Sex-Determining Region Y Box 4 Is a Transforming Oncogene in Human Prostate Cancer Cells

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Abstract

Prostate cancer is the most commonly diagnosed noncutaneous neoplasm and second most common cause of cancer-related mortality in western men. To investigate the mechanisms of prostate cancer development and progression, we did expression profiling of human prostate cancer and benign tissues. We show that the SOX4 is overexpressed in prostate tumor samples compared with benign tissues by microarray analysis, real-time PCR, and immunohistochemistry. We also show that SOX4 expression is highly correlated with Gleason score at the mRNA and protein level using tissue microarrays. Genes affected by SOX4 expression were also identified, including BCL10, CSF1, and NcoA4/ARA70. TLE-1 and BBC3/PUMA were identified as direct targets of SOX4. Silencing of SOX4 by small interfering RNA transfection induced apoptosis of prostate cancer cells, suggesting that SOX4 could be a therapeutic target for prostate cancer. Stable transfection of SOX4 into nontransformed prostate cells enabled colony formation in soft agar, suggesting that, in the proper cellular context, SOX4 can be a transforming oncogene. (Cancer Res 2006; 66(8): 4011-9)

Introduction

The sex-determining region Y (SRY) box, or SOX, family consists, in humans, of 20 highly conserved transcription factors that play important roles in development (1) and is defined by a conserved signature sequence in the high-mobility group (HMG) DNA-binding domain (DBD; ref. 2). SOX4 is a 47-kDa protein that is encoded by a single exon gene that is highly conserved in vertebrates. In fact, there is 88% identity at the DNA level between Homo sapiens and Fugu rubripes in the NH2-terminal SOX4-T-cell factor (TCF)-HMG box DBD. At the amino acid level, the SOX4 DBD is 32% identical with the DBD of the TCF transcription factor that is bound by β-catenin as a consequence of activated Wnt signaling. Other SOX factors, SOX17 and SOX3, physically interact with β-catenin and modulate expression of Wnt target genes during Xenopus embryonic development (3, 4).

SOX4 is specifically expressed in the ovary, testis, and thymus of adult mice and in mouse T and pre-B lymphocytic cell lines (5). In mice, SOX4 is also expressed in the mammary gland and uterus and is hormonally regulated by progesterone and estradiol (6). SOX4 is essential for heart, lymphocyte, and thymocyte development, and SOX4-null mice die from cardiac defects (7). Moreover, the proliferative capacity of B-cell progenitors is severely decreased in cells from SOX4 knockout mice (8). The only well-described molecular function of SOX4 is to mediate activation of transcriptional targets of the interleukin (IL)-5 receptor-α (9). Three independent studies screening for important oncogenes have found that SOX4 is commonly altered by retroviral insertions (10–12). In fact, SOX4 was the most common target of the murine leukemia virus, which stabilized the SOX4 message, producing B-cell lymphomas with increased SOX4 message levels (12).

In humans, SOX4 is expressed in the normal breast and breast cancers, and SOX4 expression is increased by progestins leading to increased SOX-mediated transcriptional activity (13). SOX4 is also overexpressed in classic medulloblastomas (14) and lung cancers (15), suggesting that SOX4 may be important in multiple tumor types.

We and others have observed by gene expression profiling that SOX4 is significantly overexpressed in prostate cancer tissues relative to benign prostate. In fact, SOX4 overexpression has been detected by microarray analysis in no less than six independent studies of prostate cancer (16–22). However, no study has gone beyond microarray analysis to evaluate SOX4 changes at the protein level in prostate cancer or to investigate the effects of loss or gain of SOX4 expression on prostate cancer cells.

We have confirmed SOX4 overexpression in prostate cancer patient tissues by quantitative real-time PCR and immunohistochemistry. Transfection of the LNCaP prostate cancer cell line with small interfering RNA (siRNA) duplexes strongly decreased cell viability and induced apoptosis. Stable overexpression of SOX4 in the immortalized, nontransformed RWPE-1 prostate cell line enabled anchorage-independent growth and colony formation in soft agar. These data suggest that, in the proper cellular context, SOX4 can be a transforming oncogene and further provide a strong rationale for consideration of SOX4 as a target for treatment of prostate cancer.

Materials and Methods

Patient samples. The methods for RNA sample preparation have been described previously (23) but are summarized here. In brief, all patients enrolled had biopsy-proven prostate cancer and had undergone radical prostatectomy: 26 patients at Emory University (Atlanta, GA)–affiliated hospitals and 15 patients at other institutions participating in the Cooperative Prostate Cancer Tissue Resource (CPCTR; Silver Spring, MD). Cases were chosen at random from 1999 to 2002. Fourteen of the tumor samples had Gleason grade sum of 6, 17 had Gleason grade sum of 7, 11...
had a Gleason grade sum of 8, and 4 had a Gleason grade sum of 9. The 11 adjacent normal samples correspond to normal prostate tissue taken from the same frozen sections as 11 of the prostate tumor specimens. Seven Gleason 6 cases, 9 Gleason 8 cases, and 4 Gleason 9 cases were obtained through the CPCTR.

**Microarray analysis.** Total RNA was prepared by Trizol extraction of serial sections from snap-frozen tissues. For each frozen section of prostate, one frozen section was stained with H&E to map areas of carcinoma and nonneoplastic prostate, after which 15-μm-thick serial sections were cut for isolation of total RNA. Histologic confirmation was obtained in all cases, and dissection of cancer foci was done to assure that ~90% of cells collected were malignant epithelial cells. Further details can be found in the Supplementary Data. Heat maps were generated using Spotfire DecisionSite 7.2 for Functional Genomics. Complete microarray datasets have been deposited in the public ArrayExpress database (accession no. E-TABM-26).

**siRNA design and transient transfection.** siRNA duplexes targeting SOX4 were designed, synthesized, annealed, and purified (MWG Biotech, High Point, NC). Optimal transfection of LNCaP cells was obtained with a combination of eight different siRNAs to SOX4 used as a cocktail at a final concentration of 100 nmol/L. All siRNA sequences were subjected to BLAST search to confirm the absence of homology to any additional known coding sequences in the human genome. The SOX4 siRNA target sequences are given in the Supplementary Data. Transient siRNA transfections were done using DMRIE-C Reagent (Invitrogen, Carlsbad, CA) for prostate cancer cells in six-well plates. At the end of 48 hours, cells were harvested for total RNA isolation and protein lysate was harvested for immunoblots, Row cytometry, and viability assays. Protein lysates were prepared as described (24), and total RNA was prepared using RNeasy kits (Qiagen, Valencia, CA) according to the manufacturer's instructions.

**Western blots.** Immunoblots were probed with monoclonal antibody (mAb) to Lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA) and our polyclonal antisera to SOX4. We synthesized three synthetic peptides specific to SOX4 (MVQQTNNAAENTAILGDESSDSC, ASPTPGSTASTGGSKADDPPWSC, and ASGGGANSKPQAGKSC), linked them to keyhole limpet hemocyanin (KLH) with maleimide-activated KLH (Pierce, Inc., Rockford, IL), and raised polyclonal rabbit antisera (Rockland Immunochemicals Inc., Gilbertsville, PA). Blots were then probed using a mAb to protein phosphatase 2A (PP2A) catalytic subunit (BD Biosciences, San Jose, CA) to normalize for equal protein loading.

**Immunohistochemistry.** The CPCTR prostate tissue microarray (TMA) 2 (Gleason TMA) was used for immunohistochemistry. Immunohistochemical staining was done using the EnVision Double-Labeling System (DakoCytofication, Carpinteria, CA) on formalin-fixed, paraffin-embedded human prostate cancer tissue. Sections were incubated with rabbit polyclonal anti-SOX4 antisera (1:2,000) for 1 hour followed by incubation with anti-rabbit secondary antibodies conjugated to hors eradish peroxidase–linked labeled polymers. 3,3'-Diaminobenzidine-positive substrate-chromogen was incubated for 5 minutes for color development. Finally, slides were counterstained with hematoxylin (Richard-Allan Scientific, Kalamazoo, MI) for 5 seconds, dehydrated, and mounted.

**Soft agar assays.** Soft agar assays were done essentially as described (25). Briefly, 10,000 cells were seeded in 0.3% Noble Agar, keratinocyte serum-free medium supplemented with bovine pituitary extract and insulin-like growth factor (Invitrogen) fungizone, penicillin-streptomycin, and l-glutamine. Colony number was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye staining as described (26).

**Chromatin immunoprecipitations.** Two p150 culture dishes (Corning, Inc., Acton, MA) each of RWPE-1 FLAG-SOX4 and parental RWPE-1 cells were grown to confluency. Cells were fixed, harvested, and lysed as described previously (27). Sonicated chromatin was precleared overnight with 50 μL Dyna M-280 sheep anti-mouse IgG magnetic beads (Invitrogen) followed by a 5-hour incubation with 50 μL magnetic beads prebound with 15 μg M5 anti-FLAG mAb (Sigma Immunochemicals, St. Louis, MO) or mouse IgG. Supernatant (400 μL) from the IgG control was saved as input. Beads were washed, antibodies were eluted, and DNA was purified as described previously (27). Purified DNA was subjected to PCR using the following primers: BCL2 binding component 3 (BBC3/PUMA), ACAC-CACGGACACACATACATA (forward) and GTTTTGGCTTGTTGTCTGGCATCA (reverse); transducin-like enhancer of split (TLE-1), GGTAGGACCCTGGATGAGGA (forward) and AATGGGCCAGATTCCATTGTTACAG (reverse); and prostate-specific membrane antigen (PSMA) intron 3, CTGGCTCTTCTTTAATGTGTTGG (forward) and TTGGGAGCTGAGTTGAGAAGA (reverse).

**Results**

**Prostate cancer patient expression profiling.** We reported previously that we have profiled 28 prostate cancer patient samples, 11 adjacent benign patient samples, and a commercial pool of normal prostate RNA (Clontech Laboratories, Inc., Mountain View, CA) using Affymetrix (Santa Clara, CA) U133 GeneChip array sets (23). We used the Significance Analysis of Microarrays (SAM) software (28) to identify 63 up-regulated and 80 down-regulated probe sets that were significantly different between prostate cancer and benign tissues. We have now extended our data to include 45 prostate cancers and 13 normal samples and identified 117 up-regulated and 613 down-regulated probe sets (Supplementary Table S1). In agreement with several previous microarray studies (16, 17, 29), 3 of these 117 significantly up-regulated probe sets corresponded to SOX4 (Fig. 1A). SOX4 was also one of the genes most highly correlated with Gleason grade and was highly significant after correction for multiple hypothesis testing (Spearman's *p* = 0.51; *q* = 0.003). Figure 1B shows the average SOX4 signal in patients with Gleason scores of 6, 7, or 8 and above. The correlation of SOX4 with Gleason score was confirmed by quantitative real-time PCR using RNA prepared from 16 prostate cancer samples and matched benign adjacent tissue also used in for expression profiling (Fig. 1C).

**Global analysis of SOX family gene expression.** To further examine the expression of SOX genes in prostate cancer, we mined our global expression profiles to analyze the gene expression patterns for all members of the SOX gene family of transcription factors. Figure 1D shows a heat map of the log_{2} transformed, robust multichip average (RMA) normalized signals of all SOX family genes present on Affymetrix U133A and U133B GeneChips. An examination of the expression levels of SOX family genes shows that SOX1, SOX3, SOX10, SOX13, SOX15, and SRY are all expressed in both benign and cancerous prostate tissues to varying degrees. However, SOX4 is the only SOX family gene that is consistently up-regulated in prostate cancer tissues compared with benign prostate tissues as measured by microarray analysis. Three separate probe sets that measure SOX4 expression produced consistently higher signals in prostate cancers relative to benign prostate samples (Fig. 1D). A total of five probe sets on the U133 set are annotated to the SOX4 gene. However, one probe set (213665_at) that had no expression and showed no difference between tumor and normal samples is actually oriented in the opposite sense to the other four probes, and, thus, is not complementary to the SOX4 message. The other probe set (213668_s_at) was not included in our dataset of significant probe sets because the maximal expression it showed for any sample was below our cutoff threshold for expressed genes. This probe set is complementary to sequences in the open reading frame of SOX4, ~3 kb upstream of the 3'-end of the transcript. Analysis of the data from this probe set showed that although the overall signal was much lower than the three significant probe sets, it was nevertheless 1.9-fold higher in the tumor samples than in the normal samples. Although one probe set for the SOX9 gene showed some increase in prostate cancers, this difference was not statistically significant by SAM analysis.
SOX4 protein is overexpressed in prostate cancer cells. To test whether the increase in SOX4 expression is also seen at the protein level, we generated polyclonal antisera to SOX4-specific peptides and immunoblotted cell lysates from primary prostate epithelial cells (PrEC, Cambrex BioScience, Walkersville, MD), immortalized RWPE-1 prostate cells, and the LNCaP and DU145 prostate cancer cell lines (Fig. 2A). SOX4 protein was greater in prostate cancer cells than in normal prostate cells. To investigate SOX4 levels in patient prostate tissues, we used our SOX4 polyclonal antisera in immunohistochemistry on formalin-fixed, paraffin-embedded prostate tissue microarrays. The tissue microarrays used contained cores from 299 cases. Cores were scored on a scale of 0 to 3 for absent, weak, moderate, or strong staining and were also scored for tumor grade (0, 3, 4, or 5). Figure 2B shows the average SOX4 staining intensity for each tumor grade, which shows that SOX4 is correlated with tumor grade at the protein level ($N = 371$; Spearman’s $\rho = 0.32$; $P = 2.4 \times 10^{-10}$). Representative examples of cores from this tissue microarray (Fig. 2C) show that reactivity for SOX4 protein is increased in prostate cancer cells relative to adjacent benign prostate epithelium and that staining is more intense in higher grade tumors. Reactivity was not detected in nonepithelial cells, except for infiltrating lymphocytes. Expression seemed to be both cytoplasmic and nuclear, consistent with previous reports of SOX4 localization in mouse pre-B-cell lines after stimulation with IL-5 (9). To verify the specificity of our antibodies in immunohistochemistry, we probed sections of embryos from wild-type and SOX4 knockout mice. Day E13.5-E14 embryos were stained because whole-mouse targeted deletion of SOX4 is an embryonic lethal mutation (7). Staining with our purified SOX4 antibodies was much more intense on wild-type embryos than in knockout embryos (Supplementary Fig. S1).

SOX4 siRNA induces apoptosis in LNCaP cells. The observation that SOX4 was the only SOX family gene that was significantly up-regulated in prostate cancer tissues prompted us to examine the effects of depletion of cellular pools of SOX4 mRNA in prostate cancer cells. We did transient siRNA transfections using pools of SOX4 siRNA duplexes in the LNCaP prostate cancer cell line and...
prepared total RNA and whole-cell protein lysates 48 hours after transfection. Parallel transfections using siRNA directed against Lamin A/C served as a positive control and against green fluorescent protein (GFP) as a negative control. Specific mRNA depletion and decreased protein levels of SOX4 and Lamin A/C were assayed by immunoblotting with SOX4 polyclonal antisera and mAbs to Lamin A/C (Fig. 3A). Densitometry scanning of immunoblots for SOX4 determined that the SOX4 siRNA transfections produced an average of 4-fold knockdown of SOX4 protein. By 48 hours after transfection, SOX4 siRNA experiments consistently produced an ~2-fold lower yield of total protein lysate and 5-fold lower yield of total RNA than the GFP control siRNA-treated LNCaP cells (data not shown), suggesting either decreased growth or increased cell death in SOX4 siRNA-treated cells. Consequently, we measured cell viability by trypan blue dye exclusion after SOX4 siRNA transfection over a time course of 5 days. SOX4 siRNA significantly (P < 0.05) inhibited cell proliferation (Fig. 3B) compared with the GFP-transfected cells. Floating, dead cells were present in the culture medium after 48 hours of siRNA treatment, suggesting that knockdown of SOX4 expression is lethal to LNCaP cells. To quantitate percentage cell death, cells were prepared 0, 48, and 96 hours after transfection with GFP or SOX4 siRNA duplexes, stained for DNA content with propidium iodide, and analyzed by flow cytometry. The sub-G0-G1 population of LNCaP cells 48 hours after SOX4 siRNA transfection was ~35% greater than the GFP siRNA control-transfected cells (Fig. 3C). By 96 hours, the dead LNCaP cell population was 234% greater in the SOX4 siRNA-transfected cells compared with GFP siRNA-transfected cells. To determine whether SOX4 siRNA-induced cell death was due to apoptosis, immunoblots were probed for the caspase-cleaved form of poly(ADP-ribose) polymerase (PARP), a marker of apoptosis, which increased in a time-dependent manner (Fig. 3C).

Because SOX4 has been detected in breast cancers (13), we also tested whether SOX4-directed siRNAs could induce apoptosis in two breast cancer lines (MCF-7 and BT-474). Figure 3D shows that siRNA directed against SOX4-induced apoptosis in both of these cell lines to varying degrees, whereas GFP control siRNA duplexes did not. Baseline levels of SOX4 were similar in the MCF-7 and BT-474 cell lines as in LNCaP and HeLa cells (Fig. 3D). Thus, sensitivity to SOX4 siRNA-induced apoptosis may be a more general phenomenon of epithelial cancers.

**SOX4 siRNA-induced apoptosis is specific.** Because there is extensive sequence homology between members of the SOX family, we examined whether SOX4 siRNA duplexes might produce off-target effects on other members of the SOX family. To ensure that the apoptotic effect due to SOX4 siRNA transfection was specific, we did quantitative real-time PCR on the RNA from LNCaP cells transiently transfected with SOX4 siRNA duplexes or GFP siRNA. The SOX family member with the greatest sequence similarity to our SOX4 siRNAs was SOX12 (17-bp perfect match). Using quantitative real-time PCR primers that are specific for SOX4 and SOX12, we determined that SOX4 was repressed 27-fold by our siRNAs (Fig. 4A) far more than SOX12. Furthermore, our analysis of the expression of SOX4 family members shows that SOX12 is

![Figure 2](cancerres.aacrjournals.org)
expressed at very low levels in prostate tissues (Fig. 1D), suggesting that the apoptotic effect is not due to decreased levels of SOX12.

Next, we cotransfected the SOX4 siRNAs complementary to the 3'-untranslated region (UTR) of SOX4 with the pcDNA-SOX4 construct lacking the 3'-UTR of SOX4 and quantitated the effect on apoptosis. Figure 4B shows that cotransfection of pcDNA-SOX4 with SOX4 siRNAs partially rescued the apoptotic effect compared with the SOX4 siRNAs alone. Cotransfection with empty vector did not significantly reduce the percentage of apoptotic LNCaP cells induced by SOX4 siRNA transfection.

Finally, we did microarray analysis on LNCaP cells treated with SOX4 siRNA duplexes or transfected with a SOX4 cDNA expression plasmid and examined the effect on all SOX family members (Fig. 4A). The only member of the SOX family that was strongly down-regulated by SOX4 siRNA transfection was SOX4. However, SOX9 seemed to be somewhat down-regulated by SOX4 siRNA treatment and up-regulated by overexpression of SOX4. It is unlikely that SOX4 siRNAs repress SOX9 expression directly because SOX9 signals were increased on overexpression of SOX4. No alignments could be made that would suggest the possibility of cross-hybridization between SOX4 mRNA and the SOX9 microarray probe sets. Thus, our data are consistent with the hypothesis that SOX9 may be a downstream target of SOX4, although we cannot conclude whether SOX4 might regulate SOX9 directly or indirectly.

**Genes affected by SOX4 expression play roles in Wnt signaling, inflammation, and apoptosis.** As noted above, we did microarray analysis of LNCaP cells treated with SOX4 siRNAs and overexpressing SOX4. For these siRNA microarray experiments, there were three sets of transfections, each including both siRNA and plasmid DNA. In the control set of transfections, the cells were treated with GFP siRNA and empty vector DNA. In the SOX4 knockdown experiment, cells were transfected with SOX4 siRNA and empty vector, whereas, in the overexpression experiment, cells were transfected with SOX4 expression plasmid and GFP siRNA. We identified 466 genes significantly altered by SOX4 levels by SAM analysis using a false discovery rate of <1% (Fig. 4C; Supplementary Table S2), but, based on these data, it is not possible to discriminate between direct and indirect targets of SOX4. These 466 genes were those that were consistently responsive both to knockdown and overexpression of SOX4. We also have analyzed these target genes for conserved SOX binding sites and found conserved SOX sites in 137 of 466 (29%) target genes. There were conserved SRY binding sites (which are less specific) in 225 of 466 target genes (Supplementary Table S2). However, this result is puzzlingly low compared with our global analysis of all human Refseq transcripts in which 59% of promoters contained conserved SOX sites, because the 29% of SOX4 targets with conserved SOX sites
represents a decrease rather than enrichment over background for SOX binding sites.

Nevertheless, we confirmed these changes by quantitative real-time PCR for five SOX4 targets (Fig. 4C). Among those genes that were activated by SOX4 overexpression and reduced by SOX4 knockdown (Fig. 4C) was the ortholog of the groucho repressor, TLE-1, which may function as a negative feedback regulator to repress SOX4 expression in the absence of Wnt signals. Other SOX4 targets identified by microarray analysis included BCL10, colony stimulating factor 1 (CSF1), BBC3/PUMA, and nuclear receptor coactivator 4 (NcoA4/ARA70). BCL10 is an adaptor protein that has been shown to activate nuclear factor-κB (NF-κB) by mediating Lys63-linked ubiquitination of NF-κB essential modulator, resulting in activation of the IκB-specific kinase complex (30). BBC3/PUMA is a downstream target of p53 that is essential for both p53-dependent and p53-independent apoptosis (31). We confirmed that BBC3/PUMA and TLE-1 are direct targets of SOX4 by chromatin immunoprecipitation assay against the FLAG epitope tag in RWPE-1 cells stably expressing FLAG-SOX4 (Fig. 4D). As negative controls, we did chromatin immunoprecipitation assays on RWPE-1 cells that do not express FLAG-SOX4 and on the PSMA gene in FLAG-SOX4 cells, which was not affected by SOX4 levels.

Fully transformed human cell lines express SOX4 and are sensitive to SOX4 siRNA-induced apoptosis. To investigate the mechanism by which loss of SOX4 can induce apoptosis in cancer cells, we probed immunoblots of HeLa and LNCaP cells depleted for SOX4 by siRNA. Figure 5A shows that depletion of SOX4 results in stabilized p53 protein and loss of survivin expression, consistent with decreased NF-κB activation. SV40 large tumor (LT) antigen binds and inactivates both p53 and Rb proteins but, combined with constitutively activated Ras-V12, it
is still not sufficient for transformation of normal human cells. To accomplish complete transformation of human cells, additional molecular changes are necessary that can be induced by expression of SV40 small tumor (ST) antigen (32). We have analyzed the transcriptional changes induced in two human embryonic kidney (HEK) cell lines. The TERST cells express telomerase, mutant Ras-V12, LT, and ST, and can form tumors in nude mice. The TERV vector control HEK cells (TERV) express telomerase, mutant Ras, and LT, but not ST, and cannot form tumors without ST expression. Each line represents pools of hundreds of clones that can capture the effects of SV40 ST on gene expression. We found that ST expression produced a dedifferentiated phenotype, with repression of cell-cell adhesion molecules, increases in stem-cell markers (33) and expression of SOX4 (Fig. 5B). Transient siRNA transfections against SOX4 induced apoptosis in the tumorigenic TERST cells but not in the control TERV cells (Fig. 5C). Thus, expression of SOX4 may be a critical component of the final pathway necessary for complete transformation of human cells and may be necessary for the survival of some fully transformed cells.

**SOX4 overexpression transforms RWPE-1 prostate cells.**

To further investigate the effects of SOX4 expression on transformation of human prostate cells, we tested whether SOX4 overexpression could yield a transformed phenotype in non-transformed prostate cells. The RWPE-1 cell line was originally established by human papillomavirus 18 (HPV18) infection of nonneoplastic primary prostatic epithelial cells (34). The RWPE-1 line is responsive to androgen, does not form colonies in soft agar, and does not form tumors when injected into nude mice (34).

We subcloned the open reading frame of the human SOX4 gene into the pcDNA vector downstream of a FLAG-tag epitope to generate a NH2-terminal FLAG-SOX4 fusion product. The pcDNA-FLAG-SOX4 plasmid and the empty pcDNA-FLAG vector were then stably transfected into the RWPE-1 prostate cell line, and multiple individual clones as well as pools of clones were obtained. Stable expression of FLAG-SOX4 was verified by immunoblotting (Fig. 6A).

The ability of cells to form colonies in anchorage-independent medium, such as soft agar, has long been used as an assay for cellular transformation. To determine whether SOX4 overexpression enabled anchorage-independent growth of RWPE-1 cells, we plated 10,000 RWPE-1 parental cells, the RWPE-1-pcDNA vector cells, and RWPE-1-FLAG-SOX4 cells. Two individual clones (RFSOX4-clone7 and RFSOX4-clone8) and a pool of dozens of individual colonies were plated in soft agar and allowed to grow for 17 days, and the number of colonies were counted. Results from all SOX4-expressing clones were identical and showed robust colony formation, whereas RWPE-1 parental cells and cells stably transfected with empty vector formed few if any colonies (Fig. 6B). Figure 6C shows the quantitation of colony growth for each soft agar assay from three independent experiments. Thus, we conclude that in the proper cellular context, SOX4 can be a transforming oncogene similar to SV40 ST, which provides some of the key changes in gene expression that are necessary for anchorage-independent growth of human cells. It is somewhat surprising that a relatively small increase in SOX4 expression can enable anchorage-independent growth, but this may be due to the fact that very high levels of expression might induce apoptosis selecting against cells with extremely high SOX4 expression.

**Discussion**

In this study, we have shown that SOX4 mRNA and protein is increased in human prostate cancer tissues. We have also shown that loss of SOX4 expression reduces viability and induces apoptosis of LNCaP cells, whereas overexpression of SOX4 enables immortalized RWPE-1 cells to grow in soft agar, suggesting that SOX4 plays a critical role in growth and survival of prostate cancer cells. The fact that overexpression of SOX4 transforms RWPE-1 cells does not imply that SOX4 alone could transform normal primary prostate cells. Because the immortalizing HPV18 E6 and E7 oncoproteins are present in RWPE-1 cells, they bind and inactivate the Rb and p53 tumor suppressor proteins. However, cells that have been compromised in regulation of the Rb and/or p53 pathways in some way may be susceptible to transformation by SOX4 as a second or third hit along the road to cancer formation. In fact, p53 inactivation may be a prerequisite for SOX4 transformation because SOX4 induces expression of BBC3/PUMA. BBC3/PUMA is an essential proapoptotic target of p53 (31), suggesting that one normal function of SOX4 may be to prime cells for apoptosis. This would explain the observed sensitivity of HEK-TERST cells to SOX4 depletion, whereas HEK-TERV cells were resistant to SOX4 siRNA. The induction of BBC3/PUMA raises the question how can SOX4 induce proapoptotic genes and still be necessary for survival? It must be noted that
SOX4 has also been shown to have proapoptotic effects when overexpressed in HeLa and HEK293 cells (35–37). This may be why we observed only an ∼2-fold overexpression of SOX4 in the RWPE-1-SOX4 cells because very high overexpression could have been selected against. Nevertheless, it is not unprecedented for transforming oncogenes to have both proliferative and apoptotic effects as has been shown in many studies of c-myc (38). Like c-myc, SOX4 is a transcription factor that affects expression of many genes, some of which (e.g., CSF1) promote cell survival, and the balance of the proapoptotic and antiapoptotic SOX4 targets may determine cellular survival. Thus, the levels of antiapoptotic SOX4 targets may be reduced more rapidly than the proapoptotic SOX4 targets on depletion of SOX4 mRNA. This hypothesis is supported by our data, showing p53 stabilization in LNCaP cells and loss of survivin expression in HeLa cells on depletion of SOX4.

We showed recently that another developmental transcription factor, homeobox C6 (HOXC6), is also overexpressed in prostate cancer tissues and that loss of HOXC6 expression can induce apoptosis of LNCaP cells (23). There is growing evidence that SOX family transcription factors are essential for embryonic development and play critical roles in cell fate determination, differentiation, and proliferation. SOX4 is important for proliferation of B-cell progenitors (8) and has been implicated in lymphoma animal models (10–12). Moreover, SOX4 is aberrantly expressed in medulloblastomas (14) and carcinomas of the lung (15), breast (13), colon (41), and prostate (16–22).

How and why is SOX4 expression increased in so many types of cancers? To gain insights into regulation of SOX4, we analyzed the genomic sequences upstream of the human and mouse SOX4 genes using our CONFAC software (42). We have identified DNA sequences and transcription factor binding sites (TFBS) that are conserved upstream of SOX4 between murine and human genomes. We identified conserved TFBS for NF-κB, signal transducers and activators of transcription, forkhead, LEF/TCF, E2F, HOX, and hypoxia-inducible factor 1-α (HIF1-α) family transcription factors. These data are consistent with reports that SOX4 is affected by HIF1-α (43), forkhead in rhabdomyosarcoma (44) estradiol (6), and tumor necrosis factor-α (45). Thus, SOX4 may be among the downstream targets of NF-κB important for transformation that also includes antiapoptotic genes, such as Bcl-2, and growth-stimulatory genes, such as c-myc, cyclin D1, and IL-6 (46). Through its activation of CSF1, SOX4 may contribute to chronic inflammation via paracrine stimulation of macrophages and to survival via autocrine activation of the AKT/mammalian target of rapamycin pathway (47). HOXC6 can repress SOX4 in LNCaP cells (23) possibly through the conserved HOX sites, thus preventing very high SOX4 expression that might induce apoptosis. This complex confluence of multiple signal transduction pathways on regulation of SOX4 expression seems to result in SOX4 expression being highly correlated with the cellular environment of a cancer cell. Proliferative, survival, hypoxic, and inflammatory signals seem to activate SOX4 expression, whereas developmental differentiation signaling seem to repress SOX4 expression.

Our data also suggest that SOX4 may be a novel potential therapeutic target for human prostate cancer. Our results show that SOX4 siRNA can effectively down-regulate SOX4 overexpression in LNCaP cells and induce apoptosis in LNCaP prostate cancer cells and BT-474 and MCF-7 breast cancer cells, and that this effect can be reversed by overexpression of SOX4. Future work to examine the effectiveness of SOX4 knockdown on prostate cancer in preclinical animal models will help determine whether this approach represents a potential avenue for clinical trials.

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