4* expression using quantitative real-time RT-PCR. Prostate-specific conditional knockout of *Pten* induces *Sox4* mRNA expression. (D) Box plots of SOX4 RNAseq level 3 normalized gene expression from 419 TCGA prostate adenocarcinoma (PRAD) patients are shown. Patients with mutations in PTEN, PIK3CA, or PIK3CB (n = 172) are shown on the left, and those without mutations (n = 247) are shown on the right. Average SOX4 expression is 1.3 fold higher in patients with mutations in PTEN, PI3KCA, or PIK3CB.

Figure 4. SOX4 expression is regulated by the PI3K-AKT-mTOR signaling pathway. (*A-C*) Human prostate cancer cell line LNCaP was serum-starved for 24 hours, followed by the treatment with 50 μM of PI3K inhibitor LY294002 (*A*) or 2 μM of AKT inhibitor MK-2206 2HCl (*B*) or 10 nM of mTOR inhibitor Rapamycin (C) for 24 hours. Whole cell lysates were prepared, and analyzed for protein levels of SOX4, phospho-AKT or phospho-p70 S6 kinase. Inhibition of PI3K, AKT or mTOR reduces SOX4 expression. (*D*) LNCaP cells were serum-starved for 24 hours, followed by the combined treatment of 2 μM of AKT inhibitor MK-2206 2HCl for 24 hours and 20 μM of proteasome inhibitor MG-132 for 4 hours. Western analysis shows that inhibition of proteasome partially restores SOX4 expression. (*E*) LNCaP cells were treated with 1 μM or 10 μM of AR antagonist MDV3100 for 24 hours. Inhibition of AR

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signaling has no effect on SOX4 expression. (*F*) LNCaP cells were treated with human EGF for

24 hours, followed by the treatment of 1 μM or 10 μM of MEK1/2 inhibitor Selumetinib.

Inhibition of MAPK/ERK pathway has no effect on SOX4 expression. (*G*) PARIS analysis of

Achilles and CCLE datasets shows that cancer cells with high SOX4 are sensitive to AKT1
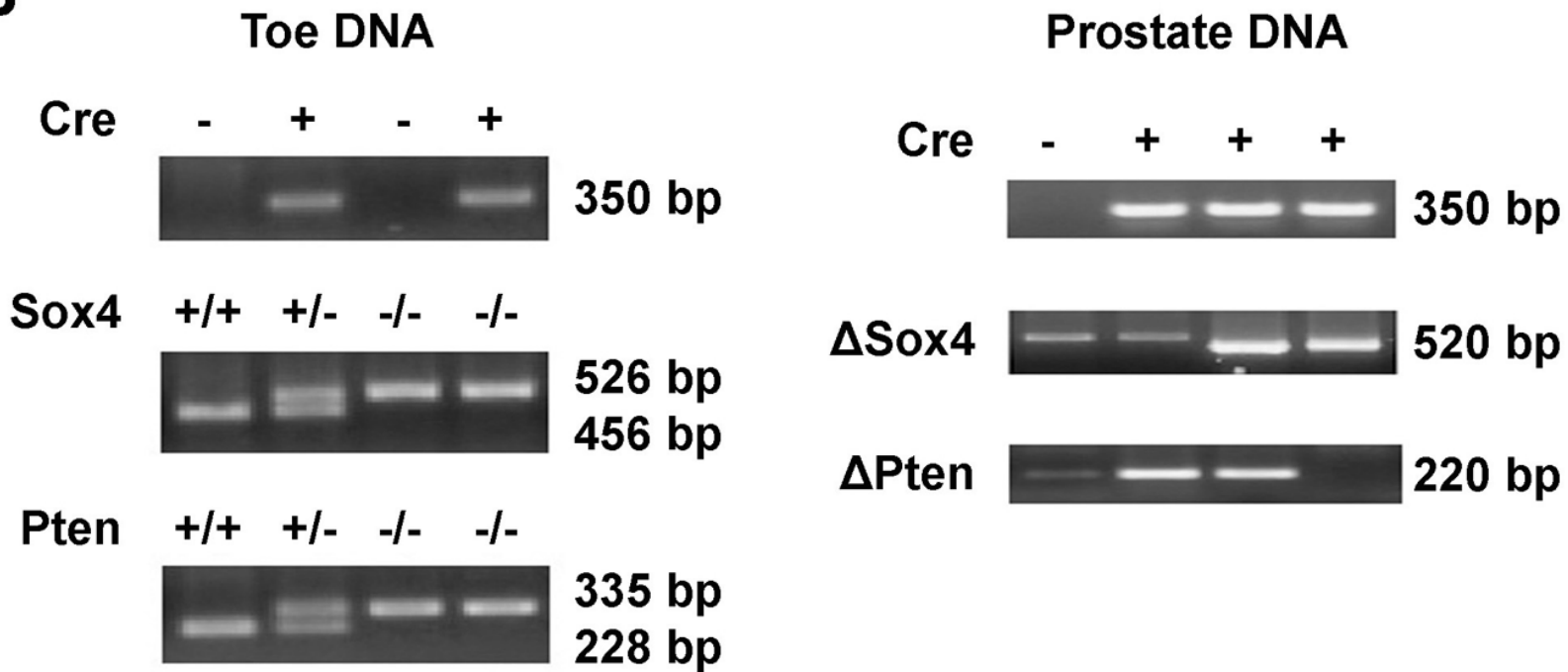
shRNA knockdown.

Figure 5. Schematic diagram of a proposed model illustrating how SOX4 interacts with PI3K-

AKT, Wnt-β-catenin, and AR signaling pathways, and how the PI3K-AKT-mTOR pathway

regulates the expression of SOX4 in prostate cancer progression. A potential positive feedback

loop is indicated by the red dashed line.

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Figure 1

A

0   3 weeks   8 weeks   32 weeks

Birth   Weaning   Tamoxifen injection   Sacrifice

B

Toe DNA

Cre	-	+	-	+	
					350 bp

Sox4	+/+	+/-	-/-	-/-	
					526 bp
					456 bp

Pten	+/+	+/-	-/-	-/-	
					335 bp
					228 bp

Prostate DNA

Cre	-	+	+	+	
					350 bp

ΔSox4					520 bp

ΔPten					220 bp

C

Wild-type   Sox4 $^{-/-}$

Figure 2

Figure 3

A

B

C
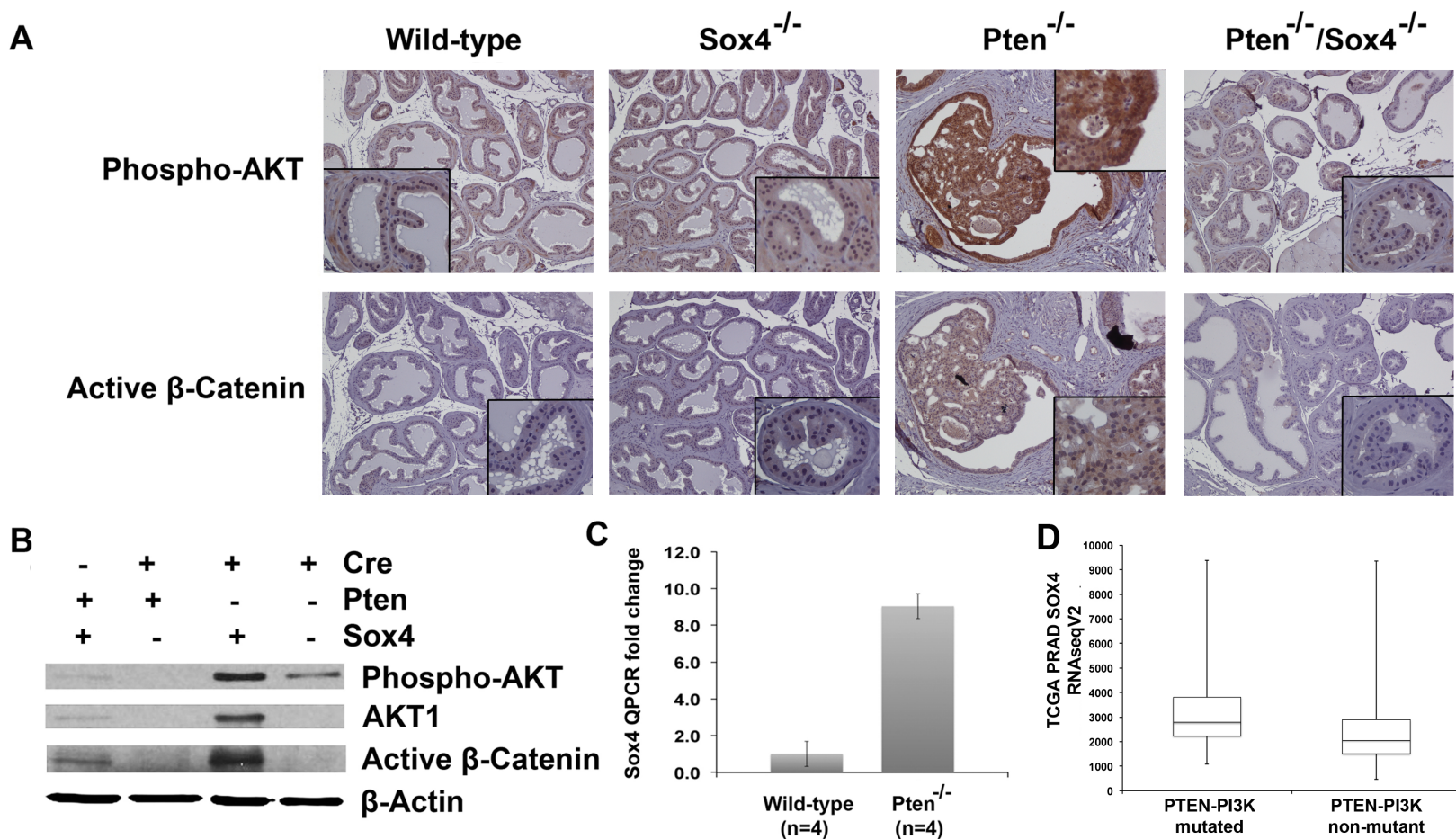

D

Figure 4
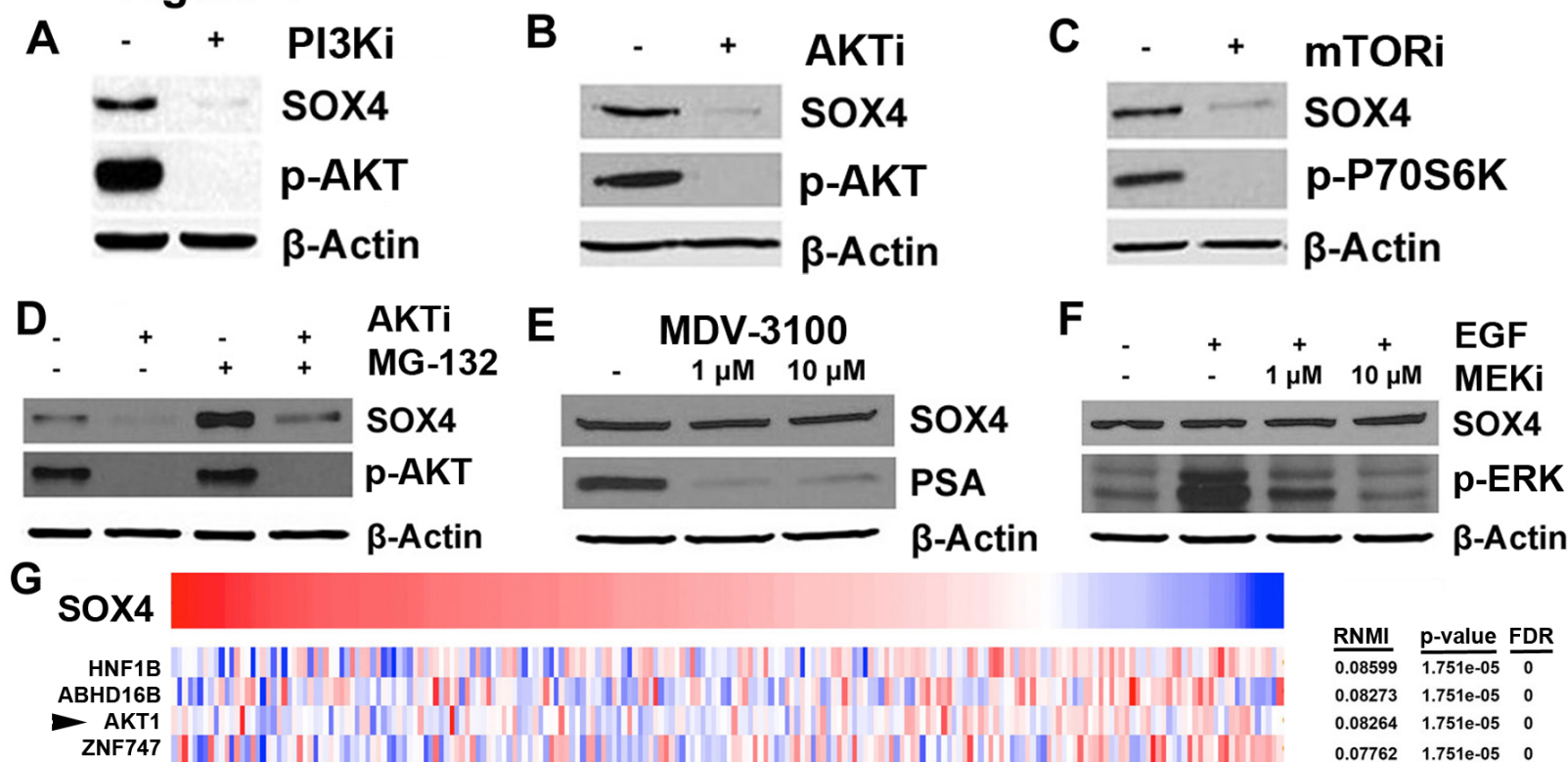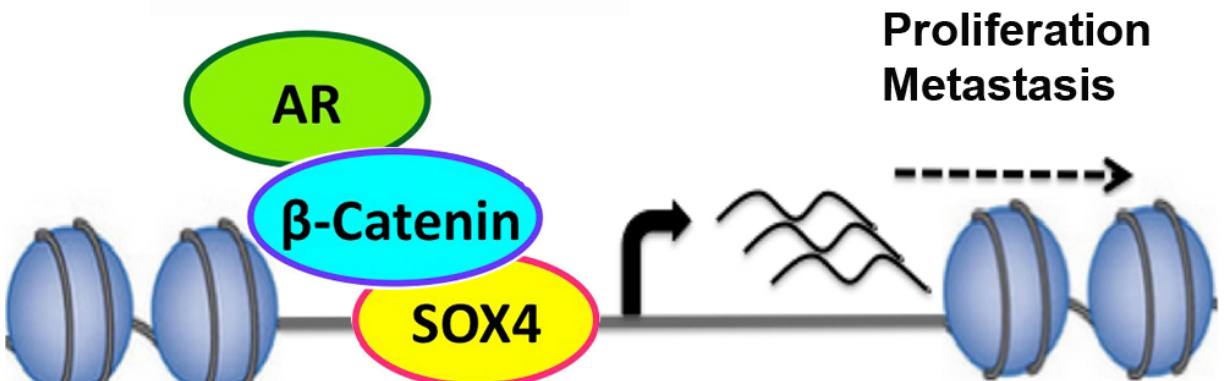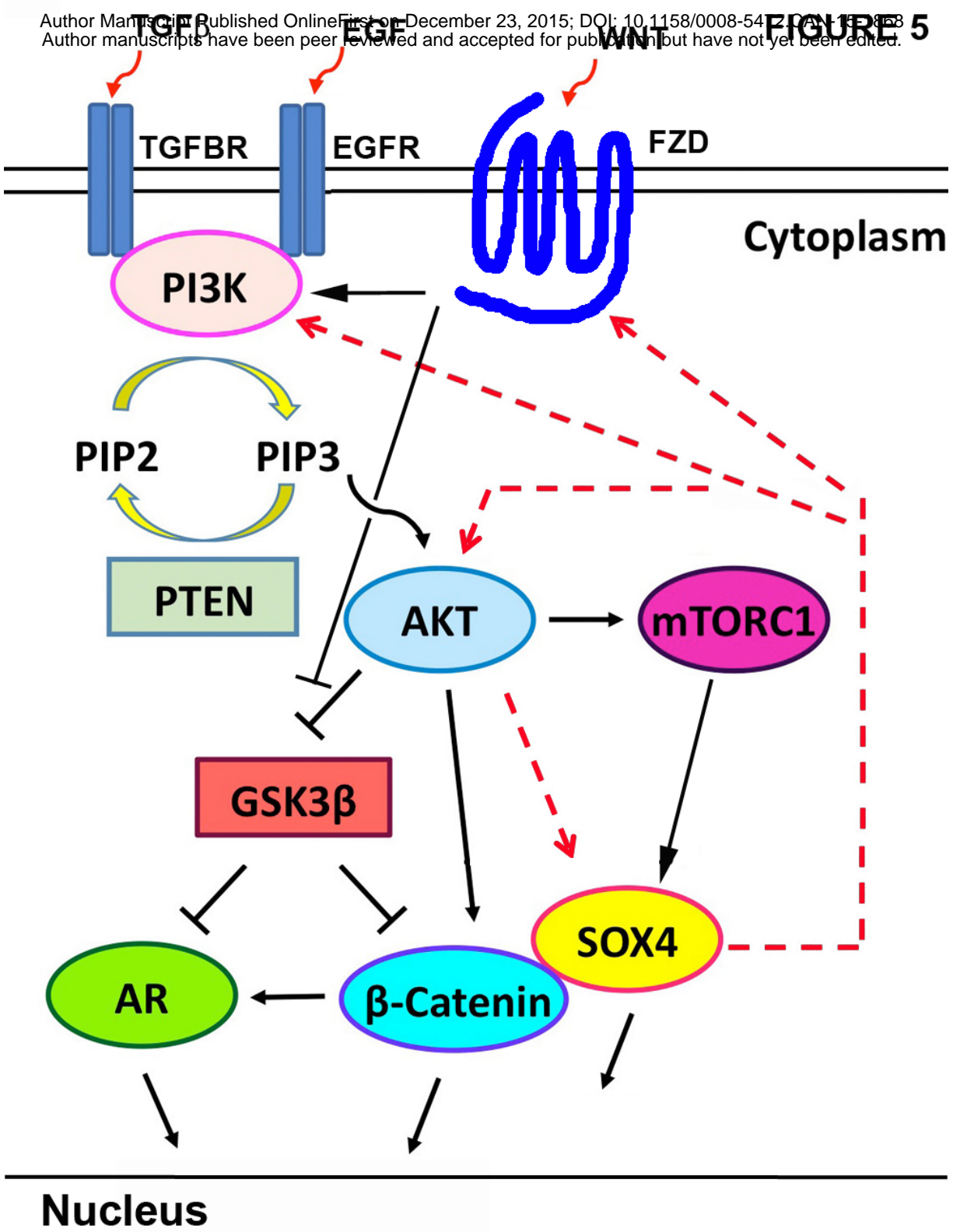
FIGURE 5

AACR American Association for Cancer Research
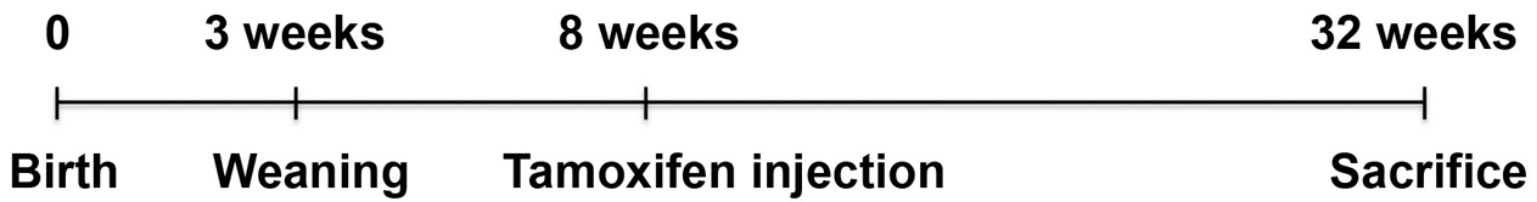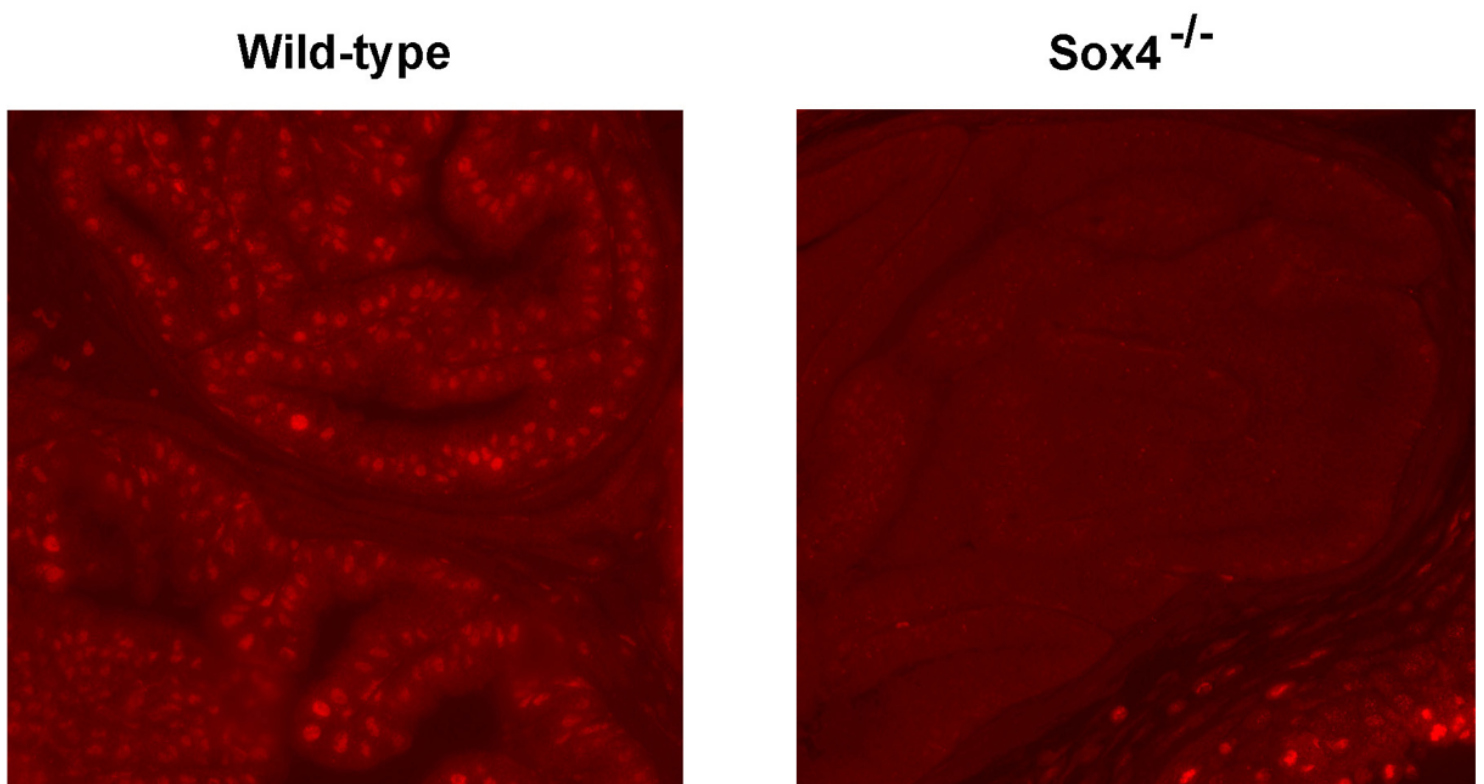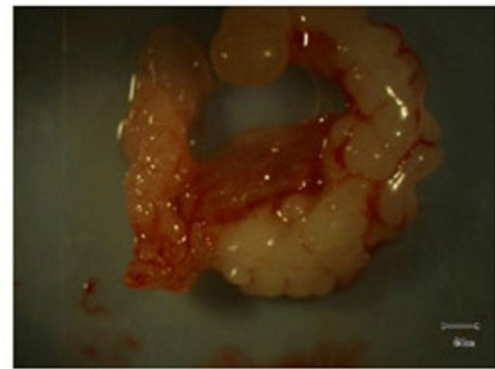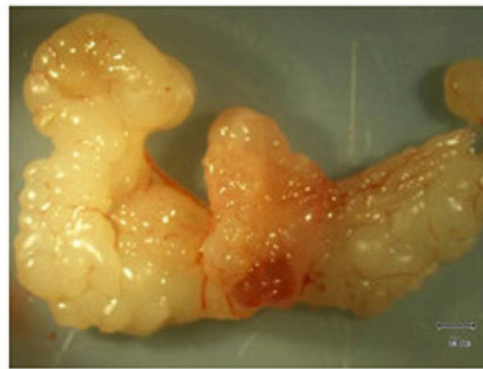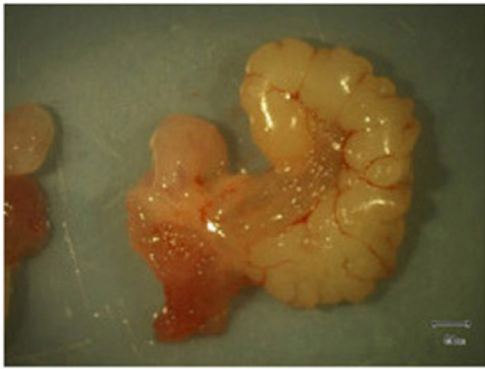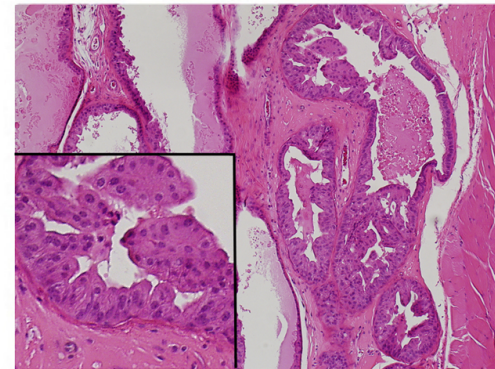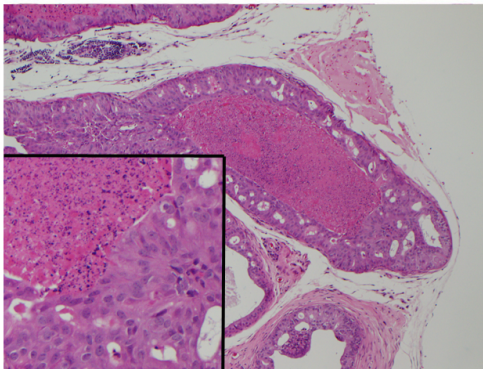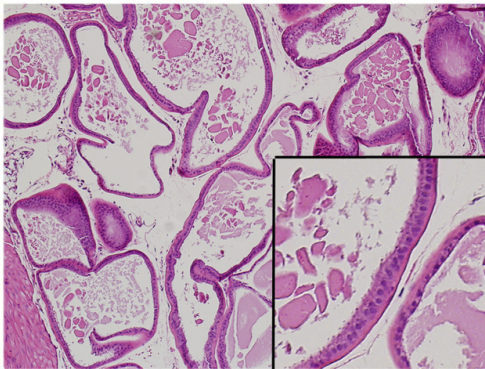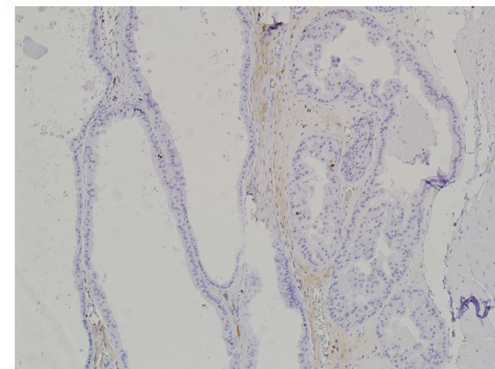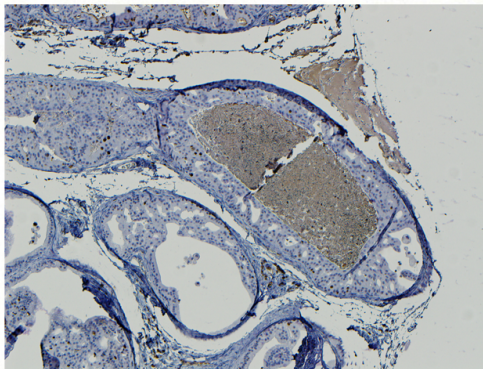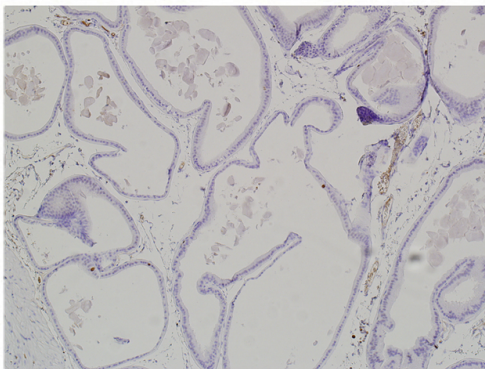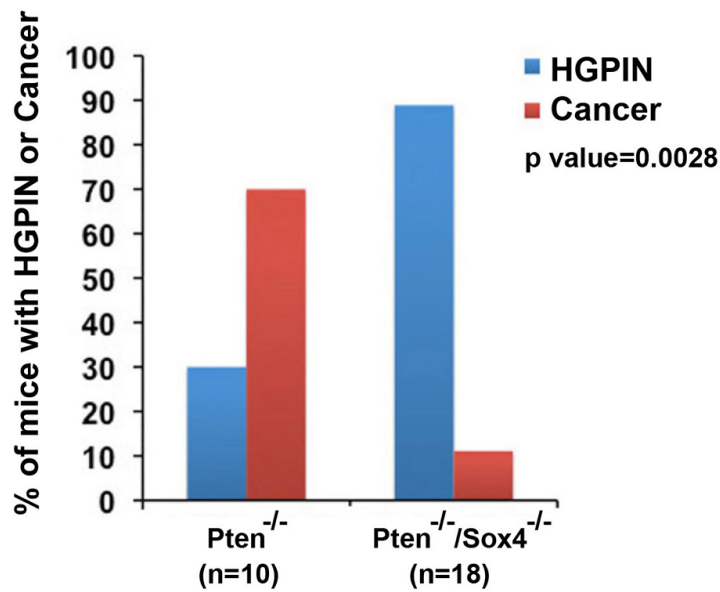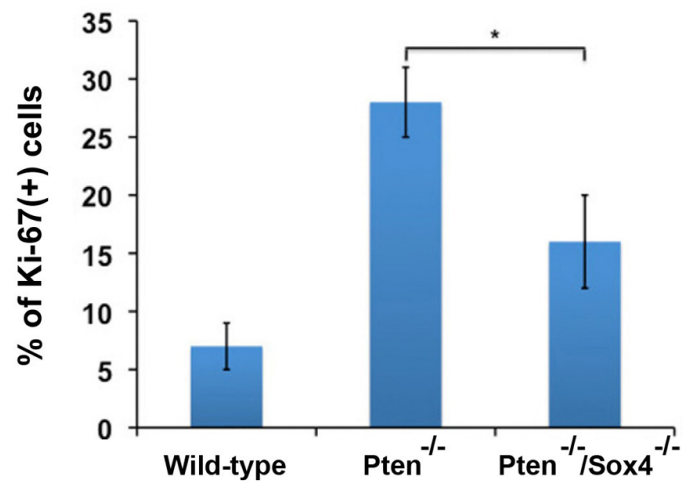
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SOX4 is essential for prostate tumorigenesis initiated by PTEN ablation

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