FOXO1A Is a Candidate for the 13q14 Tumor Suppressor Gene Inhibiting Androgen Receptor Signaling in Prostate Cancer

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Abstract

Chromosomal deletion is frequent at the region between *BRCA2* and *RB1* in the q14 band of chromosome 13 (13q14) in human cancers, including prostate cancer, suggesting the presence of a tumor suppressor gene. However, no reasonable candidate has been identified thus far. In this study, we did genetic and functional analyses to identify and evaluate the 13q14 tumor suppressor gene. Hemizygous and homozygous deletions in cell lines/xenografts of prostate cancer mapped the deletion locus to 919 kb, which harbors only one known gene, the *FOXO1A* transcription factor. Deletion at *FOXO1A* was detected in 31% to 34% in 6 cell lines, 27 xenografts, and 72 clinical specimens of prostate cancer, and was significantly more frequent than deletions at surrounding loci. In addition, *FOXO1A* was transcriptionally down-regulated in some prostate cancers. Functionally, ectopic expression of FOXO1A inhibited, and its knockdown promoted, cell proliferation or survival. Furthermore, FOXO1A inhibited androgen- and androgen receptor–mediated gene regulation and cell proliferation. Consistent with the understanding of FOXO1A biology, our findings suggest that *FOXO1A* is the 13q14 tumor suppressor gene, at least in prostate cancer. As a well-established negative effector in the phosphatidylinositol 3-kinase/AKT signaling pathway, FOXO1A inactivation in cancer would impair the therapeutic effect of phosphatidylinositol 3-kinase/AKT inhibitors in cancer treatment. (Cancer Res 2006; 66(14): 6998-7006)

Introduction

The development and progression of human cancer results from multiple genetic alterations. Chromosomal loss is one of the most common genetic alterations detected in cancer, leading to inactivation of tumor suppressor genes (1). In prostate cancer, a number of chromosomal regions have been identified for their frequent deletion by cytogenetic and molecular genetic analyses such as comparative genomic hybridization, fluorescence *in situ* hybridization, and loss of heterozygosity analysis. At present, however, only a few genes from the common regions of deletions have been identified and implicated in prostate cancer, including *NKX3-1* from 8p21, *PTEN* from 10q23, *p27/Kip1* from 12p13, and *ATBF1* from 16q21 (1, 2). Many more target genes in the deletion regions remain to be identified.

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Deletion of the q arm of human chromosome 13 is one of the most frequent deletions in prostate cancer (1, 3). Two common regions of deletion in 13q have been identified, one in 13q14 and the other in 13q21 (1). The known tumor suppressor gene *RB1* is located at 13q14, and thus has been considered as the 13q14 tumor suppressor gene in prostate cancer. Although allelic loss and somatic mutations of *RB1* have been detected in some prostate tumors, the mutation frequency is low and no correlation between loss of heterozygosity and mutation or absence of expression for *RB1* has been observed (1). In addition, several deletion mapping studies have clearly shown that, in prostate cancer, the deleted locus at 13q14 is located between *BRCA2* and *RB1* (1, 4). Deletion at 13q14 is also frequent in many other types of human malignancies (3). These findings indicate that 13q14 harbors a major tumor suppressor gene that is inactivated in different types of human cancers. The gene remains to be identified.

The phosphatidylinositol 3-kinase (PI3K)/AKT pathway is a potent oncogenic pathway regulating cell proliferation and survival as well as carcinogenesis (5, 6). The phosphatase and tensin homologue (PTEN) tumor suppressor negatively regulates this pathway by inhibiting PI3K-mediated AKT activation. As one of the key downstream mediators in the PI3K/AKT pathway, FOXO1A is inactivated by AKT-mediated phosphorylation and nuclear exclusion (7). FOXO1A is a member of the forkhead transcription factor family, which consists of at least 43 members that contain a conserved DNA-binding motif related to the *Drosophila* region-specific homeotic gene *forkhead* (8). FOXO factors play important roles in cell cycle arrest, apoptosis, stress resistance, and energy metabolism. FOXO1A-deficient mice died around embryonic day 11 because of defects in the branchial arches and remarkably impaired vascular development of embryos and yolk sacs (9). *FOXO1A* is highly expressed in normal prostate. In PTEN-deficient prostate carcinoma cell lines, FOXO1A was cytoplasmically sequestered and inactive, which seemed to contribute to increased cancer cell survival (10). In human malignancies, the PI3K/AKT pathway often becomes excessively active at least through the inactivation of *PTEN* by mutation and/or genomic deletion and the overexpression of PI3K and/or AKT (5, 6). Thus, therapeutic strategies targeting PI3K or AKT are considered a promising therapy for the treatment of human malignancies.

In this study, we mapped the minimal region of deletion in 13q14 and identified the *FOXO1A* transcription factor as a reasonable candidate for the 13q14 tumor suppressor gene in human prostate cancer using approaches established in our previous studies (2, 11). *FOXO1A* is frequently deleted and down-regulated in human prostate cancer. In addition, FOXO1A inhibited cell proliferation at least through inhibition of androgen receptor signaling. Taken together with published studies, we conclude that *FOXO1A* is a tumor suppressor gene that is frequently inactivated in human

prostate cancer. These findings provide another mechanism for the role of the PI3K/AKT pathway in human malignancies. They also underscore the importance of developing therapeutic strategies targeting PI3K or AKT.

Materials and Methods

Cell lines, xenografts, and clinical specimens from prostate cancer. Six prostate cancer cell lines (DU 145, NCI-H660, LNCaP, 22Rv1, MDAPCa2b, and PC-3) and two immortalized and untransformed prostatic epithelial cell lines (PZ-HPV-7 and RWPE1) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were propagated following standard protocols from ATCC. Twenty-seven xenografts from prostate cancer, as described in detail in our previous study (2), were also used, including CWR21, CWR22, CWR91, LAPC3, LAPC4, LAPC9, PC82, LuCaP 23.1, LuCaP 23.8, LuCaP 23.12, LuCaP 35, LuCaP 35V, LuCaP 41, LuCaP 49, LuCaP 58, LuCaP 69, LuCaP 70, LuCaP 73, LuCaP 77, LuCaP 78, LuCaP 81, LuCaP 86.2, LuCaP 92.1, LuCaP 93, LuCaP 96, LuCaP 105, and LuCaP 115. Finally, we used clinical prostate cancer specimens and matched normal cells from 72 patients who had undergone radical prostatectomies. None of the patients had lymph node involvement or distant metastasis at the time of surgery. Genomic DNA for all the samples and RNA for all the cell lines and some of the xenografts were extracted following standard procedures (2).

Detection of homozygous and hemizygous deletions. A total of 16 sequence-tagged site (STS) markers spanning the region between *BRCA2* and *RB1* ($\sim$16 Mb) in 13q were used to detect homozygous and hemizygous deletions by regular PCR and duplex PCR, as described in our previous study (12). A hemizygous deletion was considered to be present when the ratio of signal intensity for a 13q marker to that for the control marker in a tumor sample was less than half of the ratio in the normal human placenta DNA or matched normal cells (Clontech, Palo Alto, CA). The control marker was from exon 5 of the *KAI1* gene, which is rarely altered at the genomic level in human prostate cancer (2, 12).

Expression analysis. The first-strand cDNA was synthesized from total RNA by using the Iscript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). An RNA sample pooled from 19 normal human prostates (BD Biosciences Clontech, Palo Alto, CA) was used as the normal control. Expression levels of *FOXO1A* in prostate cancer cell lines/xenografts and prostate-specific antigen (PSA) in LNCaP cells were determined by real-time PCR with the ABI SYBR Green Kit and the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). Each data point was in triplicate. DNA sequences for the primers were *FOXO1A*, 5′-TTTGGACTGCTTCTCTCAGTTCCTGC-3′ and 5′-TTTGACAATGTGTTGCCCAACCAAAG-3′; *PSA*, 5′-AGAAGCATTCCCAACCCTGGCAG-3′ and 5′-GGGAAGCTGTGGCTGACCTGAAATAC-3′. Expressions of *FOXO1A* and *PSA* in each sample were calculated by averaging the readings from triplicate reactions and then indicated by a ratio to the readings for β-actin. Expressions of *FOXO1A* and *PSA* in each sample were normalized against normal controls.

In Northern blot analysis, 15 μg of total RNA for each sample were separated by electrophoresis in a denaturing gel, transferred to Hybond-C nylon membrane (Amersham, Piscataway, NJ), and hybridized with labeled probe in QuikHyb Hybridization Solution (Stratagene, La Jolla, CA) following standard protocols. The probe (636 bp) was generated by PCR amplification of *FOXO1A* cDNA with two primers (5′-GGGTGAACTTACCTGCTCACTAACCC-3′ and 5′-TCAGTATAACTGTGCGCCTGGACTC-3′) and radiolabeled using the Random Primers DNA Labeling System (Invitrogen, Carlsbad, CA).

Mutation and promoter methylation analyses. Direct DNA sequencing of PCR products was applied to determine if *FOXO1A* had sequence alterations in prostate cancer. Five pairs of PCR primers, which together amplify the entire coding region of *FOXO1A* from genomic DNA, were used to amplify *FOXO1A* in 10 prostate cancer cell lines or xenografts. Primer sequences, annealing temperature, and PCR product size were as follows: 5′-GGGTGAACTTACCTGCTCACTAACCC-3′/5′-TCAGTATAACTGTGCGCCTGGACTC-3′, 60°C, 636 bp; 5′-ATCATGACGTTCTGGCCCAGAACCC-3′/5′-GCTGCCAAGAAGAAAGCATCTCTC-3′, 58°C, 686 bp; 5′-TGTTCAGATGGAAATCTGTAGCACACACTC-3′/5′-CTTCTCCAAGATCATCCTGTTCGGTC-3′, 63.6°C, 515 bp; 5′-CCGACTTCATGAGCAACCTGAGC-3′/5′-CGAGCAAACCTGCACAGCTGC-3′, 60.5°C, 505 bp; and 5′-CCTCTTGGCTCTCCTGCGGCTGGG-3′/5′-CTTGAAGTAGGGCACGCTCTTGAC-3′, 60°C, 636 bp. PCR products were purified using the Qiaquick PCR Purification Kit (Qiagen, Valencia, CA) and DNA sequencing was done by Macrogen (Seoul, Korea).

For promoter methylation analysis, PCR amplification and DNA sequencing of bisulfite-treated genomic DNA were done following the published method (13). Briefly, bisulfite-treated DNA was subjected to PCR amplification with six pairs of primers spanning 2,960 bp of GC-rich DNA in the promoter region and exon 1 of *FOXO1A*. Purified PCR products were cloned into the pGEM-T vector (Promega, Madison, WI) and three clones for each PCR product were sequenced by Macrogen. Primer sequences, annealing temperature, and PCR product size were as follows: 5′-TTTGGATTTAGTGGATGGAGTGGG-3′/5′-AAATTCTACTCAATCACTCCCTCCTAACTC-3′, 58°C, 469 bp; 5′-GTTTAGTAAAGATATTGTGGGTGGAGTTAG-3′/5′-TACTACAACTACCAAACCACCCAACTTAC-3′, 56°C, 566 bp; 5′-TGTTTGGGTGGTGGTGTTGTATG-3′/5′-CACTCTCCTCCAACAAACTCAAATTACTC-3′, 56.8°C, 765 bp; 5′-TTGGTTGTTGTTTAGGTTGGAGTTTAG-3′/5′-CACACCAAATCCACTCACCTTCC-3′, 57°C, 577 bp; 5′-TGTAGATTTATGAGTGGATGGTTAAGAGTG-3′/5′-AACCAATCACACCCTACCTATCCC-3′, 56.6°C, 604 bp; and 5′-TGTGTTAGTTAGGAGTTTGTGTGGGATG-3′/5′-ACCAACACTCCACAAACAATAACCAC-3′, 58.5°C, 502 bp.

Colony formation assay. The *FOXO1A* expression vector pcDNA3-FLAG-FOXO1A was kindly provided by Dr. W. Bai (University of South Florida, Tampa, FL; ref. 14). This expression plasmid was transfected into 22Rv1 and LNCaP prostate cancer cells using the Lipofectamine PLUS reagent (Invitrogen). Briefly, cells were seeded in six-well plates at a density of 2×10^5 per well. On the following day, 1.6 μg of pcDNA3-FLAG-FOXO1A and negative control plasmid (pcDNA3-FLAG) were transfected in triplicate. Forty hours later, one set of cells was harvested to determine protein expression of FOXO1A by Western blotting with anti-FLAG monoclonal antibody (mAb) whereas another set of cells was grown in selection medium containing 800 μg/mL G418 for 8 to 12 days for colony formation assay. As a positive control, wild-type pcDNA3-FLAG-ATBF1, which suppressed 22Rv1 colony formation in a previous study (2), was transfected into 22Rv1 cells and analyzed in the same way. At different times after selection, cells were fixed with 10% trichloroacetic acid and stained with sulforhodamine B. Absorbances, which indicated cell numbers, were measured following the protocol in our published study (2).

Analysis of androgen receptor transactivation activity. The pSG5-AR expression construct and pGL-PSA-Luc promoter reporter construct (the latter contains the 5.8-kb promoter region of *PSA* gene) have been described in a previous study (15). 22Rv1 or LNCaP cells were plated into 12-well plates at a density of 10^5 per well in phenol red–free RPMI 1640 supplemented with 5% charcoal-stripped fetal bovine serum. On the following day, different combinations of plasmids were transfected into cells with the Lipofectamine PLUS reagent as described above. Two days after transfection, one set of cells was harvested to monitor ectopic expression of FOXO1A by Western blotting and another set of cells was treated with 2 nmol/L R1881 (Sigma, St. Louis, MO), a synthetic androgen, or ethanol (solvent for R1881) for 24 hours. Cells were then lysed in reporter lysis buffer (Promega) and luciferase activities were measured using the Luciferase Assay System (Promega) as described in our previous study (16). Protein concentrations in cell extracts were determined using the Protein Assay kit (Bio-Rad). Luciferase activity was normalized by protein concentration for each sample. The transfection and luciferase experiments were repeated twice.

Measurement of secreted PSA protein. LNCaP cells were transfected with *FOXO1A* plasmid and treated with R1881 as described above. Two days after R1881 treatment, supernatants from culture plates were collected and frozen at −20°C until analysis. In addition, cells were lysed with the radioimmunoprecipitation assay buffer (1× PBS, 0.1% SDS, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 1% Sigma cocktail proteinase inhibitors I, 1 mmol/L sodium orthovanadate, and 0.1 mg/mL phenylmethylsulfonyl fluoride) and the lysates were centrifuged to eliminate cell debris. PSA concentrations in the supernatants and cell lysates were then determined using the ELISA kit specific for PSA (Alpha Diagnostics International,

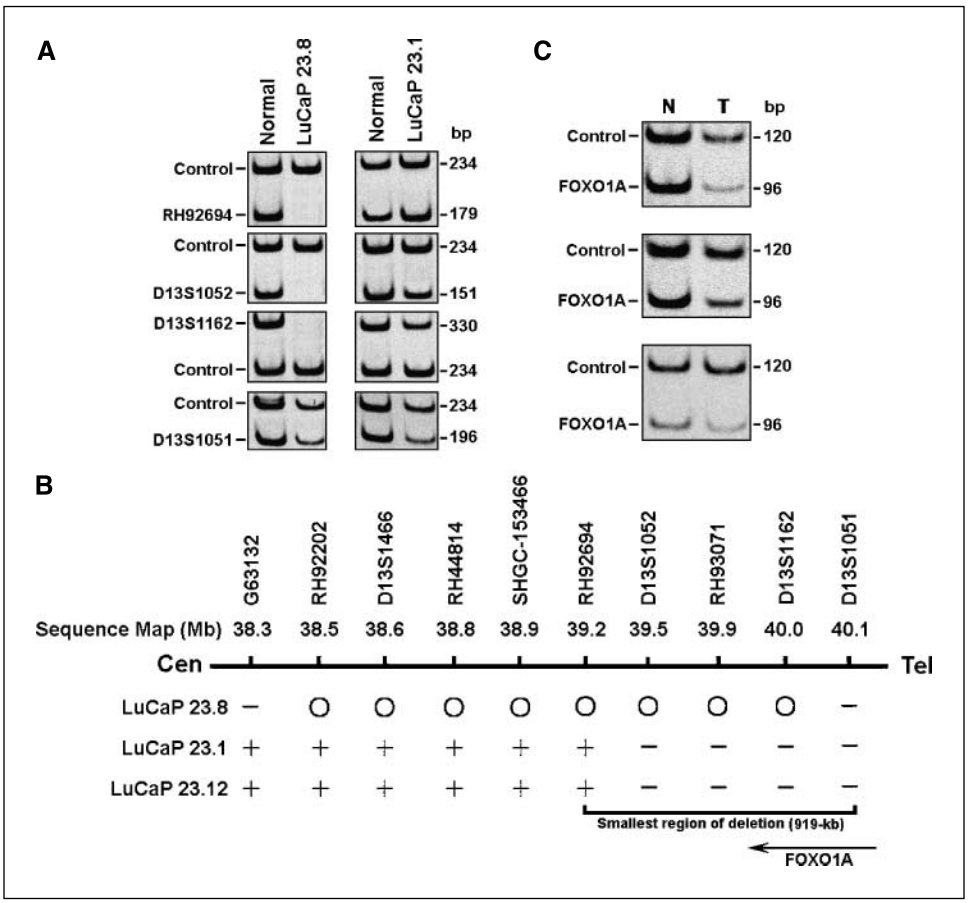

Figure 1. Mapping and analysis of the deletion region in 13q14 in prostate cancer. *A,* detection of homozygous deletion (*left*) and hemizygous deletion (*right*) by duplex PCR in xenografts of LuCaP 23.8 and LuCaP 23.1 prostate cancer, respectively. Deletion status for each marker is indicated in (*B*). *B,* definition of the smallest region of deletion at 13q14 in prostate cancer. Marker names are at the top, sample names at the bottom left, and the common region of deletion is marked by a thick line. Sequence map is indicated below each marker. ○, homozygous deletion; −, hemizygous deletion; +, no deletion. The location of *FOXO1A* is also indicated. *C,* representative images showing hemizygous deletion at *FOXO1A* in clinical samples of prostate cancer. Each pair of normal and tumor cells was from the same patient. *N,* normal; *T,* tumor. *A* and *C,* sample names are at the top, marker names at the left, and sizes of PCR products for markers are at the right.

San Antonio, TX) following the standard protocol of the manufacturer. Total protein in supernatants and lysates was also measured. PSA concentration normalized by total protein concentration was used to indicate the secreted PSA level in a sample. For each transfection, FOXO1A expression was determined by Western blot analysis.

Examination of FOXO1A effect on androgen receptor–mediated cell proliferation. In the androgen-responsive LNCaP prostate cancer cells, *FOXO1A* expression was either increased by transfecting pcDNA3-FLAG-FOXO1A expression plasmid, as described above, or knocked down by transfecting chemically synthesized small interfering RNA (Dharmacon, Chicago, IL). The sequence for *FOXO1A* small interfering RNA was 5′-GCCCUGGCUCUCACAGCAA-3′ and the negative control small interfering RNA was against the luciferase gene. The siPORT NeoFX reagent (Ambion, Austin, TX) was used to transfect small interfering RNA at 50 nmol/L into LNCaP cells in 12-well plates following the manual of the manufacturer. Two days later, cells transfected with either plasmid or small interfering RNA were treated with 0.05 nmol/L R1881 or control solution for different times. At days 5 and 8 after R1881 treatment, cells were fixed and subjected to sulforhodamine B staining as described above.

Western blot analysis. Western blotting was done as described in our published study (17). Anti-FLAG mAb and goat anti-rabbit second antibody were purchased from Sigma (St. Louis, MO) and Cell Signaling (Beverly, MA).

Statistical analysis. Student's *t* test was used to determine statistical differences between experimental and control groups; $P < 0.05$ was considered statistically significant.

Results

Deletion mapping identified *FOXO1A* as the 13q14 tumor suppressor gene. To identify the tumor suppressor gene located between *BRCA2* and *RB1* in 13q, we first analyzed 16 STS markers for hemizygous and homozygous deletions in 33 xenografts and cell lines from prostate cancer. In addition to the detection of hemizygous deletions, a homozygous deletion was detected in xenograft LuCaP 23.8 (Fig. 1*A*), within 1.8 Mb between markers *G63132* and *D13S1051* at 13q14 (Fig. 1*B*). Two other xenografts from the same patient, LuCaP 23.1 and LuCaP 23.12, showed hemizygous deletion but not homozygous deletion for the same set of markers analyzed; the hemizygous deletions further narrowed the region of deletion to a 919-kb interval between markers *RH92694* and *D13S1051* (Fig. 1*A* and *B*). The 1.8-Mb homozygous deletion spans two genes, *LHFP* and *FOXO1A*, which are involved in tumor-related chromosomal translocations in lipoma (18) and alveolar rhabdomyosarcoma (19), respectively. Whereas both genes showed a homozygous deletion region, only *FOXO1A* had hemizygous deletion, suggesting that *FOXO1A* centers the deletion at 13q14 in prostate cancer.

To further evaluate the center of 13q14 deletion, we analyzed additional markers for hemizygous and homozygous deletions in the xenografts and cell lines. We used markers from both *LHFP* and *FOXO1A*, along with four markers flanking the homozygous deletion, including *D13S1680* (33.3 Mb), *D13S994* (36.2 Mb), *G17767* (43.2 Mb), and *SHGC-149095* (45.0 Mb). Additional hemizygous deletions were detected at these markers. Deletion frequencies were 3 of 32 (9%) for *LHFP,* 11 of 32 (34%) for *FOXO1A*, 3 of 32 (9%) for D13S1680, 3 of 32 (9%) for *D13S994*, 4 of 32 (12%) for *G17767*, and 4 of 32 (12%) for *SHGC-149095*. Deletion at *FOXO1A* was significantly more frequent than other markers, further supporting the candidacy of *FOXO1A* as the 13q14 gene.

To determine the role of FOXO1A in prostate cancer, we examined *FOXO1A* deletion in prostate cancer cells microdissected from 72 clinical specimens, with their matched noncancer cells as controls. Hemizygous deletion at *FOXO1A* was detected in 22 of the 72 (31%) samples (Fig. 1*C*). These results further implicate *FOXO1A* in prostate cancer.

Transcriptional down-regulation of *FOXO1A* in prostate cancer. *FOXO1A* expression was determined in normal prostates and prostate cancer cell lines/xenografts by reverse transcription-PCR (RT-PCR), Northern blot analysis, and real-time PCR (Fig. 2). Real-time PCR analysis showed the highest level of FOXO1A expression in normal prostates but a reduced FOXO1A expression in the two immortalized nonneoplastic prostatic epithelial cell lines and most of the prostate cancer cell lines and xenografts. Relative to normal prostates, all the cell lines and 11 of 15 xenografts showed reduced *FOXO1A* expression by at least 50% (Fig. 2*B*). All the prostate cancer cell lines and xenografts with hemizygous deletion showed reduced *FOXO1A* expression. Northern blot analysis further confirmed a reduced *FOXO1A* expression in prostatic cell lines (Fig. 2*A*).

Rare mutation and promoter methylation of *FOXO1A* in prostate cancer. Each exon of *FOXO1A* was amplified by PCR and sequenced in 10 prostate cancer cell lines and xenografts to determine if *FOXO1A* had mutations in prostate cancer. No deletion, insertion, or frameshifting mutations were detected in the 10 samples. One single-nucleotide polymorphism was detected in 9 of 10 samples.

Bisulfide treatment combined with PCR amplification and DNA sequencing of *FOXO1A* promoter revealed two methylated CpG dinucleotides in the DU 145 cell line and three in the 22Rv1 cell line. However, none of the methylated CpGs occurred in both DU 145 and 22Rv1. Furthermore, treatment of both DU 145 and 22Rv1 cells with 5-aza-deoxycytidine, a demethylating agent that removes the methyl group from DNA and releases a promoter's activity, did not induce obvious *FOXO1A* expression in either cell line (data not shown). Therefore, promoter methylation is not a common mechanism for *FOXO1A* transcriptional down-regulation in prostate cancer cells.

FOXO1A inhibited colony formation. To functionally evaluate the candidacy of *FOXO1A* as the 13q14 tumor suppressor gene, we transfected *FOXO1A* expression plasmid, along with empty vector control, into prostate cancer cell lines 22Rv1 and LNCaP, both of which express reduced levels of *FOXO1A* (Fig. 2). Colony formation assay was then done. Protein expression of transfected FOXO1A was confirmed by Western blot analysis in transfected cells (Fig. 3*C*). In 22Rv1 cells, ectopic FOXO1A expression dramatically reduced colony formation (Fig. 3*A* and *B*). In LNCaP cells, FOXO1A expression also significantly inhibited colony formation (Fig. 3*A* and *B*) although the inhibitory effect was weaker than in 22Rv1 cells. As a positive control, transfection of wild-type FLAG-pcDNA3-ATBF1 into 22Rv1 cells significantly inhibited colony formation (data not shown) although the effect of ATBF1 was weaker than that of FOXO1A.

FOXO1A inhibited androgen receptor signaling in prostate cancer cells. Androgen plays an important role in the structure, function, and malignant progression of the prostate through its activation of androgen receptor (20). Previous studies showed that in prostate cancer cells, androgen signaling inhibits FOXO1A function by at least two mechanisms: one is the activation of PI3K signaling by androgen receptor interaction with the p85α subunit of PI3K (21); the other is direct inhibition of FOXO1A function by androgen receptor interaction with FOXO1A (14, 22). It is thus possible that FOXO1A also affects androgen receptor function in prostate cancer. To test this hypothesis, we first measured the ability of FOXO1A to modulate the transcriptional activity of androgen receptor in androgen receptor–positive prostate cancer cells. An androgen-responsive promoter from the *PSA* gene, which transactivates the luciferase reporter gene in an expression plasmid, was analyzed with different treatments of androgen (R1881) and FOXO1A in 22Rv1 and LNCaP prostate cancer cell

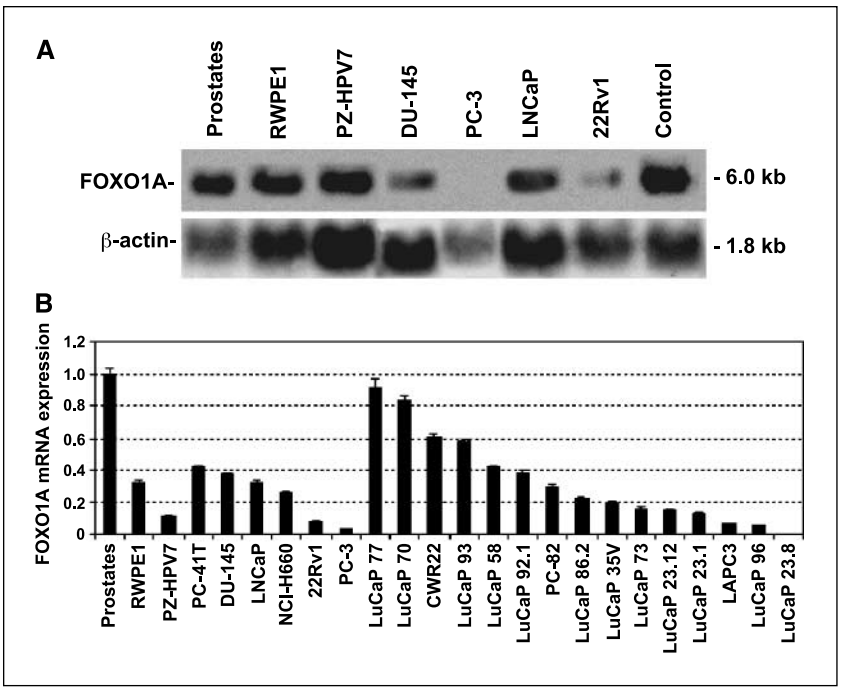

Figure 2. Reduced expression of *FOXO1A* in prostate cancer, as detected by Northern blotting (*A*) and real-time PCR assay (*B*). Sample names are at the top or bottom. *A,* the control was from *FOXO1A*-transfected 22Rv1 cells. *B, FOXO1A* expression in each sample was normalized by the reading from normal prostates.

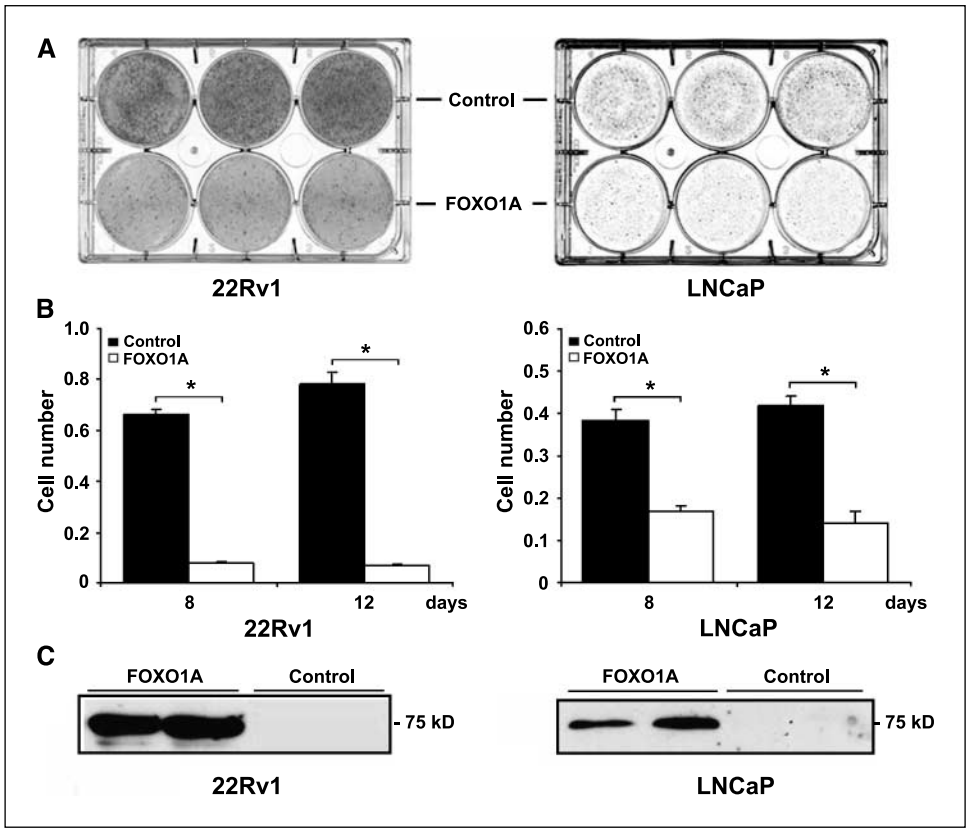

Figure 3. FOXO1A inhibited colony formation in both 22Rv1 and LNCaP prostate cancer cell lines. *A,* images of cells transfected with vector control (*top*) or FOXO1A (*bottom*), cultured for 12 days in selection medium, and stained with sulforhodamine B. *B,* estimation of cell numbers by measuring absorbances (*y axis*) of cells stained with sulforhodamine B. Cells from day 8 and day 12 posttransfection are shown. *, $P = 0.000$. *C,* verification of transfection-mediated FLAG-tagged FOXO1A expression by Western blot analysis with anti-FLAG mAb.

lines, both of which express active androgen receptor. As shown in Fig. 4, R1881 treatment induced significant activities of *PSA* promoter in both cell lines. In addition, ectopic expression of FOXO1A, as confirmed by Western blot analysis, significantly inhibited androgen receptor transactivation activities (Fig. 4*C* and *D*), and the effect of FOXO1A was stronger in 22Rv1 cells (82% inhibition) than in LNCaP cells (45% inhibition). Increased androgen receptor expression by plasmid transfection did not significantly affect the effect of FOXO1A on R1881-stimulated reporter activities (Fig. 4*A* and *B*). Whereas R1881 treatment seemed to increase androgen receptor protein expression, ectopic expression of FOXO1A did not alter androgen receptor expression (data not shown). When different amounts of plasmids were used, FOXO1A showed a dose-dependent inhibitory effect on *PSA* promoter activity in both cell lines (Fig. 4*C* and *D*).

To further test the inhibitory effect of FOXO1A on androgen receptor transcriptional activity, we measured endogenous expression of PSA, a well-established androgen receptor target gene of which the androgen receptor–mediated promoter activity is affected by FOXO1A, in the androgen-responsive LNCaP cells transfected with FOXO1A and treated with androgen (Fig. 5*A*). Both RNA and protein expressions were analyzed. Even without R1881 treatment, PSA expression was reduced by ectopic expression of FOXO1A at both the RNA and protein levels (Fig. 5*B* and *C*). R1881 treatment significantly increased PSA expression and the inhibitory effect of FOXO1A on PSA expression was also increased from 60% to 72% at RNA level and from 38% to 52% at protein level (Fig. 5*B* and *C*).

We also examined the effect of FOXO1A on androgen receptor–mediated cell proliferation. Androgen receptor signaling is indispensable and biphasic for the proliferation of LNCaP cells; thus, we first determined the optimal concentration of androgen for cell proliferation. We treated LNCaP cells with different concentrations of R1881 for 120 hours and found that 0.05 nmol/L R1881 was more potent in stimulating cell proliferation (Fig. 6*A, control curve*). With a treatment of 0.05 nmol/L R1881, we knocked down FOXO1A expression by RNA interference and conducted a colony formation assay. Real-time PCR analysis indicated that FOXO1A small interfering RNA knocked out 86% of FOXO1A expression (Fig. 6*B*). As shown in Fig. 6*C*, FOXO1A knockdown promoted cell growth by 29.7% in the presence of R1881 but only by 17.9% when R1881 was absent. The latter rate was significantly lower ($P < 0.05$). On the other hand, without FOXO1A knockdown, 0.05 nmol/L R1881 treatment enhanced cell growth by 54.2%; but when FOXO1A was knocked down, R1881 enhanced cell growth by 71.4%, a statistically significant difference ($P < 0.05$).

To further test if *FOXO1A* knockdown sensitizes LNCaP cells to androgen-mediated growth promotion, we examined different concentrations of androgen for their effect on cell growth when FOXO1A was knocked down. As shown in Fig. 6*A*, LNCaP cells became more sensitive to androgen growth stimulus when FOXO1A was knocked down. When FOXO1A was knocked down, the optimal R1881 concentration for stimulating LNCaP cell growth decreased from 0.05 to 0.025 nmol/L (Fig. 6*A*).

We then increased FOXO1A expression by plasmid transfection in LNCaP cells. When cells were treated with 0.05 nmol/L R1881, ectopic FOXO1A expression inhibited cell growth by 34.6%, which is significantly more than the 15.4% inhibition when R1881 was absent (Fig. 6*D*; $P < 0.05$). On the other hand, without

ectopic FOXO1A expression, 0.05 nmol/L R1881 increased cell growth by 100%, which is significantly more than the 54.5% increase when FOXO1A was overexpressed in LNCaP cells (Fig. 6*D*; $P < 0.05$).

Discussion

Chromosomal deletion is a hallmark for tumor suppressor genes because it can inactivate a gene by revealing recessive mutations and/or reducing gene dosage. Hemizygous deletion, in which one of the two gene copies is lost, can impair gene function through haploinsufficiency, as shown in several knockout studies in mice. Deletion at 13q14 is common in many different types of human tumors but the underlying tumor suppressor gene has been unknown. Our studies described above present the *FOXO1A* transcription factor as a strong candidate for the 13q14 tumor suppressor gene.

Our deletion mapping experiments narrowed the smallest region of deletion at 13q14 to 919 kb, and *FOXO1A* is the only known gene that is in, or overlaps with, the smallest region of deletion (Fig. 1). Consistent with previous studies (1), the smallest region of deletion is centromeric to *RB1* but telomeric to *BRCA2*, which are known tumor suppressor genes that have been excluded from being the major tumor suppressor gene at 13q14. In addition, analysis of *FOXO1A* and additional markers outside the smallest region of deletion but between *BRCA2* and *RB1* showed that deletion at *FOXO1A* is significantly more frequent than deletions at other markers in 32 xenografts and cell lines from prostate cancer (34% versus ≤12%). Furthermore, deletion at *FOXO1A* was detected in 31% of 72 microdissected primary prostate cancers. These findings indicate that hemizygous deletion at *FOXO1A* is frequent in human prostate cancer and *FOXO1A* centers the deletion at 13q14. *FOXO1A* has been shown to be haploinsufficient in adult mice because deleting one of the two *FOXO1A* alleles induced phenotypic alteration (23, 24). Therefore, frequent hemizygous deletion at *FOXO1A* in prostate cancer could inactivate *FOXO1A* function by 50%. These studies suggest that *FOXO1A* is a tumor suppressor gene in prostate cancer. We are currently testing if specific knockout of *FOXO1A* in the prostate can induce prostate cancer in mice.

FOXO1A had hemizygous deletions in LuCaP 23.1 and LuCaP 23.12 xenografts and homozygous deletion in the LuCaP 23.8 xenograft. The three xenografts were derived from the same patient but from different metastases, with LuCaP 23.1 and LuCaP 23.8 from lymph node metastases and LuCaP 23.12 from a liver metastasis (25). Although previous studies showed more frequent 13q14 deletion in recurrent and metastatic tumors than in primary tumors (26), we did not detect a significant correlation between *FOXO1A* deletion and tumor grade or tumor stage in the clinical samples analyzed.

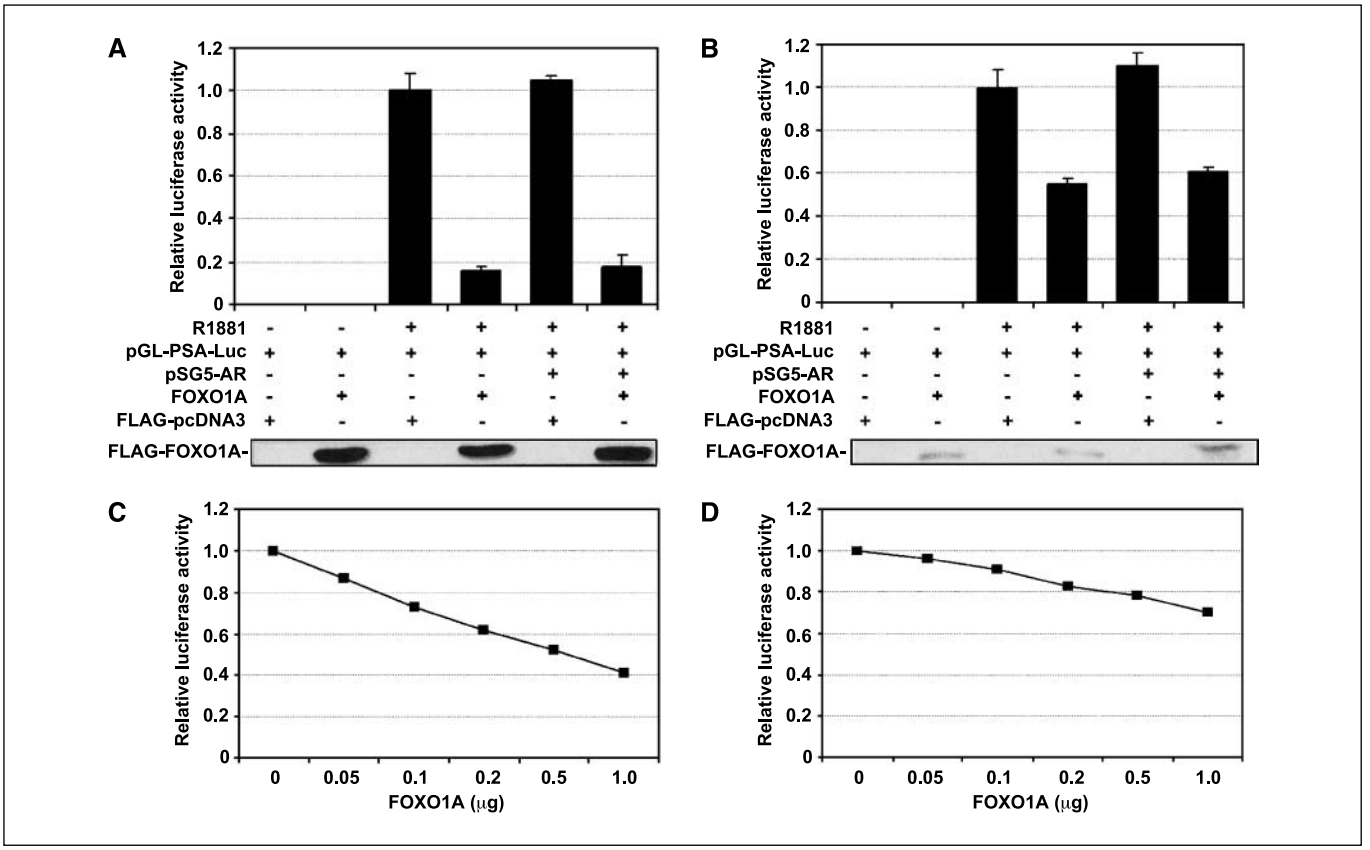

Figure 4. Inhibition of androgen receptor–mediated transcriptional promoter activity by FOXO1A, as detected by promoter-reporter luciferase assay, in androgen receptor–positive 22Rv1 (*A* and *C*) and LNCaP (*B* and *D*) cells. *A* and *B,* ectopic expression of FOXO1A significantly inhibited the promoter activity of *PSA* gene, a well-established androgen receptor target gene. Status of androgen treatment (R1881) and plasmid transfection, as well as verification of cytomegalovirus promoter-transduced FOXO1A expression by Western blotting, is indicated at the bottom. *C* and *D,* dose-dependent inhibition of androgen receptor–mediated PSA promoter activity by FOXO1A. The treatments included R1881, pSG5-AR transfection, and different amounts of FOXO1A expression plasmid (as indicated).

FOXO1A also seemed to be down-regulated in prostate cancer, as shown by RT-PCR, real-time PCR, and Northern blot analysis (Fig. 2). *FOXO1A* down-regulation is common in prostate cancer samples both with and without a hemizygous deletion, suggesting that, in addition to hemizygous deletion, other mechanisms are also involved in *FOXO1A* expression loss in prostate cancer. Promoter methylation, however, is less likely to be involved because it was rarely detected in cancer samples, and a demethylating treatment did not increase *FOXO1A* expression in cell lines. Although *FOXO1A* rarely had inactivating mutations in the cancer samples tested, it functionally inhibited not only cell growth (Fig. 3) but also the stimulatory role of androgen in cell proliferation (Figs. 4-6). Previous studies also showed that FOXO1A inhibits cell proliferation and induces apoptosis (10, 22), and activation of androgen receptor by androgen inhibits FOXO1A in prostatic cells (14). These findings further support the notion that *FOXO1A* is a tumor suppressor gene that is inactivated mainly through transcriptional down-regulation.

FOXO1A was originally implicated in human solid tumor by its fusion with *PAX3* and *PAX7* genes through translocation in alveolar rhabdomyosarcoma (19). *FOXO1A* is also at the breakpoint in some chromosomal translocations in leukemia (27). In most of the translocations, the breakpoint occurred in intron 1 of *FOXO1A*. In our studies, one boundary of the homozygous deletion in LuCaP 23.8 xenograft is also located in intron 1 of *FOXO1A*. Although the mechanism for the breakage formation is unknown, it is possible that a specific sequence in intron 1 of *FOXO1A* makes *FOXO1A* susceptible to breakage, which leads to both translocation in sarcoma and deletion in solid tumors. Although the fused genes are often amplified and act as oncogenes (28, 29), *FOXO1A* itself has been suggested as a tumor suppressor gene (30). Many studies have shown that FOXO1A causes cell death and cell cycle arrest in different types of cells, likely by inducing expression of the CDKN1B (p27) cyclin-dependent kinase inhibitor (8, 31). FOXO1A also plays a positive role in cell differentiation through interaction with other signaling pathways (8, 24). FOXO1A protein is tightly regulated by the ubiquitin proteasome pathway, at least by SKP2, an oncoprotein that also mediates the ubiquitination and degradation of several negative regulators of the cell cycle (32). Our findings are consistent with other published studies supporting the notion of FOXO1A as a negative regulator of carcinogenesis.

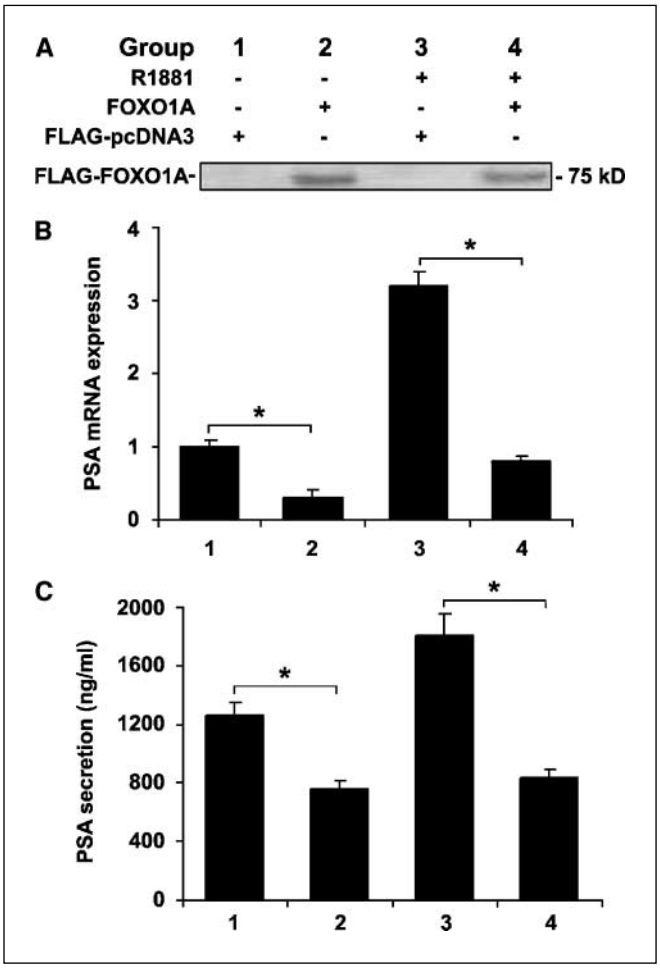

Figure 5. Inhibition of endogenous PSA expression by FOXO1A in LNCaP cells, as detected by real-time PCR assay (*B*) and ELISA measurement of secreted PSA protein (*C*). *A,* R1881 treatment, FOXO1A transfection, and verification of transduced FOXO1A expression by Western blotting for the four groups of cells analyzed in (*B* and *C*). *B*, the normalization was based on cells from group 1. *, $P < 0.05$.

Using different approaches, we found that FOXO1A inhibited androgen and androgen receptor function in the transcriptional regulation of gene expression as well as in androgen-stimulated cell proliferation (Figs. 4-6). On the other hand, several studies have shown that androgen and androgen receptor inhibit FOXO1A-mediated apoptosis and growth inhibition in prostatic cells (14, 22). Whereas androgen receptor seems to activate PI3K/AKT signaling by direct interaction (21), which can lead to inactivation of FOXO1A, androgen receptor also directly interacts with FOXO1A to inhibit FOXO1A function (14). It is likely that FOXO1A and androgen receptor signaling antagonize each other in the control of cell proliferation and death, and FOXO1A inactivation leads to enhanced androgen receptor activities in the development and progression of prostate cancer.

Protein kinase B (also named AKT) lies downstream of PI3K and mediates many of the intracellular actions of insulin and other growth factors. Several studies have shown that AKT phosphorylates FOXO1A, which inactivates FOXO1A transcriptional activity by inducing nuclear exclusion (33–35). The PI3K/AKT pathway has been well implicated in carcinogenesis as an oncogenic pathway (36). For example, constitutive activation of PI3K/AKT induces cancer and the PTEN tumor suppressor plays an inhibitory role in this process (37). Therefore, inactivating this pathway has become an attractive strategy in cancer therapy. Small molecules designed to specifically target PI3K/AKT have been developed and shown to induce cell cycle arrest or apoptosis in human cancer cells *in vitro* and *in vivo* (38). Moreover, inhibition of PI3K/AKT sensitizes cells to the antitumor effect of other therapeutic agents, likely through alteration of FOXO1A-regulated gene expression (39–41). Inhibition of PI3K/AKT also inhibits FOXO1A phosphorylation and nuclear exclusion and restores FOXO1A activity (42). When FOXO1A function is impaired by deletion, however, the effect of PI3K/AKT inhibition on restoring FOXO1A function could also be impaired. It is thus possible that the same PI3K inhibitor would be less effective in treating cancers that have less FOXO1A expression, which implicates FOXO1A in cancer therapy targeting PI3K/AKT.

In addition, molecular alterations for some components of the PI3K/AKT pathway, including activation of PI3K and/or AKT and inactivation of PTEN, have been studied as potential biomarkers for the detection and prediction of human cancers. Our results suggest that FOXO1A is another such molecule of which the

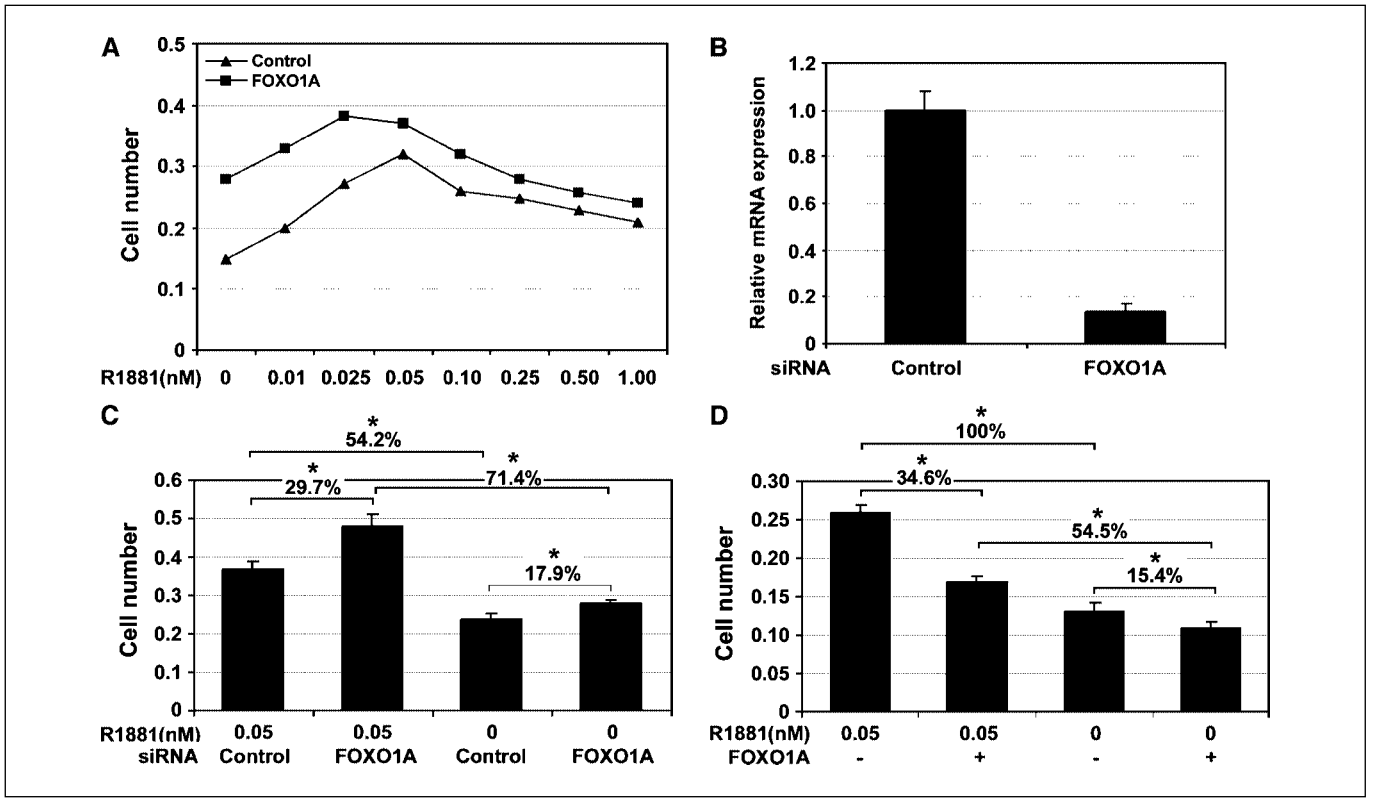

Figure 6. Inhibition of androgen receptor–mediated cell proliferation by FOXO1A in LNCaP cells, as measured by colony formation assay. *A,* FOXO1A knockdown promoted cell proliferation (*y axis*) mediated by different concentrations of androgen (R1881; *x axis*). *B,* knockdown of FOXO1A expression by small interfering RNA transfection, as verified by real-time PCR assay. *C,* promotion of cell proliferation by FOXO1A knockdown was more effective in the presence than in the absence of R1881. *D,* inhibition of cell proliferation by transduced FOXO1A expression was more effective in cells treated with R1881 than in cells without R1881. Cell numbers are indicated by absorbances. *, $P < 0.05$.

expression loss could predict cancers with an altered PI3K/AKT pathway. We are currently testing if loss of FOXO1A expression can predict the aggressiveness of prostate cancer by analyzing FOXO1A protein expression in normal and cancer cells.

In summary, we found that in prostate cancer, *FOXO1A* centered a common locus of deletion at 13q14, was down-regulated in some cancers, suppressed cell proliferation, and inhibited androgen and androgen receptor functions in gene regulation and cell proliferation. These observations suggest that *FOXO1A* is the 13q14 tumor suppressor gene of which the inactivation likely impairs the therapeutic effect of PI3K/AKT inhibitors.

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FOXO1A Is a Candidate for the 13q14 Tumor Suppressor Gene Inhibiting Androgen Receptor Signaling in Prostate Cancer

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