Sox7 Is an Independent Checkpoint for β-Catenin Function in Prostate and Colon Epithelial Cells

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Abstract

The presence of somatic β-catenin mutations in some prostate cancers implies that aberrant WNT signaling is involved in the cancer development. Although β-catenin stability is regulated by a multicomponent destruction complex, mutational alterations of β-catenin or other components of the destruction complexes are rare in prostate tumors. Therefore, β-catenin may be regulated by another protein in the prostate. In fact, recent linkage and somatic deletion analyses in prostate cancers reveal a 1.4-Mb candidate tumor suppressor locus on *8p23.1*, which includes the *Sox7* gene. Here we show that Sox7 protein expression was indeed down-regulated in 47% (15 of 32) of prostate adenocarcinomas. In addition, *Sox7* mRNA was down-regulated in 60% of snap-frozen tumors. This down-regulation was found to be due to tumor-specific promoter hypermethylation, which was present in 48% (10 of 21) of primary prostate tumors and 44% (11 of 25) of prostate cancer cell lines/xenografts. We discovered that Sox7 protein physically interacts with β-catenin and suppresses β-catenin–mediated transcription by depleting active β-catenin. Furthermore, in HCT116 colorectal cancer cell lines with *Sox7* inactivation, ectopic *Sox7* expression suppressed cell proliferation and inhibited transcription that was activated by an endogenous mutant β-catenin. Although nearly all colorectal cancers contain mutations in β-catenin or adenomatous polyposis coli/axin, epigenetic silencing of *Sox7* was still observed. These data suggest that *Sox7* is a tumor suppressor that functions as an independent checkpoint for β-catenin transcriptional activity. Inactivation of *Sox7* could promote the development of a majority of colorectal tumors and approximately half of prostate tumors. (Mol Cancer Res 2008;6(9):1421–30)

Introduction

Aberrant activation of the "canonical" WNT signaling plays an important role in the development of colorectal cancer (1). The central player in this signaling cascade is a cytoplasmic protein called β-catenin. The stability of β-catenin is regulated by a multicomponent cytoplasmic destruction complex containing adenomatous polyposis coli (APC), axin, and glycogen synthase kinase 3β (2). In the absence of WNT signaling, these proteins bind to β-catenin in the cytosol and promote its phosphorylation, which eventually triggers its degradation. In colorectal cancers, mutational inactivation of the *APC/axin* genes or mutational activation of the *β-catenin* gene disrupts the interaction between β-catenin and this cytoplasmic destruction complex, leading to the stabilization of β-catenin in the nucleus (1). Nuclear β-catenin interacts with T-cell factor/lymphoid enhancer factor and activates transcription that has been termed β-catenin/T-cell factor–regulated transcription (CRT). Activation of CRT target genes, such as *c-myc* and *cyclin D1*, is now believed to be responsible for colon carcinogenesis (3).

Several lines of evidence in humans and animal models also indicate that aberrant activation of β-catenin plays a role in the development of prostate cancer. First, studies in transgenic mice show that mutational activation of *β-catenin* in the germ line leads to nuclear stabilization of β-catenin and to hyperplasia, thereby suggesting that aberrant activation of β-catenin is responsible for the increased cell proliferation (4, 5). More importantly, in one mutant β-catenin mouse model, lesions are formed in the prostatic lobes that are reminiscent of high-grade prostatic intraepithelial neoplasia, a precursor for prostate cancer (5). Second, when nuclear β-catenin staining by immunohistochemistry is used as a surrogate marker for the activation of WNT/β-catenin signaling, it is detected in ~20% of advanced prostate cancer tissues, but not in normal prostate epithelial cells (6). Third, somatic mutations of the *β-catenin* gene that could lead to stabilization of β-catenin protein in the nucleus have been identified in only 5% of prostate cancers (7). Indeed, the molecular basis of aberrant stabilizations of β-catenin in the majority of prostate cancer cases is not due to defects in the β-catenin or even the multicomponent destruction

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complexes, as mutational alterations of β-catenin or of other known components of the destruction complexes are rare in prostate cancer cells (8, 9). Consequently, an important question about β-catenin function in prostate cancer is whether β-catenin is negatively regulated by another protein.

The short arm of chromosome 8 (8p) is one of the most frequently deleted regions in prostate cancer (10-15). Several studies indicate that chromosome 8p may contain several tumor suppressors that play different roles in the development of prostate cancer (13, 16). Linkage analyses in families with hereditary prostate cancer support this concept. A recent analysis of 206 families with hereditary prostate cancer reveals two separate linkage peaks, at *8p21.3* (linkage of disequilibrium score, 2.51; $P = 0.0007$; nonparametric linkage score, 3.14) and *8p23.1* (linkage of disequilibrium score, 1.50, $P = 0.009$; nonparametric linkage score, 2.72; ref. 17). In the same study, somatic deletion analysis done on 55 prostate tumors identifies a 3.1-Mb region at *8p21.3* and a 1.4-Mb region at *8p23.1*. The *8p21.3* region contains 37 known genes, including the putative tumor suppressor gene *NKX3.1*. In contrast, the *8p23.1* region is only 1.4 Mb in length and contains five known genes. This region covers the entire promoter and the coding regions of *Sox7*. A previous report showed that a shared sequence motif between Sox17 and Sox7 mediates the interaction of Sox17 with β-catenin (18). Hence, in this study we investigated the role of the Sox7 protein in WNT signaling in prostate and colorectal cancer and evaluated the frequency of *Sox7* inactivation in prostate cancer.

Results

Down-Regulation of Sox7 Protein and mRNA Expression in Primary Prostate Tumors

Immunohistochemistry was used to evaluate Sox7 protein expression in archived paraffin-embedded specimens. In all eight normal prostates examined, Sox7 protein was present and was mostly cytoplasmic. In contrast, in 32 cases of prostate adenocarcinomas, we found that Sox7 expression was significantly down-regulated in 15 (47%) cases. Sox7 protein was detected in 17 (53%) of the prostate adenocarcinoma cases. In addition to cytoplasmic staining, membranous staining of Sox7 protein was detected in one case. Some of these cases contained paired tumor/normal specimens from the same individual. Two examples are shown in Fig. 1A to illustrate the tumor-specific down-regulation of Sox7 expression in autologous tissues.

Next, quantitative real-time PCR was used to evaluate *Sox7* mRNA expression levels in nine paired fresh/frozen prostate specimens. In most normal prostate samples, *Sox7* was expressed at similar levels, with the exception of sample I, in which *Sox7* expression was not detectable (Fig. 1B). In contrast, *Sox7* expression was undetectable in 67% (6 of 9) of the adjacent primary prostate tumor tissues (Fig. 1B, *cases A-F*).

Tumor-Specific Promoter Hypermethylation of Sox7 in Prostate Cancers

Methylation-specific PCR (MSP) was used to determine the promoter methylation status of the *Sox7* gene in 21 paraffin-embedded primary prostate cancer tissue specimens. MSP primers were designed to interrogate the methylation status of

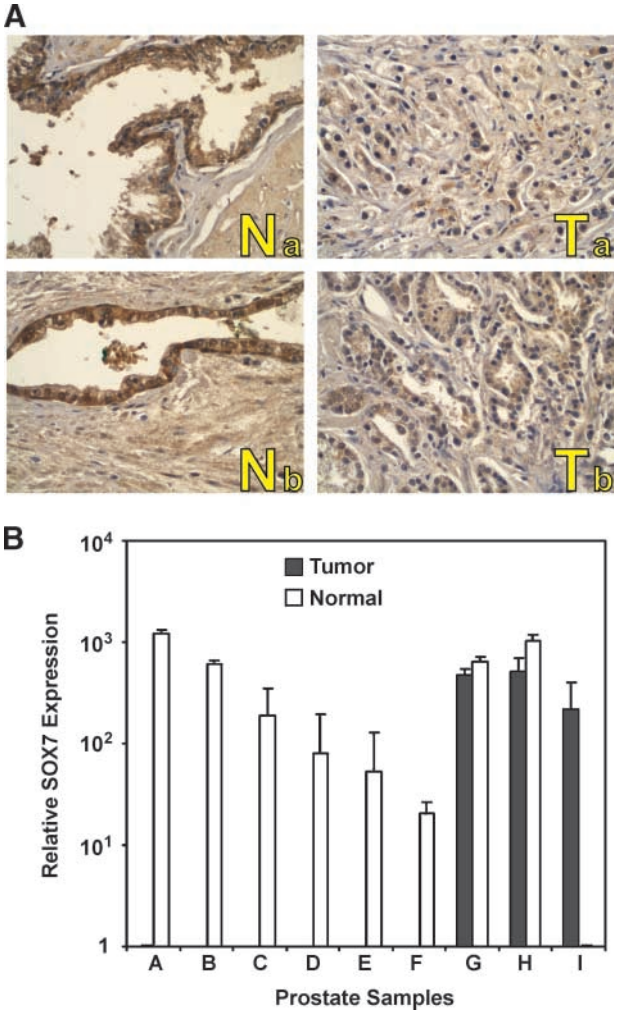

FIGURE 1. Sox7 protein and mRNA expression analyses in primary prostate tumors. **A.** Immunohistochemical analysis of Sox7 expression in two examples of paired prostate tumors with adjacent normal tissues. N, normal prostate; T, prostate tumor; a and b, case numbers. **B.** Expression of *Sox7* mRNA in paired prostate tumor and normal tissues. *Sox7* mRNA expression was measured by quantitative real-time PCR with *GAPDH* as internal control. Reactions were carried out in triplicate. Bars, SD. Filled columns, tumor; open columns, adjacent normal prostate.

CpG sites overlapping exon 1 and the CpG island of *Sox7* (Fig. 2A). Genomic DNA was microdissected from both tumor cells and adjacent normal tissues, then treated with sodium bisulfite. DNA isolated from normal human epithelial cells was used as an unmethylated control (Fig. 2B, *lanes 3* and *4*), and normal DNA methylated *in vitro* with *Sss*I (CpG) methylase was used as a methylated control (Fig. 2B, *lanes 1* and *2, IVD*). In our MSP analysis, tumor-specific *Sox7* promoter methylation was detected in 10 of 21 (48%) primary prostate tumors (Fig. 2B). Promoter hypermethylation status was also confirmed in case nos. 1 to 4 by bisulfite sequencing (Fig. 2C). In our MSP analysis, *Sox7* promoter hypermethylation was also detected in three normal prostate samples. In case no. 8, *Sox7* promoter methylation was observed in both tumor and normal tissues. Case nos. 10 and 12 had *Sox7* promoter methylation only in their normal tissues. Hence, a small percentage of

prostate cancer patients had *Sox7* promoter methylation in apparently normal prostate epithelium.

We also determined the chromosome 8p allelic status for the same panel of prostate tumors using "counting alleles," a method we had previously developed to analyze allelic imbalance in archived specimens having single nucleotide polymorphisms (19). A panel of nine single-nucleotide polymorphic markers (National Center for Biotechnology Information RefSNP nos. 1124, 3185, 3850751, 3888179, 3258, 11362, 3112, 532841, and 14879 from *8p21.2* to *8p23.2*) were used to evaluate allelic imbalance in these tumors. Three of the tumors did not have any informative markers in this panel, so their 8p allelic status could not be determined. Of the remaining 18 tumors, 11 (61%) contained allelic loss in at least one single-nucleotide polymorphic marker in this region. This result was similar to our previous finding, where 8p allelic imbalance was detected in 64% of prostate cancers (20). It is of note that Sox7 promoter methylation was detected in 64% (7 of 11) of prostate tumors with 8p allelic imbalance, but only in 29% (2 of 5) of prostate tumors without 8p allelic imbalance. Although the sample size was limited, this trend suggests that promoter hypermethylation frequently occurs in combination with loss of heterozygosity, thereby functionally inactivating both alleles of *Sox7* in prostate tumors.

We also carried out MSP analysis in a panel of prostate cancer cell lines and xenografts. *Sox7* promoter methylation was detected in 11 of 25 (44%) samples (Table 1). The expression level of *Sox7* mRNA was verified by quantitative real-time PCR, which detected *Sox7* expression in DU145 and PC-3 cells, as well as in the normal human prostate epithelial

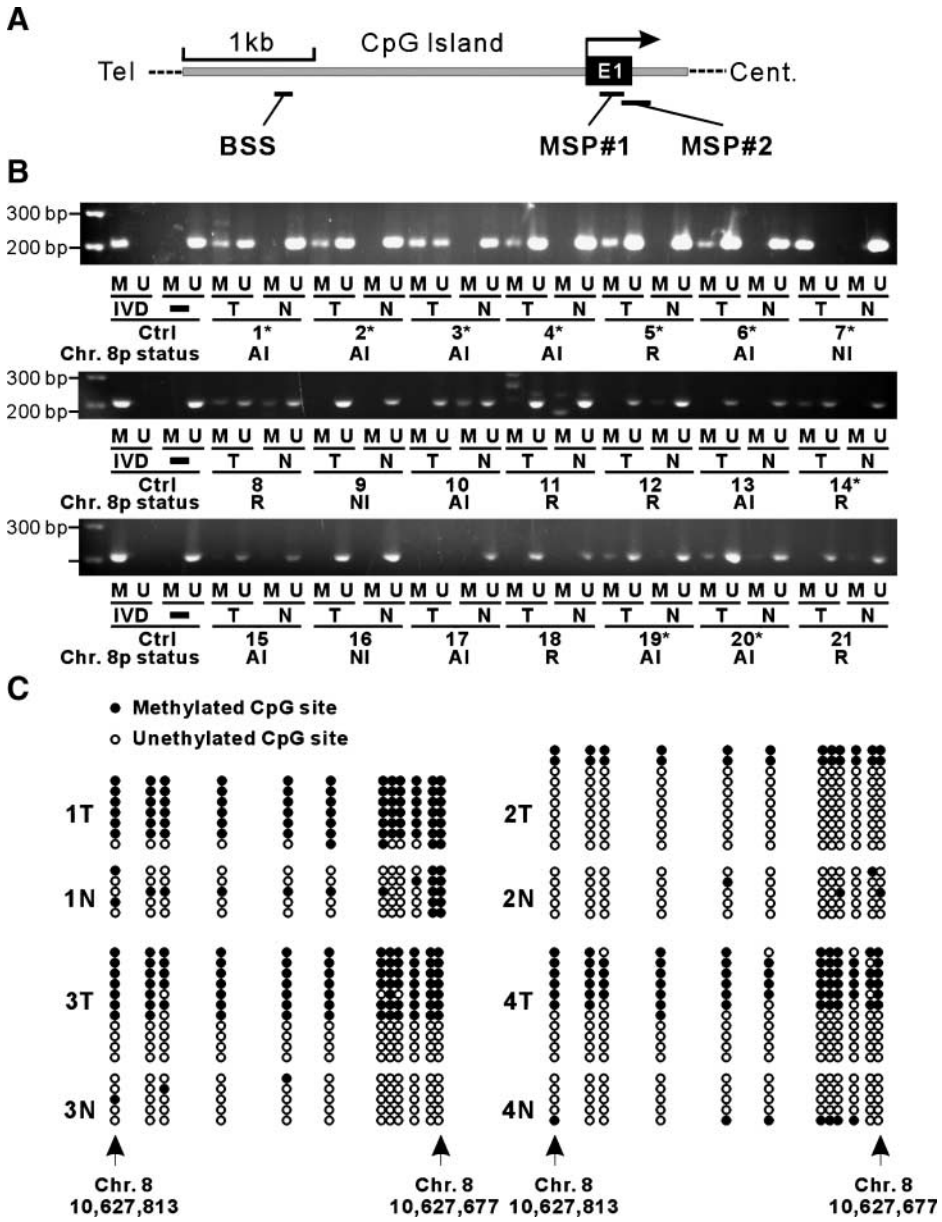

FIGURE 2. Tumor-specific promoter hypermethylation of *Sox7* in primary prostate tumors. **A.** Genomic structure of the *Sox7* promoter. Shaded region, CpG islands; arrow, direction of transcription. Tel, telomere; Cent, centromere; E1, exon 1; MSP, methylation-specific PCR product; BSS, bisulfite sequencing product. **B.** MSP analysis of the *Sox7* promoter and chromosome 8p allelic imbalance in primary prostate tumors. For the MSP analysis, genomic DNA isolated from macrodissected prostate primary tumors and adjacent normal tissues was treated with bisulfite and amplified using MSP primers. *, samples with tumor-specific methylation. IVD, *in vitro* methylated control DNA. For 8p allelic imbalance analysis, genomic DNA was used for "counting alleles" analysis. AI, tumors with 8p allelic imbalance; R, tumors retaining both parental alleles; NI, samples with no informative single-nucleotide polymorphic markers. **C.** Bisulfite-treated genomic DNA was amplified with bisulfite sequencing primers. Bisulfite sequencing PCR products were subcloned and individual subclones were isolated and sequenced. Open and filled circles, unmethylated and methylated CpG sites, respectively. Each row represents a single clone.

Table 1. Promoter Methylation of *Sox7* in Prostate Cancer Cell Lines and Xenografts

	Cell Line/ Xenografts	Promoter Methylation
1	LNCaP	Yes*
2	22RV1	Yes*
3	DU145	No*
4	PC-3	No
5	LAPC-3	Yes
6	PC-82	Yes
7	CWR21	No
8	CWR22	Yes*
9	CWR91	No
10	LuCap23.1	No
11	LuCap23.12	Yes
12	LuCap23.8	No
13	LuCap35	No
14	LuCap49	No
15	LuCap58	No
16	LuCap69	Yes
17	LuCap70	No
18	LuCap73	Yes
19	LuCap81	Yes
20	LuCap86.2	No
21	LuCap92	Yes
22	LuCap93	No
23	LuCap96	No
24	LuCap105	Yes
25	LuCap115	Yes

*Promoter methylation status in these samples was verified by bisulfite sequencing.

cell line PrEC, and revealed a significant down-regulation of *Sox7* expression in both the LNCaP and 22Rv1 cell lines (Fig. 3A). Bisulfite sequencing identified promoter methylation in LNCaP, 22Rv1, and CRW22, but not in DU145, as expected (Fig. 3B). The *Sox7* mRNA expression level also correlates with the level of Sox7 protein. Sox7 protein was detected in DU145 and PC-3 cells, but not LNCaP or 22Rv1 cells, by Western blot analysis (Fig. 6C; Supplementary Fig. S1). However, an examination of the coding sequence of the Sox7 gene did not reveal any nonsense or missense mutations in seven CaP cancer cell lines (22Rv1, LNCaP, PC-3, DU145, NCI-660, BRT41T, and PC82).

We also investigated whether demethylation of the *Sox7* promoter could restore *Sox7* expression in LNCaP cells. Both DU145 and LNCaP cells were treated with 0.5 μmol/L 5-aza-2′-deoxycytidine (5-aza-dC). Then the expression of *Sox7* mRNA was determined by quantitative real-time PCR, and the promoter methylation status in LNCaP cells was validated by MSP. We found that in LNCaP cells, 5-aza-dC treatment led to demethylation of the Sox7 promoter (Fig. 3D) and stimulation of *Sox7* expression by >250-fold (Fig. 3C). In contrast, 5-aza-dC treatment had little effect on *Sox7* mRNA expression in DU145 cells, which do not contain *Sox7* promoter methylation. Therefore, we have clear proof that *Sox7* expression can be restored in LNCaP cells by chemical demethylation of the *Sox7* promoter.

Sox7 Binds to β-Catenin, Depletes Active β-Catenin, and Inhibits CRT

Sox7 was previously shown to be able to suppress β-catenin/T-cell factor–regulated transcription (CRT; ref. 21). We analyzed the inhibitory effect of Sox7 on transiently stimulated CRT by β-catenin and the pOT-Flash reporter plasmid in H1299 cells (22). The pOT-Flash plasmid contains three T-cell factor/β-catenin binding sites upstream of a minimal promoter and the firefly luciferase reporter gene. The same plasmid, but with the binding sites mutated (pOF-Flash), was used as a negative control. A *Renilla* luciferase control reporter (pRL-CMV) was included in all experiments to normalize transfection efficiency. As expected, cotransfection of the β-catenin plasmid with pOT-Flash, but not with pOF-Flash, led to activation of firefly luciferase expression (Fig. 4B). Transfection of Sox7 (CMV-Tag2/Sox7) alone had no effect on basal transcription from the pOT-Flash or pOF-Flash reporter. However, wild-type Sox7 suppressed CRT in a dose-dependent manner in the presence of β-catenin expression, and a 77% inhibition of CRT activity was achieved with the highest dose of Sox7 in H1299 cells (Fig. 4B).

The Sox7 protein contains a DxxEFDQYL motif that is evolutionarily conserved and that may mediate the interaction of Sox7 with β-catenin (18). Therefore, we generated a Sox7 deletion construct, Sox7-Δ (DRN-S), removing this putative β-catenin interaction motif to discover its true function (Fig. 4A). We first determined whether Sox7 physically interacts with β-catenin protein through this putative β-catenin binding motif by transfecting HEK293 cells with a Flag-tagged Sox7 expression plasmid (CMV-Tag2/Sox7) and a construct expressing β-catenin (23). Indeed, β-catenin protein was detected in immunoprecipitated complexes with Sox7 (Fig. 4C, *lane 2*), indicating that Sox7 is capable of binding to β-catenin. In addition, β-catenin was not detected in cells transfected with Sox7-Δ, suggesting that the EFDQY residues within the newly confirmed β-catenin binding motif were necessary for Sox7 to bind to β-catenin (Fig. 4C, *lane 1*). A Sox7 mutant construct that only retained the DxxEF structure (Fig. 4A, Sox7-M, DRNEFGGG) was still capable of binding to β-catenin (Fig. 4C, *lane 3*), implying that the glutamic acid (E) and phenylalanine (F) within this motif might play significant roles in the binding process.

We also evaluated whether Sox7-Δ is still capable of suppressing β-catenin–mediated CRT in H1299 cells. A low dose of Sox7-Δ (32 ng of plasmid) achieved a similar level of CRT inhibition (27% inhibition) as with an equivalent amount of wild-type Sox7 (24% inhibition). Unlike wild-type Sox7, increased dose of Sox7-Δ did not result in further CRT inhibition because of a significant increase in the transcription of pOF-Flash plasmid by Sox7-Δ (Fig. 4B). Therefore, the β-catenin interaction motif of Sox7 is required for Sox7 to specifically suppress β-catenin–mediated transcription.

Because WNT signals specifically increase a subpopulation of β-catenin protein that is dephosphorylated at residues Ser^{37} and Thr^{41}, dephosphorylated β-catenin is thought to be the active form of β-catenin that participates in CRT (24). We next evaluated the level of active β-catenin protein after ectopic Sox7 expression. Sox7 or Sox-Δ was coexpressed with a S33Y mutant β-catenin in 293 cells. S33Y β-catenin mutation was initially identified in SW48 colorectal cancer cell line, and this mutant is resistant to APC-mediated degradation (1). The expression level of β-catenin protein was similar in cells transfected with either Sox7 or Sox-Δ when a polyclonal anti–β-catenin antibody was used in the analysis (Fig. 4D, *left, lanes*

1 and *2*). However, cells transfected with Sox7 had a significant decrease in active β-catenin compared with the ones transfected with Sox7-Δ (Fig. 4D, *left, lanes 1* and *2*), suggesting that ectopic expression of Sox7 specifically depletes active β-catenin and this depletion requires the interaction between Sox7 and β-catenin. In addition, MG132 treatment was capable of blocking Sox7-mediated depletion of active β-catenin (Fig. 4D, *left, lanes 1* and *3*), implying that the depletion of active β-catenin is mediated by proteasome degradation. The depletion of active β-catenin, but not total β-catenin, by ectopic Sox7 expression was also observed in COS-7 cells (Fig. 4D, *right*). In summary, these data suggest that ectopic expression of Sox7 mediates the degradation of active β-catenin in an APC-independent mechanism.

Sox7 Inactivation in Colorectal Cancers

Because Sox7 is capable of inhibiting CRT, it may act as an independent checkpoint to control β-catenin activity. If this is the case, the inactivation of Sox7 function would be necessary for developing colorectal cancer because it is known that aberrant WNT/β-catenin signaling plays a critical role in colorectal cancer development (1). We first determined the promoter methylation status of the *Sox7* gene in HCT116 (a colorectal cancer cell line with a *β-catenin* mutation) and HT29 (a colorectal cancer cell line with an *APC* mutation; refs. 1, 22) and found that the *Sox7* promoter was heavily methylated in both cell lines (Fig. 5A). The activating *β-catenin* mutation in HCT116 is a 3-bp deletion that eliminated the serine residue at codon 45 (1). This mutation disrupts the interaction of β-catenin with the cytoplasmic destruction complex (22), and CRT can be detected in HCT116 with transient transfection of the pOT-Flash plasmid alone (Fig. 5B). Ectopic expression of wild-type Sox7, however, inhibited the aberrant CRT activity mediated by this mutant β-catenin, suggesting that this mutation was not sufficient to alleviate Sox7 suppression.

Our data on HCT116 cells suggest that disruption of the Sox7/β-catenin interaction may be an additional requirement in colon carcinogenesis. To investigate this possibility, we evaluated the *Sox7* promoter methylation status of colorectal primary tumors. MSP was done using genomic DNA isolated from eight snap-frozen, paired tissues from tumor and adjacent normal specimens from colorectal cancer patients. In this tissue type, *Sox7* promoter methylation was detected in 100% (6 of 6) of the tumors, but not in any adjacent normal tissues, by MSP analysis (Fig. 5C). We failed to amplify the correct size of PCR product from tumor DNA after repeated attempts, as well as with MSP primer sets 1 and 2 for the remaining two samples. Nevertheless, this experimental data indicated that the *Sox7* gene is inactivated by tumor-specific promoter methylation in a majority of colorectal primary tumors.

Transient and Tet-Inducible Sox7 Expression Inhibits Cell Proliferation

To investigate the effect of Sox7 expression on cell proliferation in Sox7-null cells, a CMV-Tag2/Sox7 expression plasmid was transfected into LNCaP, 22Rv1, and HCT116 colon cancer cells. Following G418 selection, there was a significant reduction in colony formation after transfection with the Sox7 expression vector as compared with the empty vector control (CMV-Tag2, Fig. 6A and B), indicating that the transfected ectopic expression of Sox7 inhibited cell proliferation. As the transient transfection rate was low in the CaP cell lines, we also developed stable cell lines for the inducible

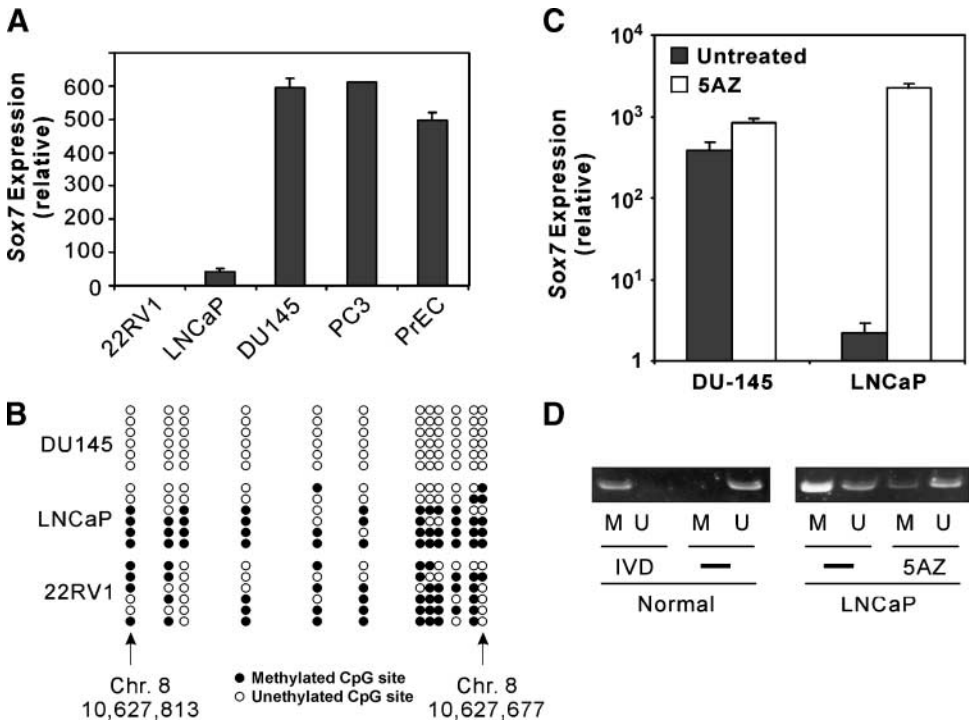

FIGURE 3. Sox7 expression in prostate cancer cell lines. **A.** Expression of *Sox7* mRNA in prostate cancer cell lines was measured by quantitative real-time PCR with *GAPDH* as an internal control. Reactions were carried out in triplicate; bars, SD. **B.** Bisulfite sequencing analysis of the *Sox7* promoter in DU145, LNCaP, and 22Rv1 cells. **C.** Promoter demethylation by 5-aza-dC (*5AZ*) restores *Sox7* expression in LNCaP cells. DU145 and LNCaP cells were treated with 5-aza-dC, then *Sox7* mRNA expression was measured by quantitative real-time PCR. **D.** Methylation status of the *Sox7* promoter in LNCaP cells, before and after 5-aza-dC treatment, as determined by MSP.

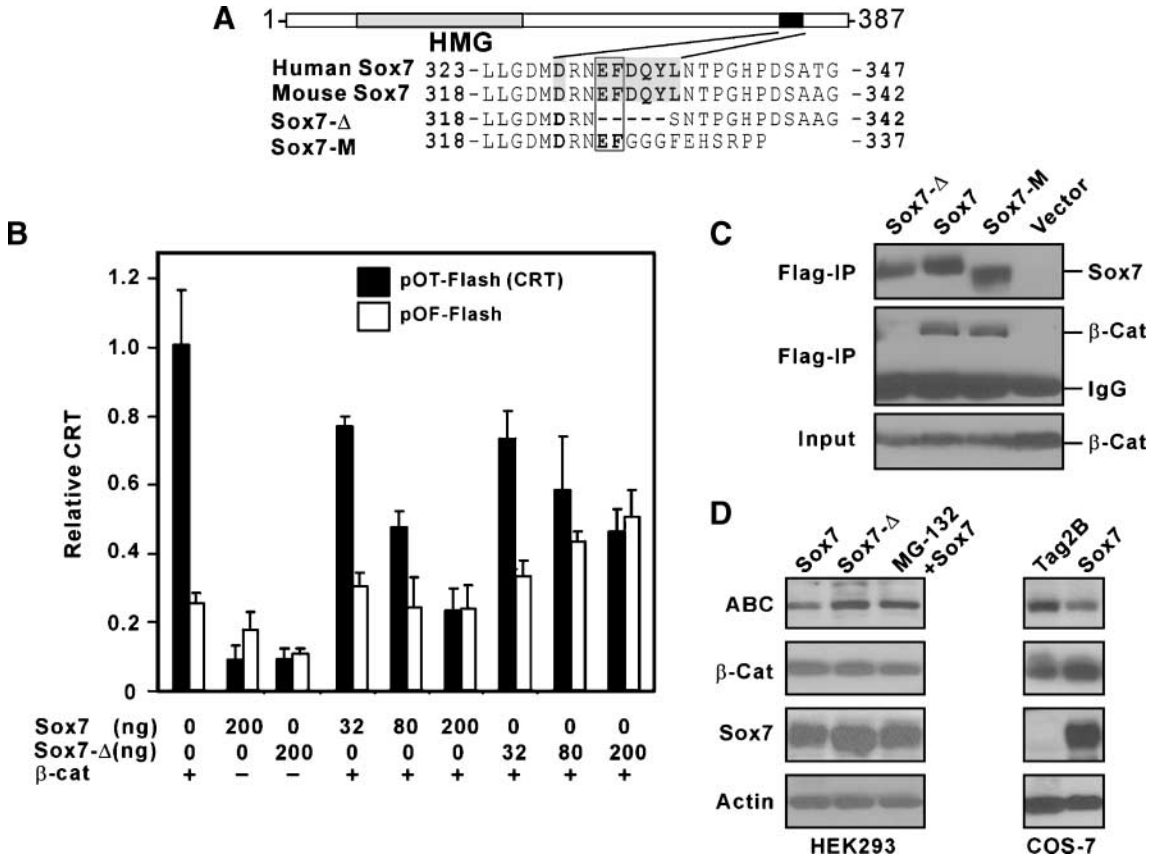

FIGURE 4. Sox7 binds to β-catenin and inhibits CRT. **A.** An illustration of wild-type and mutant Sox7 proteins. Shaded region, high-mobility group (*HMG*) box; filled box, β-catenin binding motif. Evolutionarily conserved sequences within the motif are in bold. **B.** CRT as measured using reporter gene assays with a T-cell factor–responsive reporter (pOT-Flash, 100 ng) or a negative control with a mutated T-cell factor binding site (pOF-Flash, 100 ng). CRT levels were converted to a relative value of 1.0 for cells transfected with β-catenin (100 ng). Different amounts of CMV-Tag2/Sox7 or CMV-Tag2/Sox7-Δ were cotransfected into H1299 with β-catenin. Reactions were carried out in duplicate; bars, SD. **C.** Sox7 interacts with β-catenin. CMV-Tag2B/Sox7, CMV-Tag2B/Sox7-Δ, CMV-Tag2B/Sox7-M, or CMV-Tag2B plasmid was transfected into HEK293 cells with CMV-β-catenin plasmid. Cell lysates were harvested 36 h after transfection and immunoprecipitated by anti-Flag antibody; the immunoprecipitate was probed with anti-Sox7 (*top*) or anti–β-catenin (*middle*) antibodies. Cell lysates were also probed with anti–β-catenin antibody to determine the amount of input β-catenin (*bottom*). **D.** Sox7 mediates the degradation of active β-catenin. CMV-Tag2B/Sox7 or CMV-Tag2B/Sox7-Δ plasmid was transfected into HEK293 cells with CMV-S33Y-β-catenin plasmid (*left*). Cell lysates were harvested 48 h after transfection and were probed with monoclonal anti–active-β-catenin antibody (*ABC*), polyclonal anti–β-catenin antibody (*β-Cat*), anti-Flag antibody (*Sox7*), and anti-actin antibody. For MG-132 blocking experiment, 20 μmol/L MG132 was added 4 h before the collection of cell lysate. For transfection with COS-7 cells (*right*), CMV-Tag2B or CMV-Tag2B/Sox7 plasmid was transfected into COS-7 cells with CMV-S33Y-β-catenin plasmid.

expression of Sox7. C4-2 is a prostate cancer cell line derived from LNCaP (25). A Flag-tagged mouse *Sox7* was cloned into pTRE2 and transfected into ''Tet-inducible'' C4-2 cells. Following isolation of two stable clones, the removal of doxycycline from their culture media was able to induce Sox7 protein expression after 48 hours (two examples, Fig. 6C). Similar to the transient transfection analysis, implementation of a colony formation assay for these two stable cell lines revealed significant reductions in cell proliferation in the absence of doxycycline (i.e., in colonies having Sox7 expression; Fig. 6D). Therefore, ectopic expression of Sox7 in Sox7-null cells inhibits or greatly reduces cell proliferation.

Discussion

Allelic loss of chromosome 8p is one of the most frequently observed somatic genetic alterations in human cancers (26-29). The inactivation of a putative tumor suppressor gene in this region is thought to be the reason for this loss, as tumor suppressor inactivation would be essential for carcinogenesis to occur. In 1989, this 8p allelic loss was initially observed by Vogelstein et al. (30) in colorectal cancer. However, despite rigorous efforts by various research groups over the past 18 years, the identity of the putative tumor suppressor in 8p remained unknown. Somatic deletion analyses of chromosome 8p indicate that this region may even contain several tumor suppressor genes in the case of prostate carcinogenesis (13, 16). Although *NKX3.1* is a promising candidate at *8p21.3* (31, 32), thus far, it has only been implicated in prostate cancer (33, 34). Two linkage analyses of hereditary prostate cancers predict that there is another prostate cancer susceptibility locus at *8p22-23* (35, 36), and a recent analysis by Chang et al. (17) resolves two different linkage peaks at *8p21.3* and *8p23.1*. In the same study, somatic deletion analysis refines the *8p23.1* locus to a 1.4-Mb region holding only five candidate genes, including *Sox7* (17).

Our laboratory first worked to characterize Sox7 protein expression by using an antibody from R&D Systems that is suitable for both immunohistochemistry (Fig. 1) and immunoblot analysis (Supplementary Fig. S1). An immunohistochemistry analysis revealed that Sox7 protein expression is down-regulated in ~47% of prostate adenocarcinomas. Our analyses in prostate cancer cell lines and xenografts indicated that the observed down-regulation of Sox7 protein expression may be due to *Sox7* promoter hypermethylation, which is detected in 44% of these samples. Most importantly, we showed that there was tumor-specific inactivation of *Sox7* by promoter hypermethylation in 48% of the primary prostate tumors tested. Of those prostate tumors with *Sox7* promoter hypermethylation, 78% (7 of 9) also contained a chromosome 8p allelic imbalance, suggesting that promoter hypermethylation in combination with allelic loss may be responsible for the inactivation of both *Sox7* alleles in a significant portion of prostate tumors. The combination of results from the linkage analyses and our study of the tumor-specific inactivation of *Sox7* strongly suggest that Sox7 is indeed a candidate tumor suppressor gene, located at *8p23.1*.

The *Sox7* gene encodes a transcription factor that can both enhance and inhibit transcription (21). It has been found to be essential for cardiogenesis in *Xenopus* (37) and it is known to be involved in the transcriptional regulation of several differentiation-related genes (18, 23, 38). One of more than 26 members of the Sox protein family, Sox7 is a member of the SoxF subfamily of proteins, which also includes Sox17 and Sox18 (39). In fact, Sox7 shares several homologous motifs with Sox17 and Sox18. One of these motifs enables Sox17 to bind to β-catenin and inhibit CRT in *Xenopus* (40). Because all the SoxF proteins contain this purported β-catenin interaction motif, all of them may potentially inhibit CRT. In this study, we showed that Sox7 interacts with β-catenin, and such interaction mediates the degradation of active β-catenin, which results in the suppression of CRT. In addition, we showed that ectopic expression of Sox7 in Sox7-null cells inhibits cell proliferation. The ability of Sox7 to negatively regulate CRT and inhibit cell proliferation is consistent with its role as a tumor suppressor. This information could be of use for future diagnosis and targeted therapy of prostate tumors.

We then wondered if Sox7 serves as an independent checkpoint for β-catenin function. It is known that aberrant WNT signaling plays an essential role in the initiation of colorectal cancer (3) and that the majority of colorectal cancers contain mutations in either β-catenin or the cytoplasmic destruction complex, both of which result in the stabilization of nuclear β-catenin and tumor growth (2). Emerging evidence indicates that other proteins, such as the secreted Frizzled-related proteins, which inhibit the WNT receptor, are also capable of attenuating WNT signaling even in the presence of down-stream mutations (41). Despite these advances in the understanding of the relationship between catenin, WNT signaling, and tumor growth, our current understanding of β-catenin regulation remained limited to the existence of control via the cytoplasmic destruction complex, although Zorn et al. (40) had shown back in 1999 that β-catenin function could be inhibited by Sox17, at least in *Xenopus*. The Zorn data proved to be a valuable clue to uncovering Sox7 functionality in human cells. Our data indicate that *Sox7* is inactivated in the HCT116 human colorectal cancer cell line and that ectopic Sox7 expression can suppress CRT activated by an endogenous mutant β-catenin. As Sox7 is capable of inhibiting WNT signaling even in the presence of a β-catenin mutation, the inhibition of sox7 expression may be as important as disrupting the β-catenin/cytoplasmic destruction complex interaction in colorectal cancers. Indeed, our preliminary analysis of a panel of primary colorectal tumors indicates that tumor-specific

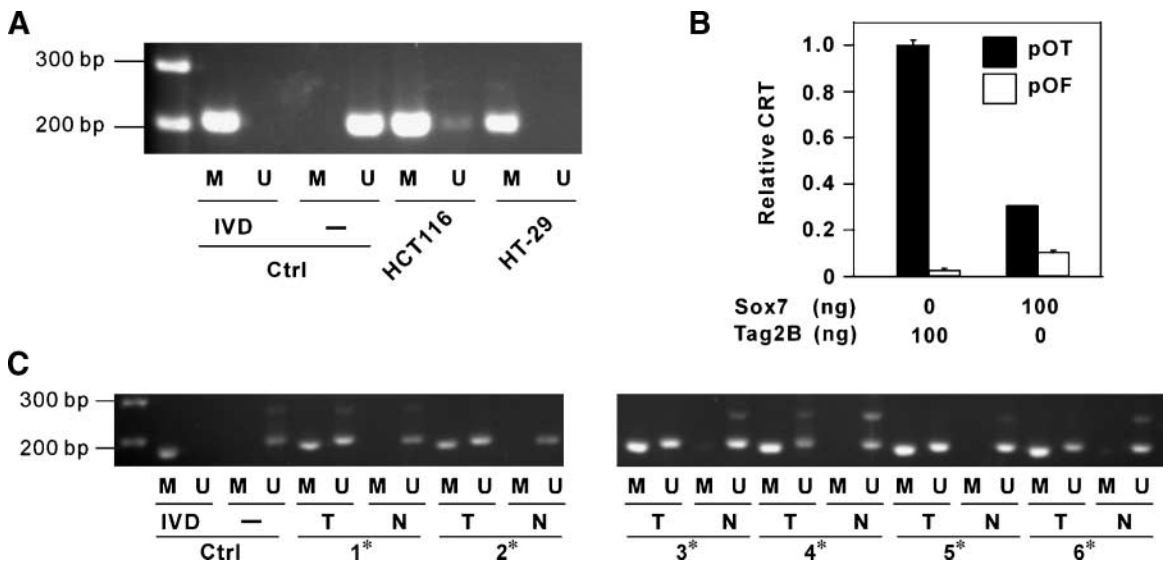

FIGURE 5. Sox7 inactivation in colorectal tumors and cell lines. **A.** MSP analysis of Sox7 promoter in HCT116 and HT29 colorectal cell lines with MSP primer set 1. **B.** CRT as measured using an experimental procedure similar to the one described for Fig. 3B, except that HCT116 cells were not transfected with a β-catenin expression plasmid. **C.** MSP analysis of Sox7 promoter in paired colorectal primary tumors with adjacent normal colon tissues, using MSP primer set 2. *, samples with tumor-specific methylation.

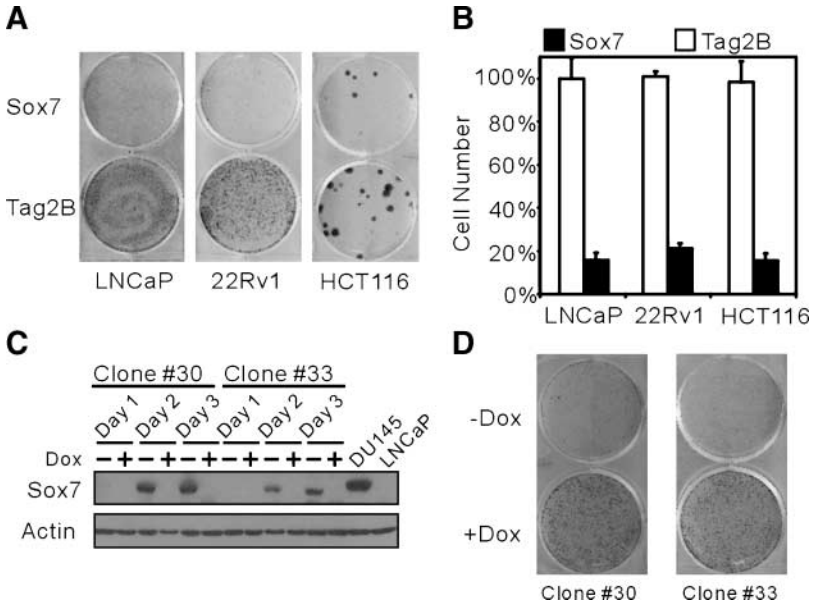

FIGURE 6. Transient and stable induction of Sox7 expression in Sox7-null cells inhibits cell proliferation. **A.** LNCaP, 22Rv1, and HCT116 cells were transfected with either CMV-Tag2/Sox7 plasmid or vector control. Equal numbers of cells were plated in triplicate 24 h after transfection, selected with G418, and stained 12 d later with sulforhodamine B. **B.** Estimation of cell numbers by measuring absorbance (*y axis*) of cells stained with sulforhodamine. Bars, SD of a triplicate assay. **C.** Induction of Sox7 expression in stable C4-2/Tet-Sox7 cell lines. Clones #30 and #33 were grown in the absence (−) or presence (+) of doxycycline (*Dox*; 50 ng/mL). Cell lysates were harvested at 24, 48, and 72 h, and then analyzed by Western blot. Cell lysates from DU145 and LNCaP cells were used as controls for the Sox7 protein level. Actin was used as a loading control. **D.** Equal numbers of cells (4×10^4) from clones #30 and #33 were plated in six-well plates, grown in culture media in the absence or presence of doxycycline for 8 d, and stained with sulforhodamine B.

promoter hypermethylation of *Sox7* is present in most colorectal primary tumors, in a manner quite similar to what was found for about half of the prostate cancers tested. These data are consistent with our observation that Sox7 mediates the degradation of active S33Y β-catenin via an APC-independent mechanism (Fig. 4D). Therefore, it is our conclusion that the *Sox7* gene most likely encodes a candidate tumor suppressor that plays an important role in suppressing various cancers, and one of the functions of Sox7 is to serve as an independent checkpoint for β-catenin transcriptional activity.

The function of Sox7 may not be limited to the regulation of β-catenin transcriptional activity because the overexpression of Sox7 lacking the β-catenin binding domain retains some inhibitory activity in colony formation assay.[7] The growth of prostate cancer cells can be stimulated by androgen (42), and some Sox proteins are involved in the regulation of androgen receptor expression in prostate cancer cells (43). In our study, Sox7 expression was not detected in the androgen-dependent LNCaP cell line, and it will be interesting to determine in the future whether the expression of Sox7 in LNCaP cells interferes with androgen-dependent cell growth.

Materials and Methods

Materials

Rabbit polyclonal anti-Sox7 antibody was purchased from R&D Systems for immunohistochemistry analysis. Rabbit polyclonal anti-Sox7 (H175) antibody was purchased from Santa Cruz Biotechnology, Inc., for immunoblot analysis. Rabbit polyclonal anti–β-catenin antibody was purchased from Cell Signaling Technology (Microarray), and anti–active-β-catenin (clone 8E7) antibody was purchased form Millipore. Rabbit polyclonal anti-actin antibody was purchased from Sigma Chemical Co. The primary tumors from human prostate and colon tissues were collected previously (20, 44). Genomic DNA and RNA were extracted as described previously (45). The pCMV-Tag2/Sox7 expression plasmid was provided by Dr. Akira Murakami (Laboratory of Molecular and Cellular Biology, Graduate School of Biostudies, Kyoto University, Sakyo-ku, Japan; ref. 23), whereas the pOT-Flash, pOF-Flash, and wild-type and S33Y β-catenin expression constructs were obtained from Dr. Bert Vogelstein (Ludwig Center at Johns Hopkins and Howard Hughes Medical Institute, Baltimore, MD; refs. 1, 22).

Cell Culture and Demethylation Treatment

Prostate cancer cell lines (PC-3, DU145, LNCaP, and 22RV1), HEK-293 cell line, non–small-cell lung cancer cell line (H1299), and colorectal cancer cell line (HCT116) were purchased from the American Type Culture Collection and propagated according to the conditions recommended by the vendor. To demethylate the hypermethylated Sox7 promoter, both LNCaP and DU145 cells were treated with 2 μmol/L 5-aza-dC for 72 h, as has been described previously (46).

Immunohistochemistry for Sox7

The formalin-fixed, paraffin-embedded tissue microarrays of prostate and colorectal primary tumors were purchased from US Biomax, Inc. These tissue arrays were immunostained

[7] D. Zhong, X.L. Liu, and W. Zhou, unpublished observations.

for Sox7 (AF2766, 1:50 dilution, goat polyclonal antibody, R&D Systems) following heat-induced antigen retrieval, then developed using horseradish peroxidase–labeled polymer and the DAKO Autostainer Staining System as described previously (45). Quantitation in immunohistochemistry was based on scoring for the number of positively stained cells, but not the intensity of staining. Scores ranged from 0 to 3+, and any samples with 2 to 3+ as an immunohistochemistry score were defined as having normal levels of Sox7 expression.

Quantitative Real-time Reverse Transcription-PCR Analysis

Total RNA was isolated from snap-frozen primary tumors as described previously (47). This total RNA was reverse transcribed to cDNA, and quantitative real-time PCR was carried out in a 20-μL volume using iQ SYBR Green Supermix (Bio-Rad) on a Bio-Rad iCycler. Primer sequences for reverse transcription-PCR and quantitative real-time PCR of *Sox7* are listed in Supplementary Table S1. A similar analysis was carried out for *GAPDH*, then the relative *Sox7* expression levels were determined by comparing the threshold cycle values of *Sox7* and *GAPDH* (45).

Methylation Analysis

Bisulfite modification of genomic DNA followed by PCR amplification was carried out as described previously (46). For bisulfite sequencing, genomic DNA was amplified using primers that were not specific for methylation status. Primer sequences for MSP and bisulfite sequencing PCR are listed in Supplementary Table S1.

Allelic Imbalance Analysis

Tissue specimens were manually microdissected, then the tumor DNA was isolated from tissue samples as previously described (20). DNA from adjacent benign prostate epithelia was also isolated for use as the autologous normal control. First, the normal DNA samples were genotyped for chromosome 8p heterozygosity using nine biallelic single-nucleotide polymorphic markers on chromosome 8p to select for an informative marker for subsequent allelic imbalance analysis. These single-nucleotide polymorphic markers (National Center for Biotechnology Information RefSNP nos. 1124, 3185, 3850751, 3888179, 3258, 11362, 3112, 532841, and 14879) are located from *8p21.2* to *8p23.2* on the cytogenetic band. The allelic status of 8p was determined using a combination of digital single-nucleotide polymorphism and sequential probability ratio tests, as previously described (19).

Luciferase Reporter Assay

Cells were plated at 5×10^4 per well on 24-well tissue culture plates 24 h before transfection. All transfections were carried out with Lipofectamine 2000 (Invitrogen) and pRL-CMV. Luciferase activity was measured in a luminometer (BD Biosciences) after 48 h, and the resulting data were normalized for the background *Renilla* luciferase activity using the Dual Luciferase Reporter Assay system (Promega). For the CRT assay, each well was transfected with 100 ng of pOT-Flash, a T-cell factor/lymphoid enhancer factor–responsive reporter, or 100 ng of pOF-Flash, a negative control with a mutated T-cell factor/lymphoid enhancer factor binding site, and the indicated amount of pCMV-Tag2B/Sox7, pCMV-Tag2B/Sox7-Δ, or pCMV-Tag2B plasmid.

Immunocoprecipitation and Immunoblot

HEK293 cells were plated at 1×10^6 per 10-cm tissue culture dish 24 h before transfection. All transfections were carried out with Lipofectamine 2000 (Invitrogen) and the indicated plasmid constructs. Cell lysates were harvested 48 h after transfection and immunoprecipitated with anti-Flag M2-agarose from mouse (Sigma-Aldrich, Inc.). For immunoblot analysis, cell lysates or immunoprecipiated complexes were separated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane. Antibodies against Sox7 and β-catenin were used for immunoblot analysis.

For the analysis of active-β-catenin in HEK293, cells were transfected with 0.5 μg of pCMV-S33Y-β-catenin and 0.5 μg of pCMV-Tag2B/Sox7 or pCMV-Tag2B/Sox7-Δ plasmids. For a similar analysis in COS-7 cells, cells were transfected with 0.5 μg of pCMV-S33Y-β-catenin and 0.5 μg of pCMV-Tag2B or pCMV-Tag2B/Sox7 plasmids. Cell lysates were harvested 48 h after transfection. For MG-132 blocking experiment, 20 μmol/L MG-132 was added to the culture 4 h before the collection of culture media. Antibodies against active β-catenin, β-catenin, FLAG, and actin were used for immunoblot analysis.

Colony Formation Assay

Cells were transfected with pCMV-Tag2B/Sox7 or an empty vector control using Lipofectamine 2000. At 24 h after transfection, these cells were trypsinized and plated onto six-well plates, then selected for 14 d with 1 μg/mL geneticin (Invitrogen).

Generation of a Stable Tet-Inducible Cell Line

The Tet-inducible C4-2 cell line was a generous gift from Dr. Leland Chung (Emory University, Atlanta, GA). Briefly, a Flag-tagged mouse *Sox7* was cloned into pTRE2 and transfected into the Tet-inducible C4-2. Cells were selected in culture media containing puromycin. Two double-stable clones were isolated 14 d after selection began. The incorporation of pTRE2-Sox7 into the cell line was validated by genomic PCR amplification of mouse-specific PCR primers, Sox7-SF (5′-TTTTTATTTGTATATTTTGGTGTAGAT-3′) and Sox7-SR (5′-CTCACTCATTCTACAAACCTTAAAC-3′). Induction of mouse Sox7 mRNA in the absence of doxycycline was also confirmed by quantitative real-time reverse transcription-PCR with *GAPDH* as an internal control.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Bert Vogelstein for providing pOT-Flash, pOF-Flash, and β-catenin expression constructs; Dr. Akira Murakami for providing pCMV-Tag2/Sox7 expression plasmid; and Dr. Leland Chung for providing C4-2/Tet-inducible cell line. We thank Kathleen Kite-Powell for manuscript editing.

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