SOX9 Is Expressed in Human Fetal Prostate Epithelium and Enhances Prostate Cancer Invasion

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Abstract
SOX9 is a transcription factor that plays a critical role in the development of multiple tissues. We previously reported that SOX9 in normal human adult prostate was restricted to basal epithelium. SOX9 was also expressed in a subset of prostate cancer (PCa) cells and was increased in relapsed hormone-refractory PCa. Moreover, SOX9 expression in PCa cell lines enhanced tumor cell proliferation and was β-catenin regulated. Here we report additional in vivo results showing that SOX9 is highly expressed during fetal prostate development by epithelial cells expanding into the mesenchyme, suggesting it may contribute to invasive growth in PCa. Indeed, SOX9 overexpression in LNCaP PCa xenografts enhanced growth, angiogenesis, and invasion. Conversely, short hairpin RNA–mediated SOX9 suppression inhibited the growth of CWR22Rv1 PCa xenografts. These results support important functions of SOX9 in both the development and maintenance of normal prostate, and indicate that these functions contribute to PCa tumor growth and invasion. [Cancer Res 2008;68(6):1625–30]

Introduction
Prostate cancer (PCa) is one of the most common noncutaneous malignancies in men and is a leading cause of cancer mortality. PCa screening using serum prostate-specific antigen has led to increased detection of early-stage PCa that can be cured by radical prostatectomy or radiation therapy. However, many PCa patients with early-stage disease will follow a prolonged and slow course and can be managed by watchful waiting, sparing them from the therapeutic side effects. Other patients will present with advanced disease, and a substantial fraction of patients who present with clinically localized PCa and undergo primary therapy will eventually recur with metastatic disease. These patients can be treated with androgen deprivation therapy, but they invariably relapse with a more aggressive form of PCa that has been termed hormone-refractory or androgen-independent PCa, for which there is currently no effective treatment (1). A better understanding of the mechanisms responsible for PCa development and progression is urgently needed to uncover better tools to diagnose patients with more aggressive disease and provide them with more effective, mechanism-based therapy.

SOX9 belongs to the SOX (Sry-related high mobility group box) family of transcription factors and is a key regulator of developmental processes including male sex determination, chondrogenesis, neurogenesis, and neural crest development (2–5). Heterozygous SOX9 mutation is the cause of campomelic dysplasia, a severe form of human dwarfism characterized by extreme cartilage and bone malformation, which is frequently associated with XY sex reversal (6). The identified major targets of SOX9 are collagens [such as type II collagen (Col2α1) and type XI collagen (Col11α2)] during chondrogenesis and the Mullerian inhibiting substance during male sex differentiation (7). In adult tissues, SOX9 is expressed in intestinal crypts and hair follicles, where it is regulated by the Wnt/β-catenin or Sonic hedgehog signaling pathways and seems to be necessary to maintain stem cell/progenitor cell populations (8, 9).

We have reported that the expression of SOX9 in normal human adult prostate is restricted to the basal epithelium (10), which seems to contain the stem/progenitor cells that are responsible for glandular epithelium maintenance and regeneration (11). We also found that SOX9 was expressed in PCa, with increased expression in advanced hormone-refractory recurrent PCa. Significantly, SOX9 expression in PCa cell lines was increased by the Wnt/β-catenin pathway, which has been implicated in the initiation and progression of many types of cancer, in addition to stem/progenitor cell maintenance in adult tissues (12). However, the detailed molecular functions of SOX9 in prostate and its target genes have not been determined.

The connection between normal development and oncogenesis has a long history. Genes and pathways important in development may also function in adult tissue homeostasis by maintaining stem/progenitor cell populations and regulating tissue repair. However, in cancer cells these genes or pathways can be subverted and contribute to malignant proliferation or invasion. To determine whether SOX9 contributes to prostate development and to assess its possible function in PCa in vivo, we studied its expression in human fetal prostate. Significantly, we found strong SOX9 expression in human fetal prostate epithelium that was expanding into mesenchyme, suggesting that SOX9 may contribute to PCa invasion. Consistent with this hypothesis, growth, invasion, and angiogenesis were increased in PCa xenografts overexpressing SOX9, whereas growth was decreased in PCa xenografts with reduced endogenous SOX9. These results indicate that SOX9 functions in normal prostate development and in the basal epithelium are subverted by PCa cells to support tumor growth and invasion.

Materials and Methods
Cell lines and cell culture. The culture condition of LNCaP and CWR22Rv1 cells and the generation of LNCaP cell lines inducibly expressing SOX9 were previously described (10). pSUPER.retro.puro vectors (Oligogen) expressing the SOX9i short hairpin RNA (shRNA) or nontargeting

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control shRNA were generated by insertion of annealed oligonucleotide pair into the BglII-HindIII sites (10). Twenty micrograms of pSUPER vectors together with 5 μg of Gag-Pol and 1 μg of vesicular stomatitis virus G were transfected by Lipofectamine 2000 (Invitrogen) into a 15-cm culture dish of subconfluent Phoenix cells (Orbigen), and culture supernatants containing retrovirus were collected at 48 and 72 h posttransfection. CWR22Rv1 clones expressing SOX9 or control shRNA were generated by infection with the Phoenix cell culture supernant plus 4 μg/mL polybrene for 72 h, and then selected with 1.5 μg/mL puromycin for 72 h.

Xenografts. Six- to eight-week-old male ICR/scid mice (Taconic) were used to generate PCa xenografts. Two million LNCaP cells mixed with 50% Matrigel were injected s.c. into each flank, and the animals were fed with either doxycycline water (1 mg/mL) to induce SOX9 expression or regular water as uninduced control. The s.c. xenografts were measured with a caliper and the xenografts were extracted when their largest dimension grew beyond 10 mm. For CWR22Rv1 xenografts, each mouse was injected with 2 million CWR22Rv1-SOX9i cells in one flank and with 2 million CWR22Rv1 control cells in the opposite flank. Tumor volumes and weights were determined by direct measurement of the tumors extracted at 17 d postimplantation. Tumors were formalin fixed and paraffin embedded for immunohistochemistry.

Immunohistochemistry. Nine human fetal prostate paraffin-embedded blocks were a part of the collection previously described (13). The immunohistochemistry was done as described (10). Neovessels were stained with a polyclonal antihuman VWF antibody purchased from DAKO. Antibody against high molecular weight cytokeratin (34βE12) was purchased from DAKO.

In vitro invasion assay. The assays were done as previously described (14). In brief, 12-well transwell chambers with 12-μm pore size filter (Costar) were coated with 300 μg/cm² Matrigel (BD Biosciences) by incubating at 37°C overnight. LNCaP-SOX9 or its parental cells were induced with tetracycline (40 ng/mL) or mock treated (uninduced) 24 h before the assay. Cells (8 × 10⁴) were mixed with diluted Matrigel (300 μg/mL) in RPMI 1640 [supplemented with 1% bovine serum albumin (BSA)] at 4°C, then seeded on the Matrigel-coated transwell top chamber. Cells were allowed to invade the Matrigel coat for 24 h at 37°C by adding 1.5 mL of complete media [RPMI 1640 with 10% fetal bovine serum (FBS)] to the lower chamber of the transwell. At the end of the incubation, noninvaded cells were scraped off from the top chamber using a cotton swab. To visualize the invaded cells that migrated to the bottom surface of the membrane, they were stained with crystal violet. To quantitate the numbers of invaded cells, cells were collected by trypsinization and measured with CyQuant (Invitrogen) fluorescence dye following the manufacturer’s instructions.

Results

SOX9 expression in human fetal prostate epithelium. SOX9 is critical for the normal development of several tissues and for male sex determination. To determine whether SOX9 is expressed during prostate development, we examined its expression in human fetal prostate. SOX9 nuclear staining was found in seven of nine fetal prostate samples collected by fetopsies at gestation weeks between 19 and 22.5 (Fig. 1A), whereas these samples were negative in control experiments stained without the anti-SOX9 primary antibody (Fig. 1B). Although the small number of cases limits a precise correlation of SOX9 expression with prostate developmental stage, some interesting patterns of fetal SOX9 expression can be noted. First, SOX9 expression is limited to the fetal prostate epithelium with essentially no expression in the surrounding stromal tissue. Second, when the epithelial glands are arranged in a compact conformation, SOX9 is expressed in almost all the cells within the gland (Fig. 1A, left). Third, when the gland begins to canalize with lumen formation, SOX9 expression is more restricted to the peripheral cells of prostatic gland (Fig. 1A, middle), which is reminiscent of its basal cell expression in adult prostate (10). Interestingly, these peripheral cells in fetal prostate glands also express high molecular weight cytokeratin, a basal cell marker of

Figure 1. SOX9 expression in human fetal prostate epithelium. A, immunohistochemistry of SOX9 in human fetal prostates of gestation age 19 to 22.5 wk. The O9-1 rabbit SOX9-specific antibody was used (4). B, negative control of SOX9 immunohistochemistry of fetal prostate with an irrelevant primary antibody and same secondary antibody. C, immunohistochemistry of fetal prostate with a high molecular weight cytokeratin (HMWC) antibody (34βE12).
adult prostate (Fig. 1C). Finally, SOX9 expression is more pronounced at the tip of branching prostate glands that are expanding into the surrounding stroma (Fig. 1A, right). These results suggest that SOX9 contributes to the developmental process that allows prostate epithelium to initially outgrow into the mesenchyme and then provide basal cell support for development and maintenance of the luminal epithelium.

**SOX9 expression enhances LNCaP PCa tumor growth and angiogenesis.** To examine the functional role of SOX9 in PCa in vivo, we established LNCaP PCa cells (which express relatively low levels of endogenous SOX9) capable of tetracycline/doxycycline inducible expression of exogenous SOX9. As previously shown, induction of SOX9 expression in these cultured cells by doxycycline-mediated inactivation of the tetracycline operator repres sor (tet repressor) is very efficient, and substantial amounts of SOX9 can be detected after 2 hours of induction compared with control parental LNCaP cells expressing only tet repressor (10). Cells from an inducible SOX9-expressing LNCaP clone (LNCaP-SOX9 #2) were injected s.c. into male scid mice to generate xenografts. Starting 2 days after injection, the mice were randomly assigned to one group fed with doxycycline water to induce SOX9 expression (#2 induced; n = 8), whereas the other group was fed with regular water (#2 uninduced; n = 3).

Beginning at 5 weeks postinjection, s.c. tumors were detectable in the induced group and grew significantly faster, reaching excision criterion much sooner (1 cm in largest dimension) than in the uninduced mice (Fig. 2A). To control for the effects of doxycycline, additional mice were injected with the parental LNCaP clone (expressing only tet repressor) and induced with doxycycline (parental induced; n = 7). Similarly to the #2 uninduced tumors, these showed a much slower rate of growth. Finally, xenograft growth was also much faster in doxycycline-fed mice injected with another independent SOX9-expressing LNCaP clone (LNCaP-SOX9 #12 induced; n = 4). The xenograft take rates among the various subgroups are comparable (#2-uninduced, 3/3; #2-induced, 7/8; #12-induced, 4/4; parental-induced, 6/7). These results indicate that SOX9 can enhance the in vivo growth of PCa.

An increase in the fraction of cells expressing SOX9 was clearly shown by immunohistochemistry in the doxycycline-induced xenografts, which showed diffusely homogenous SOX9 nuclear staining. In contrast, the parental xenografts treated with doxycycline showed a patchy pattern with clusters of SOX9-staining cells similar to the SOX9 expression pattern in wild-type untreated LNCaP xenografts (Fig. 2B, top, and data not shown). Interestingly, the intensity of SOX9 nuclear staining in each individual SOX9-positive cell was similar in the SOX9-overexpressing versus control samples, indicating that doxycycline induction in vivo was primarily increasing the fraction of SOX9-positive cells. The androgen receptor expression level was comparable between SOX9-overexpressing and parental xenografts (Fig. 2B, bottom).

One striking feature of the SOX9 induced xenografts was their grossly beefy-red color compared with the brownish gray color of control parental. Microscopically, there is a prominent increase in tumor vasculature in the SOX9-induced xenografts compared with the parental control (Fig. 2C, top). The vessels varied in size and were interconnecting (highlighted by the von Willebrand factor staining of vascular endothelium; Fig. 2C, bottom). These results suggest that SOX9 enhances tumor establishment and growth, at least in part, by increasing angiogenesis.

**SOX9 expression enhances LNCaP PCa tumor invasion.** Tumors from three of seven tumor-bearing animals in the #2

![Figure 2](www.aacrjournals.org)
induced and three of four in the #12 induced groups tightly adhered to underlying skeletal muscle when harvested. Histologic examination of these tumors showed clear evidence of invasion into the underlying skeletal muscle, with tumor cells breaking the tumor pseudocapsule and splitting of muscle bundles (Fig. 3A). This was accompanied by signs of host reaction, such as hemorrhage and accumulation of hemosiderin-laden macrophages (arrowhead), excluding the possibility of sample manipulation artifacts. The nature of the percolating tumor cells in-between skeletal muscle bundles was further confirmed by their staining for androgen receptor (Fig. 3B). In contrast, tumors isolated from parental induced or #2 uninduced animals were easily excised from the surrounding tissue and showed no evidence of local invasion (data not shown).

We further addressed whether SOX9 would enhance tumor invasion using in vitro invasion assays. As shown in Fig. 3C and D, more LNCaP-SOX9 cells migrated through Matrigel-coated filters when they were induced compared with uninduced and parental cells under either condition. These results strongly indicate that SOX9 can enhance tumor invasion.

**Down-regulation of endogenous SOX9 decreases CWR22Rv1 xenograft growth.** To further assess the biological functions of endogenous SOX9 in PCa xenograft, we initially generated LNCaP clones with inducible or stable expression of SOX9 shRNA. However, the tumors grew in only a small fraction of injected mice, even when they were uninduced, possibly due to leaky expression of low-level SOX9 shRNA in vivo. We therefore turned to another more aggressive PCA cell line, CWR22Rv1, which grows more readily as a xenograft and also expresses higher levels of endogenous SOX9 (10). Cultured CWR22Rv1 cells were infected with retrovirus encoding SOX9 or nontargeting control shRNA, and stable lines were selected that showed near depletion of SOX9 protein compared with the control (Fig. 4A). We then injected CWR22Rv1-SOX9i cells s.c. into one flank of male scid mice and injected control CWR22Rv1 cells into the other flank. The mice were sacrificed at day 17 and the SOX9i versus the control tumors on opposite flanks of the same mice were compared. Importantly, the SOX9i tumors were smaller than the control tumors in each mouse (Fig. 4B), and the overall decrease in weight of the SOX9i versus the control xenografts was significant (Fig. 4C).

Interestingly, in contrast to the control LNCaP xenografts that contained clusters of SOX9-positive cells, immunohistochemistry showed SOX9 staining of a majority of the cells in the control CWR22Rv1 xenografts (Fig. 4D, top). This is consistent with their higher level SOX9 expression in vitro and may contribute to their more rapid in vivo growth. Importantly, the fraction of SOX9-positive cells was markedly reduced in the SOX9i tumors, but there were still clearly clusters of SOX9-positive cells, suggesting there...
was selection in vivo for CWR22Rv1-SOX9i cells with retained or renewed SOX9 expression. Again, the androgen receptor level was not significantly affected by SOX9 down-regulation in these xenografts (Fig. 4D, bottom). These results support the hypothesis that endogenous SOX9 is critical in PCa growth in vivo.

Discussion

The prostate develops from the urogenital sinus, a midline structure with an endodermally derived epithelium surrounded by a mesodermally derived mesenchyme (15). The initial event in prostatic morphogenesis is the outgrowth of solid epithelial buds into the surrounding mesenchyme, followed by duct elongation, bifurcation, and branching. Ductal canalization in the solid epithelial cords is accompanied by epithelial differentiation into distinct luminal and basal cell layers expressing characteristic cytokeratins (16). The localization of SOX9 at the peripheral layers of prostate epithelial glands, both in fetal and adult prostate, suggests a role in epithelial-mesenchymal interactions, although studies in other tissues indicate that SOX9 may also contribute to cell fate determination and maintenance of stem/progenitor cell populations. Significantly, SOX9 expression can be modulated by factors that are critical for tissue development, including fibroblast growth factor (15, 17) and β-catenin (8, 10), indicating that SOX9 may function to integrate diverse signals during prostate development.

SOX9 expression in PCa may reflect an aberrantly activated developmental pathway that allows the tumors to invade into surrounding stroma and grow in the absence of basal cell support, which are defining features of PCa. SOX9 may promote the aggressiveness of PCa, at least in part, by enhancing tumor growth, angiogenesis, or invasion. SOX9 in cartilage regulates the expression of specific collagens (18) and in male gonad regulates Mullerian inhibiting substance expression (7), but the critical genes it regulates in prostate are unclear and may be tissue specific. Indeed, the tissue specificity of Sox target genes is a common theme and is attributed to Sox ability to team up with different partner proteins (19). SOX9 was reported to stimulate androgen receptor and prostate-specific antigen expression when overexpressed in M12 PCa cell lines (20). However, it remains to be...
determined whether such modulation reflects SOX9 direct or indirect effects. In any case, SOX9-regulated genes may participate in important processes such as tumor angiogenesis, growth, or invasion. Interestingly, during neural crest formation, SOX9 is required for cell survival and epithelial-mesenchymal transition, which is one of the underlying mechanisms of cancer invasion or metastasis. Studies are under way to define the target genes regulated by SOX9 in fetal prostate, normal basal cells, and PCa. The identification of these genes should provide new insights into the function of SOX9 in prostate development, homeostasis, and tumorigenesis, and may lead to the identification of new targets for mechanism-based therapy of PCa.

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