A Transcription-Independent Function of FOXO1 in Inhibition of Androgen-Independent Activation of the Androgen Receptor in Prostate Cancer Cells

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Abstract

Increasing evidence suggests that aberrant activation of the androgen receptor (AR) plays a pivotal role in the development and progression of androgen depletion–independent prostate cancer (PCa) after androgen deprivation therapy. Here, we show that loss of the PTEN tumor suppressor gene is associated with hyperactivation of the AR in human PCa cell lines. This effect is mediated primarily by its downstream effector FOXO1. In addition to the inhibition of androgenic activation of the AR, forced expression of FOXO1 in PTEN-negative PCa cells also inhibits androgen-independent activation of the AR in a manner independent of FOXO1 transcriptional function. In contrast, silencing of FOXO1 in PTEN-positive cells not only increases the basal activity of the AR in the absence of androgens, it also markedly sensitizes the AR activation by low levels of androgens or nonandrogenic factors such as interleukin-6. FOXO1-mediated inhibition of the AR is partially attenuated by the histone deacetylase (HDAC) inhibitor trichostatin A. Accordingly, FOXO1 interacts with HDAC3 as shown by coimmunoprecipitation assays, and cotransfection of cells with FOXO1 and HDAC3, but not HDAC1 and HDAC2, results in a greater inhibition of AR activity than in cells transfected with FOXO1 or HDAC3 individually. Together, our findings define a novel corepressor function of FOXO1 in inhibition of androgen-independent activation of the AR. [Cancer Res 2008;68(24):10290–9]

Introduction

Prostate cancer (PCa) cells depend on androgens for proliferation and survival (1–4). Since the seminal work of Huggins and Hodges (1941), androgen deprivation therapy (ADT) has been the standard treatment for advanced PCa. However, this treatment has significant shortcomings as the majority of PCas invariably evolve into androgen depletion-independent (ADI) disease (5), from which most patients eventually die. In virtually all cases, ADI progression is accompanied by a recurrence of prostate-specific antigen (PSA), a well-studied androgen receptor (AR) target gene. Additionally, many of these tumors are sensitive to further hormonal manipulations such as the discontinuation of steroidal and nonsteroidal hormones, or antiandrogens plus enzymatic inhibitors of the adrenal androgen synthetic pathway. These clinical observations indicate that the AR remains active even under androgen-deprivation conditions. Consistently, studies on patient specimens show that the AR protein is expressed in nearly all cancers of the prostate both before and after hormonal therapy (6). Moreover, after ADT, dihydrotestosterone, a major form of androgens, remains in the prostate tissue at a certain level that is sufficient to activate the AR (7). Previous studies also suggest that the AR can be activated by nonandrogenic factors, such as growth factors and cytokines, in the absence or presence of low levels of androgens (8). Indeed, androgen-independent activation of the AR seems to be critical in PCa proliferation and progression under androgen deprivation conditions (8–11). Finally, both in vitro and in vivo studies indicate that the function of the AR is required for the proliferation of ADI-PCa cells (12, 13). Together, these findings accentuate the importance of the AR in ADI progression of PCa. However, the molecular mechanisms underlying activation of the AR by low levels of androgens or nonandrogenic factors are largely unknown.

Phosphatase and tension homologue deleted on chromosome 10 (PTEN) is one of the most frequently mutated/deleted tumor suppressor genes in human cancers. Although PTEN gene deletions and/or mutations are found in ~30% of localized prostate tumors, it is mutated/deleted in over 60% of metastatic PCas, indicating its role in PCa progression (14). Indeed, complete or partial loss of PTEN protein in PCas is associated with ADI progression (15). The notion that PTEN is a bona fide tumor suppressor in the prostate is further supported by the findings that inactivation of PTEN in mice with various genetic backgrounds inevitably leads to the development of prostatic intraepithelial neoplasia and PCa (16, 17). A salient feature of murine PTEN-mutated prostate tumors recapitulating human PCa is that they are initially sensitive to castration but eventually become castration resistant (17, 18). Importantly, similar to human ADI PCa, growth of PTEN-null murine PCa cells is ADI but AR dependent (18, 19). These observations suggest that loss of PTEN may prompt androgen-independent activation of the AR in vivo.

The tumor suppressor function of PTEN is primarily mediated by its antagonistic effect on the phosphatidylinositol-3-OH kinase/Akt pathway (20). Indeed, loss of PTEN leads to the activation of Akt. Previous studies showed that the activity of AR is regulated by the PTEN/Akt signaling axis (21–25). Although some studies suggest that Akt inhibits AR activity (22), the majority of reports show that Akt enhances AR function (21, 24, 25). Earlier studies propose that Akt phosphorylates AR at serine 213 (S213) and serine 791 (S791) in vitro (21, 22). However, other groups failed to detect AR phosphorylation at these residues in cells by mass spectrometry (26, 27). Importantly, forced expression of PTEN inhibits the transcriptional activity of both wild-type and S213A/S791A-mutated AR in PC-3 cells (23), suggesting that the inhibition of AR activity by PTEN is independent of Akt-mediated phosphorylation of the AR. In accordance with this observation, Akt has been shown to synergize with the AR in promoting PCa growth in mice; however, this effect was shown to be independent of Akt.
phosphorylation of the AR at these residues (25). Thus, previous studies in human and mouse PCas suggest that PTEN loss and/or Akt activation are important for AR activation and PCa progression. However, the precise mechanism through which the PTEN/Akt signaling axis regulates AR function in PCa cells remains unclear.

Here, we show that loss of PTEN is associated with hyperactivation of the AR in various PCa cell lines. In contrast, restoration of PTEN in PTEN-null cells blocks activation of the AR by low doses of androgens. We further show that FOXO1 is a critical downstream effector of PTEN-mediated inhibition of AR activation and that its function is independent of its transcriptional activity. We also provide evidence that FOXO1 not only inhibits androgenic activation of the AR, but it also abolishes androgen-independent activation of the AR. Consistently, forced expression of FOXO1 inhibits the growth of ADI-PCa cells.

Materials and Methods

Cell lines and cell culture. LNCaP, DU145, and PC-3 cells were purchased from American Type Culture Collection. These cell lines were cultured in RPMI 1640 (Invitrogen) containing 10% fetal bovine serum (FBS; Hyclone), 100 μg/mL streptomycin, 100 units/mL penicillin, and 0.25 μg/mL amphotericin B (Invitrogen). 22Rv1 cells were obtained from Dr. C. Y. Young (Mayo Clinic, Rochester, MN) and maintained in RPMI 1640 plus 10% FBS. The LAPC-4 cell line was kindly provided by Dr. C. L. Sawyers (Memorial Sloan-Kettering Cancer Center, New York, NY) and cultured in IMEM with 10% FBS. The C4-2 cell line was purchased from UroCorporation and grown in RPMI 1640 supplemented with 10% FBS.

Expression vectors for the wild-type and truncated mutants of FLAG-tagged FOXO1 were described previously (28). The mutants of FLAG-FOXO1-HR-537, FLAG-FOXO1-HR-537-A3, and FLAG-HR-537-A3-NLSm (conversion of three arginine residues (RRR, 251-253) in the nuclear localization signal [NLS] into SAS) were generated by means of PCR-based mutagenesis (Stratagene). V5-FOXO1-C (282-655), V5-AR-N (1-667), V5-AR-C (554-919) were cloned in the pCDNA3.1/D/V5-His-TOPO vector (Invitrogen). All the mutants were verified by sequencing. Expression vectors for the enhanced green fluorescent protein were purchased from BD Biosciences (Clontech). The renilla luciferase reporter vector and pGL3-basic and pGL3-promoter firefly luciferase vectors were purchased from Promega. The PSA promoter luciferase reporter (PSA-Luc) containing an ~5.8-kb genomic fragment from the promoter of the PSA gene, and the luciferase reporter construct containing three copies of androgen responsive element (ARE) derived from the PSA gene (3× ARE-Luc) were obtained from Dr. C.Y. Young (Mayo Clinic, Rochester, MN). Expression vectors for AR shRNA and AR shRNA/ARsr were kindly provided by Dr. S.M. Dehm (University of Minnesota, Minneapolis, MN; ref. 29). Expression vectors for FLAG-HDAC1, FLAG-HDAC2, and FLAG-HDAC3 were kindly provided by Dr. J.J. Westendorf (Mayo Clinic, Rochester, MN). Pools of siRNAs for the human FOXO1 and PTEN genes and nonspecific siRNAs were purchased from Dharmacon. Methyltrienolone (R1881) was purchased from DuPont. Recombinant human IL-6 was purchased from R&D Systems.

Cell transfection and luciferase reporter assay. Transient transfection of PCa cells was performed by electroporation as described (28). For siRNA transfection, cells were transfected with 200 pmol of siGenome SMART pool siRNAs specific for FOXO1 or PTEN genes (Dharmacon), control SMART pool siRNA, or mock transfected. For luciferase reporter assays, cells were harvested 36 to 48 h after transfection, and firefly and renilla luciferase
activities in cell lysates were measured using a dual luciferase kit (Promega). Renilla luciferase activities of cells were used as internal controls.

**Immunoprecipitation, Western blot, and antibodies.** Protein immunoprecipitations (IP) were carried out using an IP kit (Roche Applied Science) as described (28), and Western blot analysis was performed as described (28). The antibodies used were as follows: anti-FOXO1 and anti-phospho-Akt (Ser473, Akt-p; Cell Signaling Technology), anti-PTEN monoclonal (6H2.1; Cascade BioScience), anti-FLAG (M2; Sigma-Aldrich), anti-AR polyclonal (N-20 and C-19), anti-p27 KIP1 (F8) and anti-Erk2 (Santa Cruz Biotechnology), anti-PSA mouse monoclonal (Dako), and anti-V5 (Invitrogen).

**Immunofluorescence chemistry and confocal microscopy.** LNCaP cells were transfected by electroporation with plasmids of FLAG-tagged FOXO1-HR-537 or its mutants and plated onto coverslips in 6-well plates. At 48 h after transfection, cells were subjected to immunofluorescence chemistry (IFC) as described (28). Cellular localization of endogenous AR and FLAG-tagged FOXO1 proteins were detected by a rabbit anti-AR polyclonal antibody and a mouse anti-FLAG monoclonal antibody, respectively. Cells were counterstained with Vectashield (Vector Laboratories) containing 4',6-diamidino-2-phenylindole and analyzed with an Olympus Fluoview 1000 laser-scanning microscope.

**Cell viability assays.** C4-2 cells were seeded at a density of 2,000 cells per well in culture medium in 96-well plates. At indicated time points, the number of viable cells was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reduction (CellTiter 96 Aqueous One Solution kit; Promega), which was measured at 490 nm.

**Statistics.** Data are expressed as mean ± SD from at least three experiments, and statistical analyses were performed by Student's t test. P values of <0.05 were considered statistically significant.

**Results**

**Loss of PTEN is associated with hyperactivation of the AR in human PCa cell lines.** To gain insight into the molecular mechanism underlying the regulation of AR activation by the PTEN/Akt pathway, we compared expression of the AR protein and its downstream target gene PSA in various human PCa cell lines that either express or lack the PTEN protein (Fig. 1 A–D). As shown in Fig. 1B, expression of endogenous PSA protein was highly induced by the synthetic androgen methyltrienolone (R1881) in LNCaP cells, which do not express endogenous PTEN protein (Fig. 1A). In contrast, androgen-stimulated expression of PSA was very limited in 22Rv1 and LAPC-4 PCa cell lines, both of which express the PTEN protein (Fig. 1A, C, and D). Next, we assessed
androgen-induced transactivation of the AR in these cell lines using PSA promoter–based luciferase reporter assays. Androgen treatment of LNCaP cells resulted in a maximal 30-fold increase in PSA reporter gene activity (Fig. 2A). In two PTEN-positive 22Rv1 and LAPC-4 cell lines, however, the androgen-induced increase in PSA promoter activity was <5-fold (Fig. 2B). Importantly, these results were not likely due to the different levels of the AR protein in these cells as AR levels in LNCaP and 22Rv1 cells are comparable (Fig. 1A). The low AR activity in LAPC-4 cells could be due to either low levels of the AR protein (Fig. 1A) or a high ratio of the FOXO1 protein to the AR protein relative to other cell lines (see the results below; Fig. 1A). Because endogenous AR proteins in LNCaP and 22Rv1 are mutated, we next examined the effect of PTEN on activation of ectopically expressed wild-type AR in two AR-null PCa cell lines, PC-3 and DU145. PC-3 cells are PTEN-negative, whereas DU145 cells express a functional PTEN (Fig. 1A). Androgen treatment induced a maximal 10-fold increase in AR transcriptional activity in the PTEN-negative PC-3 cells (Fig. 2C), whereas a maximal 3-fold induction in AR transcriptional activity was detected in the PTEN-positive DU145 cells (Fig. 2D). The levels of ectopically expressed AR proteins in these two cell lines were comparable (data not shown). Thus, we conclude that loss of endogenous PTEN is highly correlated with AR hyperactivation in human PCA cell lines.

Role of FOXO1 in PTEN-mediated inhibition of AR activation. Next, we sought to determine a causal role of PTEN loss in the androgen-induced hyperactivation of AR in human PCA cells. To this end, endogenous PTEN was knocked down by a pool of PTEN-specific siRNAs in 22Rv1 cells (Fig. 3A, left). Silencing of PTEN significantly increased the basal activity of the AR in cells without androgen stimulation (Fig. 3A, right). Importantly, AR activity stimulated by 0.01 nmol/L R1881 in PTEN knockeddown cells was much higher than that induced by 1 nmol/L R1881 in cells with functional PTEN (Fig. 3A, right). In contrast, ectopic

Figure 3. FOXO1 is a critical mediator of PTEN inhibition of androgenic activation of the AR. A, silencing of PTEN in 22Rv1 cells increases the basal or androgen-stimulated activity of the AR. 22Rv1 cells were transfected with plasmids for PSA-Luc firefly and renilla luciferase reporter genes and siRNAs as indicated. At 48 h after transfection, cells were mock treated or treated with different concentrations of R1881 for 24 h. Cells were harvested and subjected to Western blot analysis of expression of PTEN and AR proteins (left) and measurement of luciferase activities (right). B, silencing of FOXO1 diminishes PTEN-mediated inhibition of AR activity. LNCaP cells were cotransfected with plasmids for PSA-Luc firefly and renilla luciferase reporter genes, PTEN, and siRNAs as indicated. At 48 h after transfection, cells were treated with vehicle (ethanol) or different doses of R1881 for 24 h. Cells were harvested and subjected to Western blot analysis for expression of FOXO1 and AR proteins in PTEN-transfected cells (left) and measurement of luciferase activities (right). Similar results were obtained from three independent experiments. C, silencing of FOXO1 in 22Rv1 cells increases the basal or androgen-stimulated activation of the AR. 22Rv1 cells were transfected with siRNAs as indicated for 48 h after transfection, and then cells were mock treated or treated with different concentrations of R1881 for 24 h. Cells were harvested and subjected to Western blot analysis for expression of FOXO1 and AR (left) and luciferase measurement (right). Points, mean among three individual experiments; bars, SD.
expression of PTEN in PTEN-null LNCaP cells decreased the sensitivity of the AR to androgens (Fig. 3B, right). Previous findings from both in vitro and in vivo studies suggest that PTEN inhibition of the AR is independent of Akt-mediated phosphorylation of the AR (23, 25). To define the mechanism underlying the inhibitory effect of PTEN on the sensitivity of AR transactivation, we focused on the role of FOXO1 in the regulation of the AR by PTEN because it is known that FOXO1 is both a critical downstream effector of PTEN (30) and an inhibitor of AR in PCa cells (31, 32). siRNA-mediated silencing of FOXO1 (Fig. 3B, left) largely abolished PTEN-induced inhibition of AR activity in LNCaP cells (Fig. 3B, right). Like knocking down of PTEN, silencing of endogenous FOXO1 in 22Rv1 cells also markedly increased the sensitivity of the AR to low levels of androgens (Fig. 3C). Combined, these data suggest for the first time that FOXO1 is a key mediator of PTEN inhibition of the AR in PCa cells.

FOXO1 represses AR transactivation via a corepressor function. It has been reported recently that forced expression of FOXO1 inhibits both the promoter activity and mRNA expression of PSA, a well-studied AR target gene (31, 32). Because expression of PSA can also be regulated by other transcription factors such as nuclear factor-κB, FOXA1, and FOXA2, we sought to determine whether the inhibitory effect of FOXO1 on PSA expression is solely mediated through repression of the AR. Similar to the previous report (31), forced expression of FOXO1 inhibits PSA promoter activity in a dose-dependent manner (Fig. 4A, left). Importantly, expression of FOXO1 also decreased the activity of a composite AR reporter gene (3xARE-Luc) that contains only three copies of the ARE derived from the promoter of the PSA gene (Fig. 4A, right). This finding strongly suggests that FOXO1 inhibition of PSA transcription is mediated through the repression of AR. FOXO1 is known to primarily function as a transcription factor. Next, we sought to determine whether the transcriptional function of FOXO1 is required for its inhibition of the AR. To this end, we assessed the inhibitory effect of transcription-deficient mutants of FOXO1 on AR transactivation. It has been shown previously that mutation of the histidine 215 residue to arginine (HR) in the forkhead domain (FKHL, also called DNA binding domain) of FOXO1 (see Fig. 4B) abolishes binding of FOXO1 to the forkhead responsive element in its target genes (33). Moreover, there is a transactivation domain within the COOH terminus (∼100 amino acids) of the FOXO1 protein. Deletion of this domain (FOXO1-357) abrogates the transcriptional activity of FOXO1 (34). In contrast to wild-type FOXO1, forced expression of the FOXO1-HR-357 double mutant (Fig. 4B) failed to induce the expression of p27KIP1 (Fig. 4C, left), a known FOXO1 target gene (35), confirming that FOXO1-HR-357 is a transcription-deficient mutant. Importantly, expression of this mutant decreased the expression of endogenous PSA protein (Fig. 4C, left) and the activity of the 3xARE-Luc reporter gene (Fig. 4C, right) in a degree similar to wild-type FOXO1. Ectopic expression of FOXO1-01-HR-357 also inhibited the luciferase reporter activity of the promoter of hK2, another AR-regulated gene (data not shown). These findings suggest that FOXO1 inhibits AR activation via a novel corepressor function that is independent of its transcriptional activity.

Role of histone deacetylases in FOXO1 inhibition of AR activation. Some corepressors possess intrinsic histone deacetylasles (HDAC) activity and some repress gene expression through recruitment of HDAC proteins (36). Therefore, we sought to determine whether HDAC proteins are involved in FOXO1-mediated inhibition of AR activation. LNCaP cells were transfected with the PSA luciferase reporter gene and either FOXO1 or empty expression vectors, and then they were treated with the HDAC inhibitor trichostatin A (TSA) or vehicle. TSA treatment markedly increased AR activity in LNCaP cells as shown by luciferase assays (Fig. 4D, left). In the absence of TSA treatment, ectopic expression of FOXO1 resulted in ∼60% inhibition of AR activity and the inhibition is statistically significant (P = 0.026). In TSA-treated cells, however, expression of FOXO1 resulted in only ∼15% inhibition of AR activity, which is not statistically significant (P = 0.275; Fig. 4D, left). Thus, FOXO1-mediated inhibition of AR can be partially attenuated by TSA, suggesting that this function of FOXO1 is likely mediated by both HDAC-dependent and HDAC-independent mechanisms. To further explore the role of HDAC proteins in FOXO1-mediated inhibition of AR activation, we transfected LNCaP cells with expression vectors for FOXO1 and/or class I HDAC enzymes HDAC1, HDAC2, or HDAC3. Transfection of cells with HDAC1, HDAC2, or HDAC3 alone resulted in moderate, but not statistically significant, inhibition of AR activity (Fig. 4D, middle). Importantly, cotransfection of FOXO1 with HDAC3 significantly decreased AR activity relative to that of cells transfected with FOXO1 or HDAC3 alone (Fig. 4D, middle). No such inhibitory effect on AR activity was observed in cells cotransfected with FOXO1 and HDAC1 or HDAC2 (Fig. 4D, middle). Moreover, ectopically expressed FOXO1 formed a protein complex with HDAC3 in LNCaP cells (Fig. 4D, right). Thus, these findings suggest that HDAC3 may contribute to FOXO1-mediated inhibition of AR activity in PCa cells.

FOXO1 inhibition of the AR requires the nuclear localization of FOXO1. AR proteins are primarily localized in the nuclei of PCa cells after androgen stimulation (37). We therefore sought to determine whether FOXO1 inhibits AR activation in the nucleus. As shown in Fig. 5A (second row), ectopically expressed transcription-deficient FOXO1 proteins (FLAG-FOXO1-HR-537) were localized either in the cytoplasm (∼58% of transfected cells), the nucleus (∼25%), or both (∼17%). Similar to the previous report (30), when three Akt phosphorylation sites were mutated to alanine, the mutated FOXO1 proteins (FOXO1-HR-537-A3) were mainly localized in the nucleus (∼85%; see Fig. 5A, third row). The inhibitory effect of FOXO1-HR-537-A3 on AR activity was considerably higher than that mediated by FOXO1-HR-537 in which the Akt phosphorylation sites are not mutated (Fig. 5B). When three arginine residues within the NLS were replaced with neutral residues (serine, alanine, and serine) in FOXO1-HR-537-A3, the function of NLS was largely impaired. As a result, there were only <5% of transfected cells exhibiting exclusive nuclear localization of this mutant (FOXO1-HR-537-A3-NLSm; Fig. 5A, bottom row), which is consistent with a previous report (38). Importantly, no significant inhibition of the AR by FOXO1-HR-537-A3-NLSm was observed (Fig. 5B). Together, these data suggest that nuclear localization of FOXO1 is required for its inhibition of the AR.

FOXO1 inhibits androgen-independent activation of the AR in PCa cells. Increasing evidence suggests that AR activity is required for the growth and survival of ADI PCa (12, 13). Given that the AR proteins are primarily located in the nuclei of ADI-PCa cells in the absence of androgens (37) and that FOXO1 inhibits AR activity in the nucleus (Fig. 5), we were interested in determining whether FOXO1 could also inhibit androgen-independent activation of the AR. Similar to previous reports (9, 10), treatment of LNCaP cells with interleukin-6 (IL-6) increased the transcriptional activity of the AR (Fig. 6A, top). However, this effect was inhibited by ectopic expression of FOXO1-HR-537-A3, the transcription-deficient...
Figure 4. FOXO1 inhibits AR activation independent of its transcriptional activity. A, LNCaP cells were transfected with PSA-Luc and renilla luciferase reporter genes and plasmids for FOXO1 at different doses (left). At 48 h after transfection, cells were harvested and subjected to measurement of luciferase activity. Columns, mean among three individual experiments; bars, SD. Right, LNCaP cells were transfected with 3xARE-Luc and renilla luciferase reporter genes and plasmids for FOXO1 at different doses. Luciferase measurement and data analysis were performed as described above. B, schematic diagram of expression constructs for FLAG-tagged wild-type (FLAG-FOXO1) and the transcription-deficient mutant of FOXO1 (FLAG-FOXO1-HR-537). C, LNCaP cells were transfected with empty vector, wild-type FOXO1, and the transcription-deficient mutant of FOXO1 (left). At 48 h after transfection, cells were harvested and subjected to Western blot analysis for expression of proteins as indicated. The level of PSA was quantified and normalized with the amount of PSA proteins in mock transfected cells. Right, LNCaP cells were transfected with 3xARE-Luc and renilla luciferase reporter genes and plasmids as indicated. At 48 h after transfection, luciferase measurement and data analysis were performed as described in A. Middle, LNCaP cells were transfected with luciferase reporter genes and plasmids as indicated. At 36 h after transfection, luciferase measurement and data analysis were performed as described in A. *, P < 0.05 comparing PSA-Luc activity in cells cotransfected with FOXO1 and HDAC3 with that in cells transfected with FOXO1 or HDAC3 alone. Right, FOXO1 interacts with HDAC3 as shown by co-IP in LNCaP cells transfected with FLAG-HDAC3 and V5-FOXO1. M2, anti-FLAG antibody. a.a., amino acid.
mutant of FOXO1 (Fig. 6A, top). The IL-6–induced increase in AR activity was also inhibited by forced expression of FOXO1-HR-537-A3 in C4-2 cells (Fig. 6A, bottom), an ADI derivative of LNCaP cells (29). In contrast with the results obtained in PTEN-negative LNCaP and C4-2 cells, no significant increase in AR activity, as indicated by PSA luciferase reporter gene activity, was observed in PTEN-positive 22Rv1 cells after IL-6 treatment (Fig. 6B). However, AR activity was significantly increased by high doses of IL-6 in 22Rv1 cells transfected with a pool of FOXO1-specific siRNAs (Fig. 6B). Thus, FOXO1 can inhibit IL-6–induced AR activation in both androgen-dependent and androgen-independent PCa cells.

Mounting evidence implies that the function of the AR NH2-terminal domain (NTD; also called activation function-1, AF1) plays a pivotal role in ADI activation of the AR and progression of PCa (29). The AR is active in C4-2 cells under androgen deprivation conditions (e.g., cultured in CSS medium; Fig. 6A, bottom), and this AR activity is believed to be mediated by the AF1 function of the AR NTD (29). Importantly, this ligand-independent AR activity was largely inhibited by ectopic expression of FOXO1 in a manner independent of its transcriptional activity (Fig. 6A, bottom). To directly test whether expression of FOXO1 inhibits the AR NTD function, AR-negative PC-3 cells were cotransfected with expression vectors for an AR NH2-terminal mutant (AR-N; Fig. 6C, top) and FOXO1-HR-537-A3. As expected, in the absence of androgen treatment, the transcriptional activity of wild-type AR was relatively low in PC-3 cells, whereas the AR-N mutant was constitutively active in the absence of androgens (Fig. 6C, middle). The androgen-independent activity of the AR-N was largely inhibited by forced expression of the transcription-deficient mutant FOXO1-HR-537-A3 (Fig. 6C, middle). In addition to the observation that endogenous FOXO1 forms a protein complex with endogenous AR in LNCaP cells (Supplementary Fig. S1A), the transcription-deficient mutant of FOXO1 (FOXO1-HR-537-A3) also

Figure 5. Nuclear localization of FOXO1 is important for its inhibition of AR. A, LNCaP cells were transfected with plasmids as indicated. At 48 h after transfection, cells were fixed and subjected to IFC. Cellular localization of FLAG-tagged FOXO1 was quantified and presented on the right. N, nucleus; C, cytoplasm; N+C, nuclear and cytoplasmic localization of FLAG-FOXO1. These experiments were repeated thrice and similar results were obtained.

B, LNCaP cells were transfected with PSA-Luc reporter gene and plasmids as indicated. At 48 h after transfection, cells were harvested and luciferase activities were measured. Columns, mean among three individual experiments; bars, SD.
Figure 6. Inhibitory effects of FOXO1 on androgen-independent activation of the AR and growth of ADI PCa cells. A, LNCaP cells were transfected with plasmids for renilla and PSA-Luc luciferase reporter genes, and plasmids as indicated and plated in CSS-medium (top). At 24 h after transfection, cells were either left untreated or treated with 0.1 nmol/L R1881 or IL-6 (50 ng/mL) for 24 h. Cells were harvested and subjected to measurement of luciferase activity. Columns, mean among three individual experiments; bars, SD. Bottom, ADI C4-2 cells were transfected with plasmids as indicated and plated in CSS-medium. At 24 h after transfection, cells were either treated with 0.1 nmol/L R1881 or IL-6 (50 ng/mL) or left untreated for 24 h and luciferase activity was measured. B, 22Rv1 cells were transfected with siRNAs as indicated. At 48 h after transfection, cells were treated with different doses of IL-6 for 24 h, and Western blot analysis and luciferase measurement were performed. C, schematic diagram of expression constructs for wild-type AR (AR-WT) and NH2-terminal truncated AR (AR-N; amino acids 1–667; top). Middle, PC-3 cells were transfected with plasmids as indicated. At 48 h after transfection, cells were collected and lysed. Luciferase measurement and data analysis were performed as described in A. Bottom, PC-3 cells were transfected with plasmids as indicated. Cells were harvested and lysed at 48 h after transfection. Lysates were subjected to IP and Western blot analysis. D, C4-2 cells were transfected with plasmids as indicated. At 0 and 72 h after transfection, relative viable cells were measured by an MTS assay (top). Columns, mean from six replicate experiments; bars, SD. Expression of FLAG-FOXO1-HR-537-A3, V5-FOXO1-C, and endogenous AR was examined by Western blot (bottom).
interacts with the ectopically expressed full-length AR in PC-3 cells (Fig. 6C, bottom). Importantly, this mutant of FOXO1 interacts with the AR-NH2-terminal domain (Fig. 6C, bottom) but not the AR-COOH-terminal domain (Supplementary Fig. S1B), which is consistent with the finding that this mutant of FOXO1 inhibits the constitutive activity of the AR-NH2-terminal domain in PC-3 cells. Together, we have identified a novel transcription-independent function of FOXO1 that antagonizes the androgen-independent activation of the AR in ADI-PCa cells.

**FOXO1 inhibits growth of ADI PCa cells.** The AR is required for the growth of ADI-PCa cells (12, 13). Indeed, knocking down endogenous AR by a small hairpin RNA (shRNA) in C4-2 ADI-PCa cells decreased viability as shown by both MTS and trypan blue exclusion assays (Supplementary Fig. S2A; Fig. 6D). Restoration of AR levels by expressing an AR shRNA-resistant form of AR (ARsr) rescued AR silencing–induced inhibition of cell viability (Supplementary Fig. S2A; Fig. 6D). Consistent with its ability to inhibit AR activity, ectopic expression of FOXO1-HR-537-A3 decreased the viability of C4-2 cells (Supplementary Fig. S2A; Fig. 6D). In contrast, forced expression of the COOH terminus of FOXO1 (FOXO1-C, amino acids 282–655), which does not interact with the AR (data not shown), had no inhibitory effect on C4-2 cell viability (Supplementary Fig. S2A; Fig. 6D). In line with these findings, transfection of C4-2 cells with FOXO1-HR-537-A3 or an AR shRNA modestly induced apoptotic cell death as shown by Annexin V uptake (Supplementary Fig. S2B). Similar analyses were carried out in AR-negative PC-3 cells, growth of which is independent of AR. Transfection of PC-3 cells with FOXO1-HR-537-A3 or an AR shRNA failed to inhibit PC-3 growth (data not shown). These data suggest that FOXO1 inhibits growth of ADI-PCa cells via inhibition of AR activity.

**Discussion**

Resistance to ADT represents a major challenge for the treatment of advanced PCa. Increasing evidence from clinical and cell culture studies suggests that the AR is required for growth and survival of ADI PCa (5). Loss of the PTEN tumor suppressor gene is also often associated with progression of human PCas in the clinic (15), pinpointing the importance of PTEN loss in PCa relapse during ADT. Most importantly, both in vitro and in vivo studies inevitably suggest a functional interaction between the AR signaling and the PTEN/Akt pathway in PCa cells. However, how the PTEN/Akt signaling axis regulates AR activity is debatable (21–23). In the present study, we show that loss of PTEN is highly associated with hyperactivation of the AR in the presence of low levels of androgens. We provide evidence that this effect of PTEN is mediated primarily through FOXO1, a key downstream effector protein of PTEN (30). It has been well-established that loss of PTEN or activation of Akt leads to phosphorylation and cytoplasmic localization of FOXO1 (30). We show that FOXO1-mediated inhibition of AR transcriptional activity requires the nuclear localization of FOXO1. Indeed, blockade of the FOXO1 nuclear localization achieved by introducing mutations in its NLS domain abrogates FOXO1-mediated inhibition of AR activity. Thus, our findings suggest a working model in which loss of PTEN or activation of Akt promotes nuclear exclusion of FOXO1, thereby abolishing the AR inhibitory function of FOXO1 in the nucleus. This model is further supported by our observation that expression of endogenous PSA and PSA reporter gene activity were highly induced by androgens in PTEN-null (FOXO1 resides primarily in the cytoplasm) but not in PTEN-positive cells (FOXO1 resides primarily in the nucleus).

It has been reported recently that FOXO1 inhibits androgen-induced expression of PSA (31) and that FOXO1 forms a protein complex with the AR in the promoter of the PSA gene (32). We show in the present study that forced expression of FOXO1 largely inhibited the activity of a composite AR report gene (3xARE-Luc) that contains only three copies of androgen response element. Thus, our data show for the first time that FOXO1-induced inhibition of PSA promoter activity is mediated through the AR. Moreover, we show that FOXO1-mediated inhibition of AR activity can be partially attenuated by the HDAC inhibitor TSA, suggesting that FOXO1 may inhibit AR activity by cooperating with HDAC proteins. We further show that HDAC3, but not HDAC1 and HDAC2, can enhance FOXO1-induced inhibition of AR function in LNCaP cells. Co-IP experiments indicate that HDAC3 associates with FOXO1 in the same protein complex. Thus, our results reveal an HDAC-mediated mechanism that contributes to FOXO1 inhibition of the AR in PCa cells. Of note, our data cannot rule out the possibility that FOXO1 may also inhibit AR transactivation via HDAC-independent mechanisms.

In addition to the inhibition of androgenic activation of the AR, we show that expression of FOXO1 also blocks AR activity induced by nonandrogenic factors such as IL-6 as well as ligand-independent activation of the AR. Moreover, expression of a functional nuclear mutant of FOXO1 diminishes the growth of ADI-PCa C4-2 cells. In light of the finding that FOXO1 interacts with HDAC proteins, we postulate that under a homeostatic scenario when FOXO1 proteins reside mainly in the nucleus, the association of FOXO1 with AR and HDAC proteins inhibits AR activity. During PCa progression, however, FOXO1 inhibitory proteins, such as Akt, become highly activated due to various genetic and epigenetic alterations, which include deletion or mutation of PTEN, overexpression/amplification of Her2/Neu, or increased secretion of insulin-like growth factor in the serum. Activated Akt subsequently causes nuclear exclusion of FOXO1, which thereby prevents association of FOXO1 with the AR in the nucleus and eventually promotes aberrant activation of the AR by residual/castration levels of androgens, promiscuous activation of the AR by nonandrogenic factors, or ligand-independent and constitutive activation of the AR. Further studies are warranted to test this hypothesis under in vivo settings.

In summary, we show that in addition to the inhibition of androgenic activation of the AR, FOXO1 also inhibits androgen-independent activation of the AR in a manner dependent on the nuclear localization of FOXO1 but independent of its transcriptional function. This function of FOXO1 may be mediated, at least in part, by its interaction with HDAC proteins such as HDAC3. Given that nuclear localization of FOXO1 is often deregulated due to frequent loss of PTEN in ADI PCas, loss of FOXO1 inhibition of the AR may promote ADI PCa progression by favoring aberrant activation of the AR under androgen-deprivation conditions.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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