Molecular and Cellular Pathobiology

Cancer Research

SOX9 Elevation in the Prostate Promotes Proliferation and Cooperates with *PTEN* Loss to Drive Tumor Formation

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Abstract

Dysregulation of tissue development pathways can contribute to cancer initiation and progression. In murine embryonic prostate epithelia, the transcription factor SOX9 is required for proper prostate development. In this study, we examined a role for SOX9 in prostate cancer in mouse and human. In *Pten* and *Nkx3.1* mutant mice, cells with increased levels of SOX9 appeared within prostate epithelia at early stages of neoplasia, and higher expression correlated with progression at all stages of disease. In transgenic mice, SOX9 overexpression in prostate epithelia increased cell proliferation without inducing hyperplasia. In transgenic mice that were also heterozygous for mutant *Pten*, SOX9 overexpression quickened the induction of high-grade prostate intraepithelial neoplasia. In contrast, Sox9 attenuation led to a decrease proliferating prostate epithelia cells in normal and homozygous *Pten* mutant mice with prostate neoplasia. Analysis of a cohort of 880 human prostate cancer samples showed that SOX9 expression was associated with increasing Gleason grades and higher Ki67 staining. Our findings identify SOX9 as part of a developmental pathway that is reactivated in prostate neoplasia where it promotes tumor cell proliferation. *Cancer Res; 70(3); 979–87. ©2010 AACR.*

Introduction

Prostate cancer is a leading cause of mortality in older men of the western world (1). Understanding the processes that lead to prostate neoplasia is essential for the assessment of disease progression and choice of treatment. It is frequently the case that pathways involved in embryonic development are reactivated in cancer. Gene expression studies have indicated that programs that regulate prostate development and growth are reactivated in prostate cancer (2, 3).

The mouse has been used as a model for the study of prostate development and cancer. Although spontaneous prostate tumors do not arise in mice, several genetically modified mice have been shown to develop prostate intraepithelial neoplasia (PIN) and invasive carcinoma that is similar to the human neoplasia. An example of this is mice lacking the tumor suppressor gene *Pten* (phosphatase and tensin homolog deleted on chromosome 10) in the prostate that develop PIN at 6 weeks and invasive carcinoma after 9 weeks (4). Mutations in *PTEN* have also been associated with prostate cancer in humans (5).

The transcription factor SOX9 has been shown to be essential for many processes during embryogenesis, including early prostate development (6). Studies on mice lacking *Sox9* in the prostate showed a requirement in ventral prostate development and proper anterior prostate differentiation (7). High levels of SOX9 were associated with the prostate epithelia from the first stages of bud development from the urogenital sinus. In the ventral prostate, the absence of *Sox9* gave rise to a decrease of proliferation within the epithelia and a lack of expression of genes specific to prostate bud development that led to a complete loss of the structure.

Studies on prostate cancer cell lines have implicated SOX9 in prostate cancer, although its role has not been clearly determined. One study showed that it can suppress growth and tumorigenesis (8). In contrast, other studies have shown that it can enhance the proliferation and invasion of prostate cell lines in a xenograft model (9, 10). Analysis of human tissue samples showed an association between SOX9 expression and prostate cancer, with a higher protein level in samples from patients with hormone-refractory cancer (2, 9). SOX9 expression has been associated with other cancers such as colorectal cancer and melanoma (11, 12). However, in some

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cases expression is associated with tumor formation, but in others it is downregulated in neoplasia. This suggests that the role of SOX9 is distinct in different cancers.

In this study, we have performed an extensive analysis of the role of SOX9 in prostate cancer in mouse and human. We show that high levels of SOX9 associate with PIN lesions of all grades in mice and correlate with high Gleason score in prostate cancer in humans. Overexpression of SOX9 in adult mouse prostate epithelia gave rise to an increase in proliferation and induced early high-grade PIN lesions when mice were also heterozygous for *Pten*. Our studies show that high levels of SOX9 contribute to regulate proliferation within the prostate epithelia and can cooperate with *PTEN* loss to accelerate prostate neoplasia.

Materials and Methods

Mouse strains. The mouse strain containing the *Sox9* conditional allele was kindly provided by Andreas Schedl (Institut National de la Santé et de la Recherche Médicale U470, Nice, France; ref. 7). Mice with the mutant *Pten* allele and the PbCre4 transgene were obtained from the Mouse Models of Human Cancers Consortium repository unit (13, 14), and mice with the conditional allele of *Pten* were obtained from The Jackson Laboratory (15). Mice deficient for *Nkx3.1* were obtained from Michael Shen (Columbia University, New York, NY; ref. 7). The animals were bred on a mixed genetic background.

Human and mouse samples. Details of the human prostate samples containing the three different zones are given in Supplementary Fig. S1. Tissue arrays were constructed from unselected transurethral resections as described previously (16). Assignment of areas of cancer and noncancer in each core was carried out after antibody staining on the basis of histopathologic examination. The primary end points were time to death from prostate cancer and time to death from any cause. Univariate and multivariate analyses were done by proportional hazard (Cox) regression analysis (17). All follow-up times commenced at the point of 6 mo following diagnosis as in the previous report (18). The association between SOX9 expression and Gleason score and Ki67 staining was examined using the χ^2 test. The following variables were included in the multivariate analyses: centrally reviewed Gleason score, baseline PSA (last PSA value within 6 mo of diagnosis), extent of disease, and age at diagnosis.

For the mouse studies, the histologic phenotypes of all samples used for immunohistochemistry were initially assessed on H&E-stained sections. The assessment was based on published guidelines and assisted by a pathologist (19, 20). PIN lesions noted as low grade were equivalent to PIN I to II and those noted as high grade were equivalent to PIN III to IV as defined by Park and colleagues (20).

Immunohistochemistry. Antibody stains were done on paraffin sections as described previously (7). The following antibodies were used: For SOX9, two antibodies were used with amplification protocols, one was a gift from Francis Poulat (Centre National de la Recherche Scientifique, Montpellier, France; 1/2000 dilution) and was used for the human tissue array samples and the other was a gift from Robin Lovell-Badge (National Institute for Medical Research, London, United Kingdom; 1/16,000); Ki67 (TEC-3, Dako) was used at 1/25 and 1/200 dilutions (with amplification protocols); pAKT (736E11, Cell Signaling Technologies) was used at 1/25 and 1/200 dilutions (with amplification protocols); p63 (4A4, Santa Cruz Biotechnology) was used at 1/50 dilution; green fluorescent protein (GFP; ab290-50, Abcam) was used at 1/3,000 dilution. Secondary fluorescent antibodies were obtained from Molecular Probes and were used at 1/1,000 dilution. The ABC vector kit (Vector Laboratories) was used for amplification and stained with 3,3′-diaminobenzidine chromogen or with the use of the TSA kit for fluorescence (NEL741, Perkin-Elmer).

Quantification of proliferation. Ki67 staining was done on a number of sections per animal and positive cells were counted from four to six different fields from different sections. The number of total cells per animal ranged from 600 to 2,500.

Quantitative RT-PCR. Tissues from the distal regions of the different prostatic lobes were dissected and RNA was isolated using the Rneasy Micro Kit (Qiagen). cDNA synthesis was primed with oligo dT and done with Superscript II reverse transcriptase (Invitrogen). Real-time PCR was done using the TaqMan system (ABI7900) with the following primers/probe sets: Mm00448840_m1 for *Sox9* and Mm00486906_m1 for e-cadherin. Relative mRNA accumulation was determined by the $\Delta\Delta$Ct method and *Sox9* levels were normalized to E-cadherin levels.

Results

SOX9 expression in the adult mouse and human prostate. Using an antibody to SOX9 that showed high levels of protein in the prostate epithelia of mouse embryos (7), we investigated the expression of this factor in the adult mouse prostate. This analysis determined that SOX9 was expressed in the epithelia of the adult mouse prostate but at different levels between lobes. Highest levels were found in the lateral and ventral prostates (Fig. 1A). This difference was confirmed by real-time RT-PCR (Fig. 1B). SOX9 was expressed in both luminal and basal cells of the lateral and ventral prostates (Supplementary Fig. S1A). In contrast, all zones in the human expressed similar levels of SOX9. Basal cell staining was predominantly found, with all zones showing some luminal cell staining (Fig. 1A; Supplementary Fig. S1B).

The mouse prostatic lobes can be divided into three regions relative to the urethra: distal, intermediate, and proximal. Recent studies have shown that the proximal region harbors cells that have properties of adult stem cells (21, 22). Staining with an antibody to SOX9 revealed high levels of this protein within the proximal region relative to the intermediate and distal regions (Fig. 1C). Higher levels of SOX9 were also found in the epithelia of all lobes of prostates from mice that had been castrated when compared with intact animals (Fig. 1D).

SOX9 expression in mouse models of prostate neoplasia. SOX9 has been implicated in human prostate cancer, and therefore, we investigated the levels of SOX9 in various mouse models of prostate neoplasia. *Nkx3.1*-deficient mice

have been shown to develop hyperplasia and dysplasia at 12 months that will progress to PIN in mice over 18 months old (23, 24). Sections of 12-month-old anterior prostates from these mice showed a general hyperplastic lobe with cells filling the lumen. SOX9 antibody staining of these samples revealed discrete areas of high SOX9 expression within the epithelia (Fig. 2A).

Mice heterozygous for a deletion in *Pten* develop spontaneous tumors in various organs (13, 25–27). Within the prostate, they show hyperplasia that progresses to PIN after 10 months. Staining with an antibody to SOX9 of 6-month-old anterior prostates from these mice showed that high levels of this factor were associated with areas of hyperplasia (Fig. 2A).

Mice with a conditional allele of *Pten* (*Pten*^{fl}) and the prostate-specific Cre expressing construct PbCre4 develop PIN at 6 weeks of age that progresses to invasive carcinoma after 9 weeks (4). Antibody staining showed high levels of SOX9 expression associated with low- and high-grade PIN (ref. 20; Fig. 2B). Therefore, these studies show that an increase in SOX9 levels was observed at early stages of prostate hyperplasia and was associated with progression to high-grade PIN lesions.

Overexpression of SOX9 in prostate epithelia. The association between increased levels of SOX9 and prostate neoplasia indicated that this factor may contribute to prostate cancer initiation and/or progression. To investigate this possibility, we created mice that overexpressed high levels of SOX9 in the adult prostate. For this, we used transgenic mice that were hemizygous for a construct where *Sox9* and GFP, through the use of an internal ribosomal entry site, were driven by the CAG (cytomegalovirus early enhancer/chicken β-actin) promoter in tissues where Cre recombinase is expressed (Z/Sox9 mice; see Supplementary Fig. S2 for details of the Z/Sox9 construct). These mice were mated to the PbCre4 mice, creating mice that expressed high levels of SOX9 within the adult prostate epithelia. A variable number of GFP positive cells could be found in all lobes in a mosaic pattern, with highest levels in the lateral and dorsal prostates. As expected, cells that expressed GFP showed high levels of SOX9 (Fig. 3A). These cells were mainly luminal, although some positive basal cells could be found (Fig. 3A; Supplementary Fig. S2A). In the dorsal prostate, these mice expressed up to 3.75-fold more *Sox9* than control animals without the PbCre4 construct (Supplementary Fig. S2B). Analysis of the prostates of these mice of various ages (6-12 months) showed no major histologic abnormalities when compared with wild-type animals (five animals analyzed). However, staining with an antibody to Ki67 revealed a 2.8-fold increase in the number of proliferating prostate epithelial cells in the dorsal lobes of overexpressing animals when compared with animals without PbCre4 (three mice per genotype analyzed; $P < 0.05$; data not shown). Costaining with Ki67 and GFP antibodies showed that, within the dorsal lobe, the few Ki67-positive cells were 4.7-fold more likely to express transgenic SOX9 (Fig. 3A). These studies indicate that high levels of SOX9 lead to an increase in cell proliferation. However, this increase is not sufficient to induce hyperplasia within the prostate epithelia in animals up to 12 months of age.

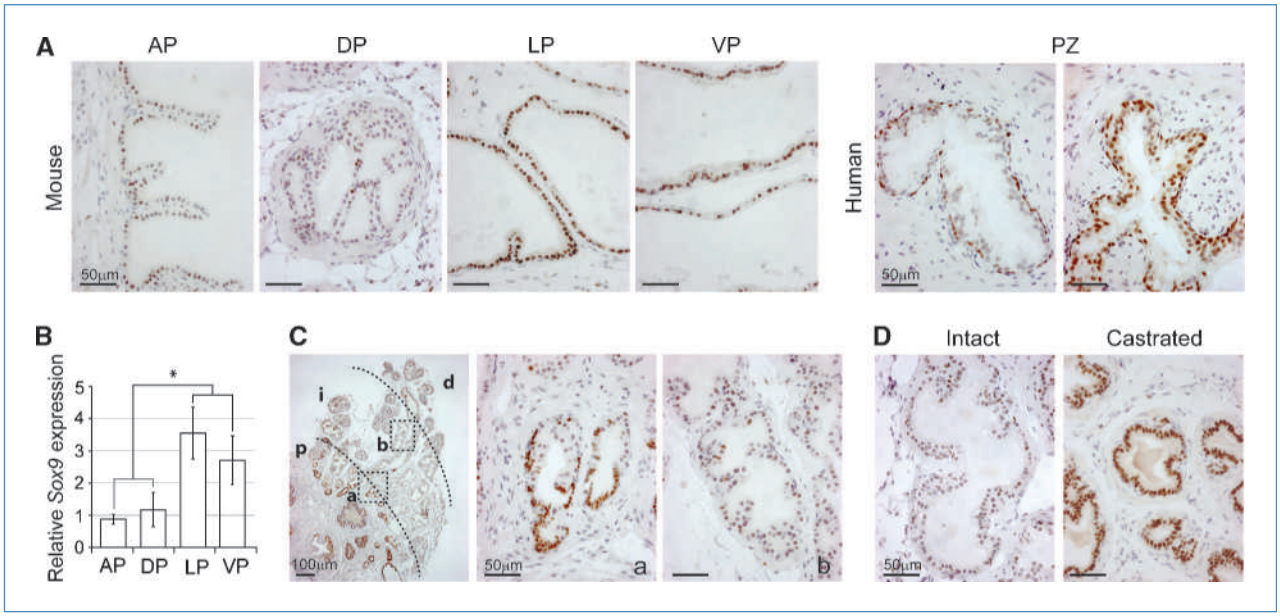

Figure 1. SOX9 expression in the mouse and human adult prostate. A, sections from mouse and human prostate were stained (brown) with an antibody to SOX9 and counterstained with hematoxylin. The different lobes and zones are indicated. *AP,* anterior lobe; *DP,* dorsal lobe; *LP,* lateral lobe; *VP,* ventral lobe; *PZ,* peripheral zone (10 mice and 4 human samples analyzed). B, quantitative RT-PCR for *Sox9* expression was done on RNA from the different mouse prostate lobes (4 mice per lobe analyzed). *, $P < 0.05$ (Student's *t* test). C, SOX9 levels are highest in the proximal region. The dashed lines in the left image indicate the proximal (p), intermediate (i), and distal (d) regions of the dorsal lobe. Images a (proximal region) and b (intermediate) show magnified pictures of the boxed regions in the left (10 mice analyzed). D, SOX9 levels are high in castrated mice. Staining of sections from the dorsal prostates from adult mice that were intact and castrated (10 mice analyzed).

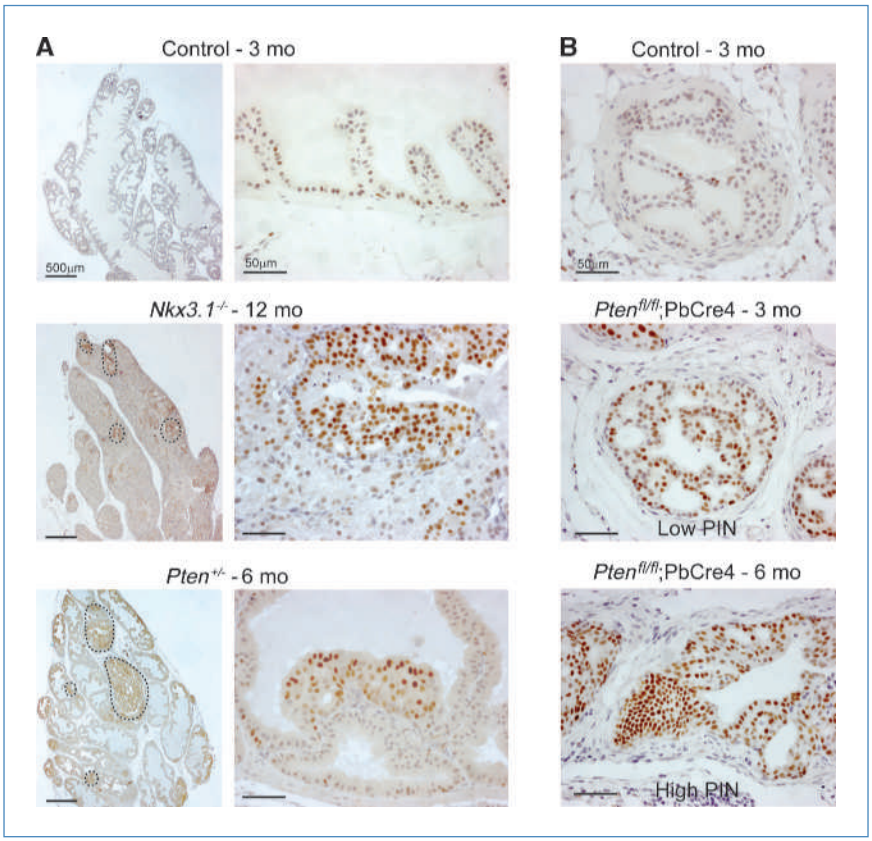

Figure 2. Upregulation of SOX9 is associated with early and late stages of prostate neoplasia. A, sections from the anterior prostates of mice that were wild type (control), *Nkx3.1* deficient (*Nkx3.1*$^{-/-}$; 10 mice analyzed), and heterozygous for *Pten* (*Pten*$^{+/-}$; 5 mice analyzed) were stained (brown) with an antibody to SOX9 and counterstained with hematoxylin. Right, higher magnifications of the samples on the left. The ages of the animals are indicated. Dashed lines, regions of high SOX9 expression. B, SOX9 staining of sections from the dorsal prostates of mice that were wild type (control) or mutant for the conditional allele of *Pten* (*Pten*$^{fl/fl}$;PbCre4; 10 mice analyzed). Areas of low- and high-grade PIN are indicated.

To establish if high levels of SOX9 can contribute to the prostate neoplasia observed in *Pten* mutant animals, we created mice that overexpress SOX9 and are heterozygous for the conditional *Pten*^{fl} allele. Analysis of prostates from 6- to 7-month-old heterozygous *Pten*^{fl} mutant animals without the Z/Sox9 construct showed few areas of mild hyperplasia in the anterior prostate (six animals analyzed). In contrast, two of four mice containing the Z/Sox9 construct revealed areas of high-grade PIN in the anterior prostate (Fig. 3B). These areas showed high levels of GFP and Sox9 (Fig. 3C; Supplementary Fig. S2C). Staining with an antibody to Ki67 showed a clustering of positive cells associated with these areas (Fig. 3D). Homozygous loss of *Pten* is associated with high levels of phosphorylated AKT (pAKT) in prostate neoplasia (4). Therefore, to investigate if increased levels of SOX9 could contribute to a *Pten* loss of heterozygosity phenotype, we stained sections from these animals with a pAKT antibody. We found that high levels of pAKT were specifically associated with the PIN lesions (Fig. 3D). These studies show that increased levels of SOX9 lead to an increase in proliferation and can contribute to the process of neoplastic transformation in cells that have lost one allele of *Pten*.

Effect of* Sox9 *deletion on normal and neoplastic prostate epithelia. Our studies on mice that overexpress SOX9 indicate a role for this factor in promoting proliferation. To investigate if absence of SOX9 in the adult prostate epithelia could lead to changes in cell proliferation, we created mice that contained the conditional loss-of-function allele of *Sox9* (*Sox9*^{fl}) and the PbCre4 construct. These mice showed no obvious phenotype in the lateral and ventral prostates, where SOX9 levels were highest in normal prostate. Analysis of Ki67 expression showed a significant 3.1-fold decrease in positive cells in the prostate epithelia of the mutant mice (Fig. 4A). Therefore, these studies show a strong correlation between SOX9 levels and the number of Ki67-positive cells within the prostate epithelia.

To establish if SOX9 can contribute to the phenotype observed in homozygous *Pten* mutant animals, the conditional allele of *Sox9* (*Sox9*^{fl}) was included in our breeding program. Two types of mice were generated that contained PbCre4 and were homozygous for *Pten*^{fl} (*Pten*$^{fl/fl}$): those that were heterozygous for *Sox9*^{fl} (*Sox9*$^{fl/+}$) and those that were homozygous for *Sox9*^{fl} (*Sox9*$^{fl/fl}$). Histologic analysis of mice at 3 and 6 months showed no major difference in the overall prostate neoplasia phenotype between these two groups. Prostates from mutant *Pten* animals have been shown to have an increase in proliferation as measured by Ki67 antibody staining (4). Interestingly, we observed a difference in the number of Ki67-positive cells in the prostate between the two groups. The *Sox9*$^{fl/fl}$-containing animals showed a reduction in the number of Ki67-positive cells (Fig. 4B). Consistent with previous studies, most of the Ki67-positive cells were found near the stromal compartment of the gland (28).

To confirm that *Sox9* has been deleted in the *Sox9*$^{fl/fl}$-containing animals, we stained prostate sections with an antibody to SOX9. As expected, most cells of the prostates of

these animals lacked SOX9; however, cells with high levels of SOX9 were found in the region near the stromal compartment of the prostates of these mice (Fig. 4B). Staining with an antibody to pAKT showed that the SOX9-positive cells in the $Sox9^{fl/fl}$-containing animals lacked an upregulation of this protein in the plasma membrane (Fig. 4B). Upregulation of pAKT levels has been shown to be associated with loss of *Pten* in the prostate (4); therefore, these studies indicate that these cells did not express Cre recombinase, and no recombination between flox sites at the *Pten* and *Sox9* loci had occurred. Double staining with Ki67 and either SOX9 or pAKT antibodies showed that most of the Ki67-positive cells in the $Sox9^{fl/fl}$ animals were also positive for SOX9 and negative for pAKT (Fig. 4B). Consistent with this, quantification of cells that were positive for Ki67 and negative for pAKT between the two groups showed a significant 2-fold decrease in proliferation in $Sox9^{fl/fl}$ animals compared with the $Sox9^{fl/+}$ animals (Fig. 4B). These data show that loss of *Sox9* does not inhibit neoplastic progression in the prostate of homozygous mutant *Pten* mice but leads to a reduction in the number of proliferating prostate epithelial cells that have retained phosphoinositide 3-kinase (PI3K) signaling.

SOX9 expression in human prostate cancer. Our studies in mice suggested that high levels of SOX9 in the prostate might be associated with prostate cancer. Therefore, to establish the relationship between SOX9 levels and prostate cancer in humans, we stained 880 prostate cancer cores with an antibody to SOX9. These cores were derived from 387 patients with localized disease and Gleason scores ranging from 4 to 10. In contrast to our mouse studies, not all cancer samples showed SOX9 expression, with 54% of cores (N = 478) showing no staining and the remaining cores showing either low or high levels of nuclear staining (Fig. 5A and B). The level of SOX9, either 0 or some positive staining (>0), was determined for each patient and used in statistical analysis. There was a significant positive correlation between SOX9 expression and Gleason score (46% of SOX9 positive for Gleason <7, 67% for Gleason 7, and 61% for Gleason > 7; Pearson's $\chi^2 = 13.6$, $P = 0.001$; Table 1). In univariate analysis, SOX9 expression correlated with poorer prognosis and

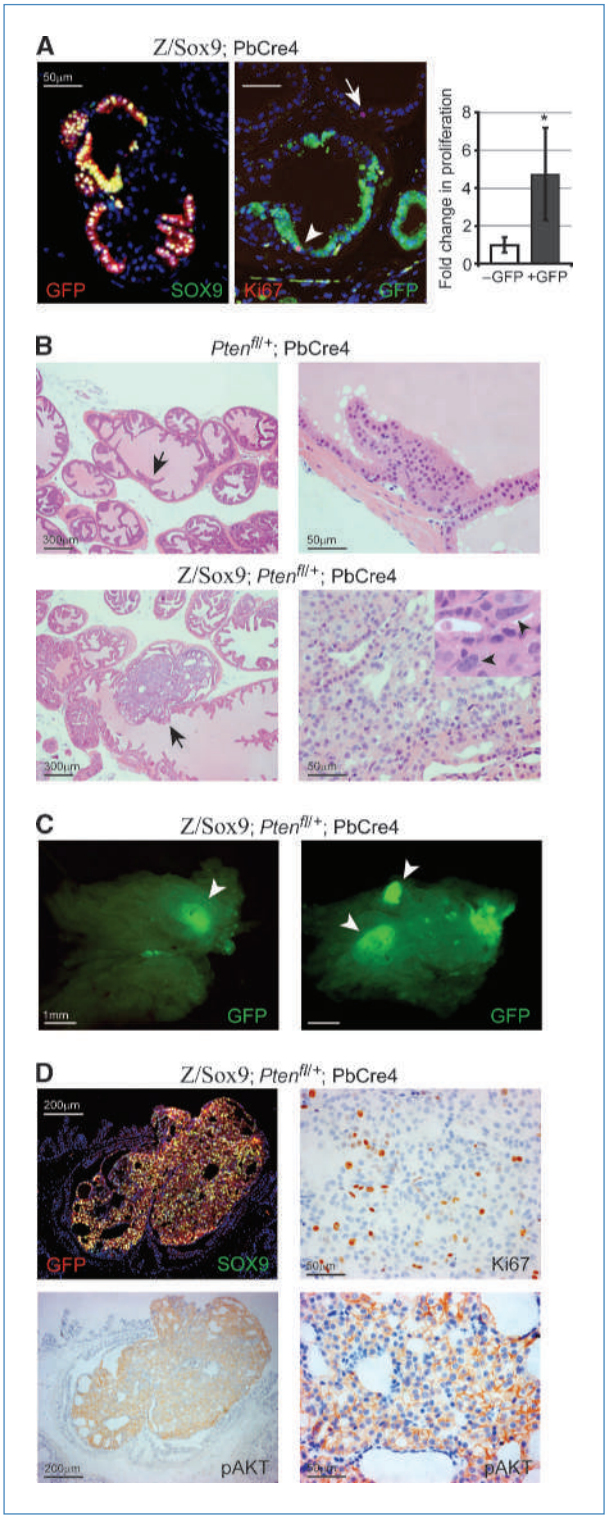

Figure 3. Overexpression of SOX9 leads to an increase in proliferation in normal mice and PIN in heterozygous *Pten*-deficient mice. A, immunofluorescent staining was done on sections of the dorsal prostates of mice that were hemizygous for the Z/Sox9 and PbCre4 constructs with antibodies to GFP (red and green, as indicated), SOX9 (green), and Ki67 (red), as indicated. Arrowhead, a cell that is positive for Ki67 and GFP; arrow, a cell that is Ki67 positive and GFP negative. Blue staining, nuclei stained with 4′,6-diamidino-2-phenylindole. The graph depicts the quantitative analysis of Ki67-positive cells that were either GFP positive or GFP negative within the dorsal lobe (5 mice analyzed). *, $P < 0.05$ (Student's t test). B, H&E staining of sections from the anterior prostates of mice that were heterozygous for the $Pten^{fl}$ allele and PbCre4 and were either positive or negative for the Z/Sox9 construct. Right, higher magnifications of regions in the left image indicated with arrows. Features of high-grade PIN were observed in mice that overexpress SOX9. *Inset,* atypical cells are indicated with arrowheads. C, whole-mount image of fluorescence in the anterior prostates from mice that contained the Z/Sox9 construct. White arrowheads, areas that showed PIN on histologic sections. D, sections from the anterior prostates of Z/Sox9-containing mice that contained areas of PIN were stained with antibodies to SOX9 (red), GFP (green), Ki67, and pAKT, as indicated. Bottom right, higher magnification of the left image (pAKT staining).

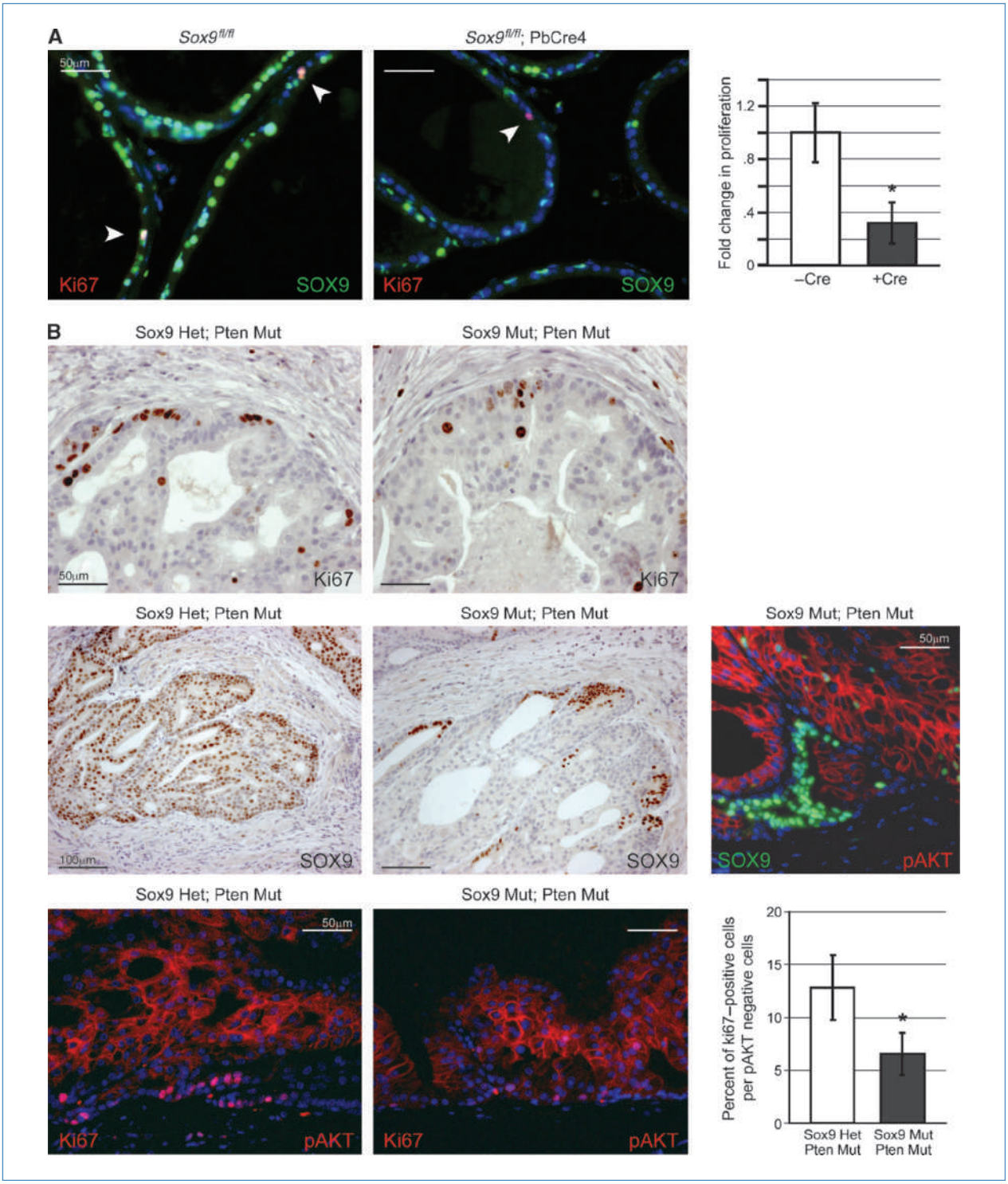

Figure 4. Loss of *Sox9* leads to a decrease in proliferation in normal and neoplastic prostate epithelia. A, immunofluorescent staining with antibodies to SOX9 (green) and Ki67 (red) of sections from the lateral prostates of mice that were homozygous for the conditional allele of *Sox9* (*Sox9*$^{fl/fl}$) and either contained the PbCre4 construct (+*Cre*) or did not (–*Cre*). Blue staining, nuclei stained with 4′,6-diamidino-2-phenylindole. The graph depicts the quantification of the number of Ki67 cells for each genotype (5 control and 6 mutant mice analyzed). *, $P < 0.05$. Cells that were Ki67 and SOX9 positive in the +Cre group were not counted. All Ki67 cells in the –Cre group were SOX9 positive. B, sections from the anterior prostates of mice that were mutant for the conditional allele of *Pten* (*Pten Mut*; *Pten*$^{fl/fl}$;PbCre4) and were either heterozygous (*Sox9 Het*; *Sox9*$^{fl/+}$) or homozygous (*Sox9 Mut*; *Sox9*$^{fl/fl}$) for the conditional *Sox9* allele were stained with antibodies to Ki67, SOX9, and pAKT, as indicated. The graph shows the percentage of the number of cells that were positive for Ki67 and negative for pAKT (six mice for each genotype analyzed). *, $P < 0.05$ (Student's *t* test).

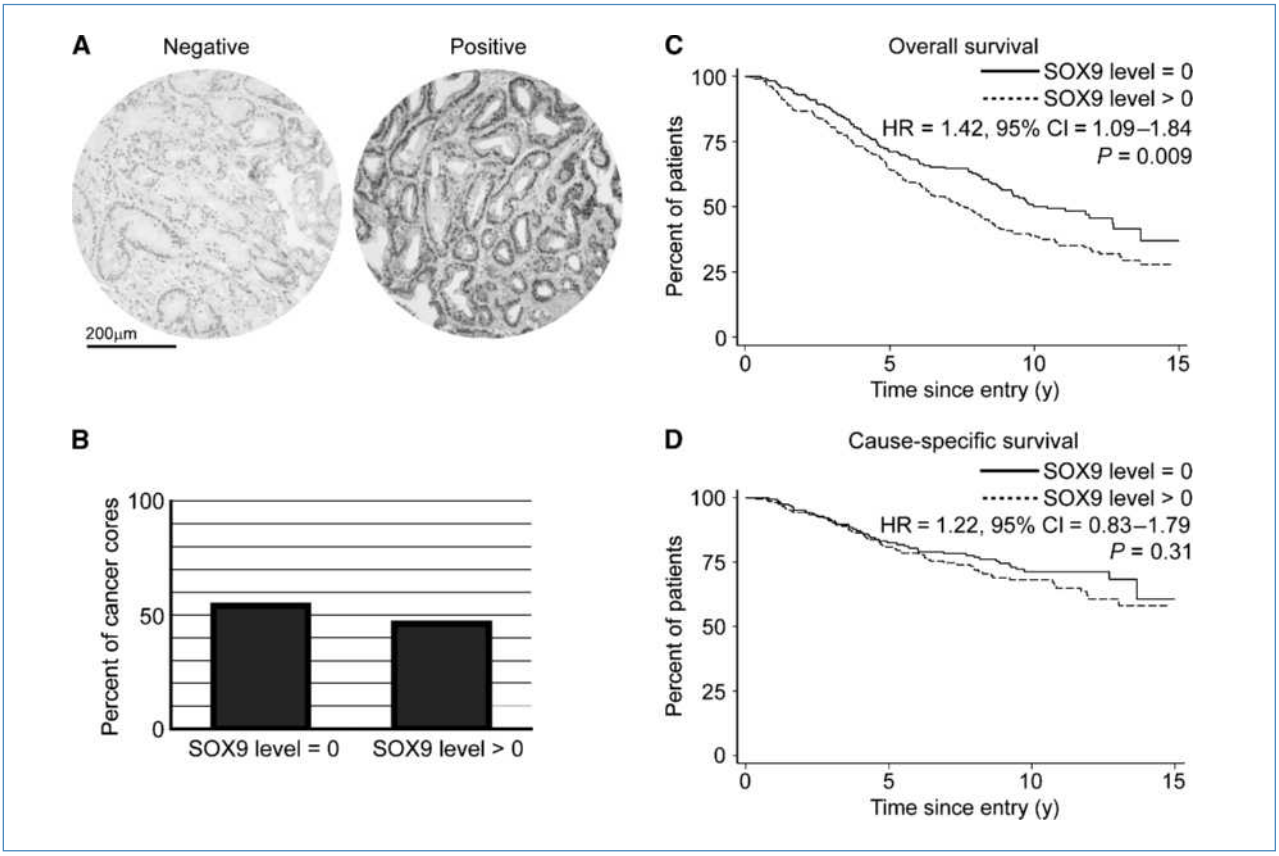

Figure 5. SOX9 expression in tissue arrays from human prostate cancer samples. Sections of 880 prostate cancer cores derived from 387 patients were stained with an antibody to SOX9 and counterstained with hematoxylin. A, staining of two prostate cancer cores showing negative and positive staining. B, percent of cancer cores that were negative (54%) and positive (46%) for SOX9 staining. C and D, Kaplan-Meier curves for overall survival (C) and cause-specific survival (D). (*HR,* hazard ratio; *95% CI,* 95% confidence interval.)

was significant for overall survival (Fig. 5C) but not for cause-specific survival (Fig. 5D). However, SOX9 expression did not remain significant independently of age, Gleason score, PSA, and extent of disease in a multivariate model [$\Delta\chi^2$ (1 degree of freedom) = 0.17, P = 0.19]. Higher levels of Ki67 have been shown to correlate with lower chance of survival in this group of samples (29). Consistent with our mouse studies, expression of SOX9 was significantly positively associated with Ki67 in the prostate cancer samples (χ^2 for trend = 7.25, P = 0.007; Table 1). These studies indicate that expression of SOX9 correlates with increased proliferation and tumor progression and decreased survival in humans.

Table 1. Distribution of Gleason score categories and the percentage of Ki67-positive cells between SOX9-negative (0) and SOX9-positive (>0) cancer cores

	SOX9 level, *n* (%)	
	0	>0
Gleason score		
<7	93 (54)	79 (46)
7	36 (33)	73 (67)
>7	41 (39)	65 (61)
Ki67 staining (%)		
0–1	89 (53)	80 (47)
>1–5	53 (39)	82 (61)
>5–10	14 (29)	34 (71)
>10–100	12 (40)	18 (60)

Discussion

Various studies have implicated SOX9 in prostate cancer. However, the data are in conflict with studies indicating that it can promote as well as repress cell proliferation, and that it is involved in metastasis and may be required in the early stages of prostate carcinogenesis (2, 8, 10, 30). Our results in mice show that SOX9 expression is upregulated within the epithelia in areas where neoplastic abnormalities are first observed, and high levels of SOX9 are associated with all stages of PIN progression. This is consistent with our data on human prostate cancer where SOX9 expression was more frequent in samples with higher Gleason score. Our functional data show that high levels of SOX9 in the prostate

contribute to an increase in proliferation that can lead to PIN in certain mutant genetic backgrounds. Therefore, our results are consistent with a cooperative role for SOX9 in the progression of the early stages of prostate cancer formation.

Our expression data had suggested that high levels of SOX9 could promote early stages of prostate neoplasia. Our overexpression studies showed that a high level of SOX9 on its own is not sufficient to induce hyperplasia and neoplasia *in vivo*, although increased proliferation was observed. However, in the context where one allele of *Pten* was lost, focal areas of high-grade PIN were detected, where all cells showed high GFP and SOX9 expression. This indicates that cells with increased levels of SOX9 and decreased levels of *Pten* are more likely to become neoplastic. The focal nature of this event could be due to a stochastic process or the nature of the cell of origin, which, if relatively rare in Cre-expressing cells, would be made even more infrequent if both transgenic SOX9 expression and loss of one $Pten^{fl}$ allele needed to occur in the same cell. The areas of high-grade PIN in these mice showed increased pAKT in most cells. This phenotype was not observed in GFP-positive cells that were not part of the PIN lesion. This suggests that a high level of SOX9 does not directly regulate pAKT levels, rather it increases the likelihood of an event that leads to an increase in cells with high PI3K signaling. A similar situation occurs in mice that are heterozygous for *Pten* and *Nkx3.1* mutations (31). Our data suggest that an increase in proliferation is a potential mechanism in which SOX9 can contribute to the acceleration of neoplasia progression. Overall, our studies indicate that SOX9 does not act like a classic oncogene, but rather it cooperates with other events such as the loss of *PTEN* to induce carcinogenesis.

Our genetic data show that *Sox9* deletion leads to a decrease in proliferation in wild-type and homozygous $Pten^{fl}$ mutant mice. However, this decrease did not inhibit prostate neoplasia progression in the mutant animals. Presumably, other pathways regulating proliferation are also active in these mice and are sufficient to ensure neoplastic progression. In the $Pten^{fl}$ mutant mice, the proliferating cells were more likely to harbor cells where Cre recombination had not occurred (see Fig. 4B) and therefore contained wild-type *Pten* and *Sox9*. This suggests that the neoplastic epithelia associated with *Pten* loss produce non–cell-autonomous factors that regulate proliferation in neighboring cells. Paracrine effects from the epithelia have been observed to control processes within the stroma in models of prostate cancer (32). Our results are consistent with a contribution of SOX9 to the regulation of the factors controlling proliferation. This effect was also observed in the early neoplastic lesions where Ki67-positive cells tended to surround the areas of high SOX9 in the prostates of *Nkx3.1* mutant mice (Supplementary Fig. S3). This suggests that the production of paracrine factors regulating proliferation might be a general mechanism associated with the initial stages of carcinogenesis.

A striking difference between mouse and human in our studies is that we observed high expression of SOX9 in prostate neoplasia in mouse models, but only 46% of human cancer cores were positive for this factor. One technical explanation is that the SOX9 antibody we used did not react with all the samples in the same way due to the tissue array process. We do not think this is the case because our studies with a different SOX9 antibody and two other studies using different antibodies and samples found a similar percentage of cancer samples that were negative for SOX9 (2, 9). Cancer samples that were positive for SOX9 tended to have a higher Gleason score and increased Ki67-positive cells. A multivariate analysis of our human data shows that expression of SOX9 is not an independent prognostic factor of survival. Therefore, our analysis suggests that SOX9 expression correlates with disease progression in a subset of prostate cancers and that other SOX9-independent pathways can also lead to a low survival prognosis. Classification of prostate cancers into different subtypes may be required to uncover whether expression of SOX9 has any predictive value. Screening of other mouse models of prostate cancer for SOX9 levels may also uncover differences in the dependence of SOX9 in the different pathways that lead to neoplasia.

SOX9 has been implicated in other cancers such as melanoma in humans (12). In this example, higher levels of SOX9 were associated with normal melanocytes and were downregulated in premalignant and malignant cells. In addition, overexpression of SOX9 inhibited proliferation and led to cell cycle arrest. This shows that the function of this factor in cancer is context specific. This is consistent with its many roles during embryonic development, in which it can cooperate with tissue-specific factors to initiate different cell fates such as Sertoli cell differentiation, chondrogenesis, and neural crest development (33–35). This also highlights the value of *in vivo* studies to delineate the role of SOX9 in specific tissues.

In summary, this study identifies SOX9 as a factor involved in the regulation of proliferation of prostate epithelial cells, which can contribute to neoplastic transformation in specific contexts. Future identification of different prostate cancer subtypes may uncover the contributions of SOX9 to specific pathways that lead to neoplasia.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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