Androgens negatively regulate forkhead transcription factor FKHR (FOXO1) through a proteolytic mechanism in prostate cancer cells

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Summary

The ability of androgens to inhibit apoptosis in both normal and malignant prostatic cells has been well documented. However, the underlying mechanisms are understood poorly. Here we demonstrated that forkhead transcription factor FKHR (FOXO1)-induced death of LNCaP cells was blocked by a synthetic androgen R1881. Androgen treatment also resulted in a reduction in transcriptional activity of FKHR in these cells. Moreover, treatment of LNCaP cells with R1881 led to a decrease in the intact FKHR protein (70 kDa) and an increase in a faster migrating protein band (60 kDa). Androgen-enhanced appearance of the 60 kDa protein was diminished specifically by lysosomal acidic cysteine protease inhibitors. Mass spectrometry analyses of the purified FLAG-tagged 70 kDa and 60 kDa proteins demonstrated that the 60 kDa species is a FKHR protein product that lacks about 120 amino acid residues of the C-terminal end. Mutagenesis of the basic amino acid Arg 537 in the protease cleavage region, as suggested by mass spectrometry, abrogated both the androgen-induced accumulation of the 60 kDa product and decrease in cell death induced by FKHR, suggesting the residue R537 is a potential protease cleavage site. Finally, ectopic expression of the first 537 amino acids of FKHR produced an inhibitory effect on transcriptional activity of the intact protein. Together, these results suggest that androgens induce increased activity of an acidic cysteine protease, which in turn cleaves FKHR. This provides a mechanism by which androgens protect prostate cancer cells from the killing effect of FKHR.
Introduction

Androgens are critical for proliferation and apoptosis in both normal and malignant prostatic epithelial cell (1). Orchitectomy results in extensive apoptosis and involution of the rat ventral prostate and human prostate cancer xenografts (2-4). Regression of prostatic tumors in patients following androgen ablation therapy is associated with apoptotic death in malignant prostatic epithelium (5,6). These findings suggest that androgens function as anti-apoptotic factors in both normal and malignant prostatic cells.

The tumor suppressor gene PTEN (also known as MMAC1/TEP1) is mutated frequently in a variety of tumors including prostate, brain, and endometrium (7-9). Although the overall prevalence of PTEN mutations in primary PCa is low relative to other tumors (10), the gene product is lost frequently in advanced prostate tumors (11,12). Inactivation of PTEN through different mechanisms such as deletion, methylation, or protein degradation has been implicated in progression of a number of tumors (7,8,13-17). Heterozygous disruption results in hyperplasia of the prostate, skin, and colon. Prostate-specific homozygous deletion of PTEN alleles in mice results in metastatic prostate cancer (18). PTEN acts as a tumor suppressor protein primarily via its phosphatidylinositol phosphatase activity, which antagonizes the PI3K/Akt pathway (19,20). Loss of PTEN in prostate cancer cells results in the constitutive activation of the oncoprotein Akt. Moreover, restoration of PTEN expression in PTEN-mutated prostate cancer cell lines abolishes the activation of Akt and subsequently induces cell death (12,21-23).

Active Akt phosphorylates many downstream pro-apoptotic proteins, which include Bad, caspase-9, and members of the FOXO subfamily of forkhead transcription factors FKHR (FOXO1), FKHRL1 (FOXO3a), and AFX (FOXO4) (24-26). Phosphorylated forkhead proteins
remain inactive in the cytoplasm. The forkhead proteins undergo dephosphorylation due to the inhibition of Akt activity by factors such as PTEN or PI3K inhibitors. Activated forkhead proteins translocate from the cytoplasm to the nucleus and subsequently bind to promoters of their target genes (27).

Recent studies have suggested that FOXO forkhead transcription factors may play important roles in regulating many cellular functions including proliferation, cell survival, and DNA damage. The members of this family regulate G1 cell cycle progression by modulating expression of the cyclin-dependent kinase inhibitor p27kip1 and D type cyclins (23,28,29). They also mediate transition from M to G1 of the cell cycle by directly regulating expression of mitotic genes such as cyclin B and polo-like kinase (plk) (30). Forkhead transcription factors affect the expression of several other genes that are involved in the cell cycle including Wip1, EXT1, and cyclin G2 (31). Expression of scavenger proteins such as cytosolic catalase and superoxide dismutase and the DNA damage response gene Gadd45 are regulated by FOXO forkhead transcription factors, suggesting that these proteins play a role in surveillance of DNA damage (31-33). A number of pro-apoptotic proteins such as Fas ligand (FasL), the IGF binding protein-1 (IGFBP1), Bim, NIP3, and legumain are transcriptionally regulated by members of this subfamily (31,34-37). FOXO1 (FKHR) regulates cell survival in hepatic cells through modulation of gluconeogenesis by interacting with PGC-1 (38). Foxo3a (FKHRL1) knockout female mice exhibit a distinctive ovarian phenotype of global follicular activation leading to oocyte death (39). Expression of active FKHR induces death in different types of mammalian cell lines including LNCaP prostate cancer cells (23,35). Thus, regulation of FKHR function may be a critical factor for survival of prostate cancer cells.
Our laboratory and others have shown previously that androgens act as survival factors by antagonizing PTEN activity in LNCaP cells (40,41). In the present study, we demonstrate that the FKHR-induced decrease in cell viability and increase in cell death is blocked by androgen treatment, which is concomitant with inhibition of transactivaton of FKHR in androgen-treated cells. We further provide evidence that the inhibitory effect of androgens on FKHR is mediated primarily through a proteolytic mechanism.

**Experimental Procedures**

**Materials**—N-terminal FLAG tagged pcDNA3 expression plasmids for wild-type FKHR, FKHR(WT), a constitutively active form, FKHR(AAA), and the luciferase reporter construct 3xIRS-Luc were kindly provided by Drs K.L. Guan and E.D. Tang (35). The substitution mutants of R537G, R554G, K559G, and P538G were constructed by polymerase chain reaction mutagenesis (Stratagene). Two truncation mutants WT(Δ) and AAA(Δ) were constructed from FKHR(WT) and FKHR(AAA), respectively by deleting a C-terminal fragment after the residue R537. These mutations were verified by sequencing. Construction of the mammalian expression vector for PTEN has been described previously (12). A 2.5-kb fragment of the human FasL promoter was kindly provided by Dr. C.V. Paya (42). The fragment of the FasL promoter containing the FKHR response element located between nucleotide –930 to –821 (23) was amplified by PCR and subcloned between the Mlu I and Bgl II site of the pGL3-promoter vector (Promega). A polyclonal antibody against FKHR was purchased from Cell Signaling Technology (Beverly, MA). A monoclonal antibody against PTEN (6H2.1) was purchased from Cascade BioScience (Winchester, MA). Erk2 (D-2) monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz). Mouse monoclonal antibodies against α-FLAG were purchased from Sigma. R1881 (Dupont), bicalutamide (Zeneca Pharmaceuticals), and
cycloheximide (Sigma) were dissolved in ethanol. LY294002 (Calbiochem), MG132 (Calbiochem), lactacystin (Calbiochem), ALLnL (Sigma) was prepared in DMSO (Sigma). Chloroquine (Sigma), ammonium chloride (Sigma), leupeptin (Sigma), and EGTA (Sigma) were prepared in water. Phenylmethysulfonyl fluoride (PMSF) (Sigma) was dissolved in isopropanol (Sigma).

**Cell lines and cell culture**- The prostate cancer cell line LNCaP (purchased from the American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium containing 10% FBS. In the experiments where LNCaP cells were treated with the synthetic androgen R1881 and/or the antiandrogen bicalutamide, these chemicals were refreshed every 48h. The immortalized prostatic epithelial cell line BPH-1 was kindly provided by Dr. S.W. Hayward (43) and cultured in RPMI 1640 medium (Invitrogen) containing 5% FBS.

**Cell transfections**- Transient transfection of LNCaP cells was performed by electroporation as described previously (12). Cells were mixed with DNA in 400 ul of RPMI 1640 medium. The DNA-cell mixture was transferred into a 4-mm cuvette (BTX Inc., San Diego, CA) and electroporated with a 305V/10ms pulse using a BTX T820 square wave electroporator (BTX Inc., San Diego, CA). Transfection efficiency was monitored 12 h after transfection with green fluorescence protein (GFP) by examining aliquots of cells under a Zeiss fluorescence microscope with a wavelength of 488 nm. Transfection efficiency was determined by the percentage of the green cells in the whole cell population. Routinely, transfection efficiency met 60-90% was used for experiments.
Western blot analysis-Protein samples were prepared by lysing cells in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 mM Na3VO4, and 1 mM NaF). Equal amounts of protein (30-80 µg) from cell lysates were denatured in sample buffer, subjected to 4-12% SDS/PAGE gels (Invitrogen), and transferred to nitrocellulose membranes (Bio-Rad). The filters were immunoblotted with specific primary antibodies, HRP-conjugated secondary antibodies, and visualized by enhanced chemilluminescence (Amersham Pharmacia Biotech).

RT-PCR and Northern blot analysis-Total RNA was isolated from cells treated with R1881, bicalutamide, or vehicle by TRIZOL (Invitrogen). The first-strand cDNAs were synthesized by SuperScript II reverse transcriptase (RT) (Invitrogen). The forward 5’-AAGAGCGTGCCCTACTTCAA-3’ and reverse primer 5’-CAGTTCCTGCTGTCAGACAATC-3’ were used for polymerase-chain reaction (PCR). Samples of 15 ug total RNA from each treatment were separated on 1.2% denatured formaldehyde-agarose gels and transferred to nylon membranes (Bio-Rad). Filters were analyzed for expression of FKHR, PSA or GAPDH by using an isotope-labeled cDNA fragment of the FKHR coding region, or probes as described previously (40,44).

Luciferase reporter assay-LNCaP cells were harvested after transfections, and cell lysates were prepared by adding lysis buffer directly to the cells on ice. Firefly luciferase and renilla luciferase activities in cell lysates were determined using a dual-luciferase kit (Promaga, Madison, WI). Renilla luciferase activities of cells were used as internal controls.
**Immunofluorescence chemistry and confocal microscopy**—For cell viability assays, cells were re-plated into 6-well plates after transfection. After 12 h, cells were treated with 1 nM R1881 or ethanol for additional 36 h. Cells were examined under a Zeiss LSM-510 confocal laser microscope (Carl Zeiss, Inc., Germany). Cells were photographed with a wavelength of 488 nm for GFP. For immunofluorescence chemistry, cells on coverslips (Eppendorf Scientific, Inc., Hamburg, Germany) were washed briefly in 1xPBS and fixed for 20 min in 2% paraformaldehyde (Toussins) in 1 x PBS. Cells were permeabilized by incubating with 0.3% Triton X-100 for 15 min. Cells were washed three times in 1xPBS, and incubated in blocking buffer (5% goat serum in 1xPBS) for 1 h at room temperature. Cells were incubated with a rabbit anti-AR polyclonal antibody (1:500) and a mouse anti-FLAG monoclonal antibody (1:1000) for 2 h at room temperature. After washing with 1xPBS three times for 5 min each, cells were incubated for 1 h at room temperature with the following secondary antibodies: Alexa Fluor 594 goat anti-rabbit IgG conjugate (Molecular Probes, Inc.) (1:1000), and Alexa Fluor 488 goat anti-mouse IgG conjugate (Molecular Probes, Inc.) (1:1000) prepared in blocking buffer for one hour at room temperature. Coverslips were washed with three changes of 1xPBS for 5 min each and mounted in ProLong (Molecular Probes, Inc.). Cells were analyzed with a laser-scanning microscope LSM510. Argon Ion and HeNe lasers were used to excite FITC and Texas red fluorescence, respectively, and UV laser was used to excite DAPI.

**Cell death assay**—Nuclear fragment and chromatin condensation were measured as described previously (40,43). Briefly, cells treated for 48h after transfections were collected at 4°C. Supernatant media was aspirated, and cells were treated with fixative solution (4% formaldehyde in 1xPBS). Bis-benzimide was added at a final concentration of 1 µg/ml and incubated for 10 min at room temperature. Cell aliquots were placed on slides and viewed under UV and a
wavelength of 488 nm (Carl Zeiss Axiophot). Green cells with signs of chromatin condensation and/or nuclear fragmentation (apoptotic) were scored as dead.

**Purification of FLAG-tagged FKHR proteins**—LNCaP cells grown in fifty 150-mm dishes were transfected with FLAG-tagged FKHR by electroporation. All subsequent steps were performed at 4 °C. Cells were washed once with ice-cold 1xPBS and lysed in Triton lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) plus 1% of protease inhibitor cocktail (Sigma). Cells were lysated at 4 °C for 30 min. Whole cell lysates were cleared at 12,000 x g for 20 min. Protein samples were incubated with anti-FLAG M2 affinity resins (Sigma) in protein binding buffer (190 mM NaCl, 50 mM Tris-HCl, pH 7.4, 6 mM EDTA, 2.5% Triton X-100) for 2 h. The beads were washed three times with washing buffer (150 mM NaCl, 10 mM Tris-HCl, pH 9.0, 5 mM EDTA, 0.1% Triton X-100) and once with Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 150 mM NaCl). Proteins were eluted with SDS-PAGE sample buffer (Invitrogen), analyzed by SDS-PAGE, and stained with GelCode blue (Pierce) and Western blotting using anti-FKHR antibody (Cell Signaling).

**In-gel trypsin digestion of FLAG-tagged FKHR**—The GelCode blue-stained gel bands were destained with 50% acetonitrile/ 50mM Tris pH 8.1 for 30 min at room temp, then reduced with 20mM DTT / 50mM Tris pH 8.1 for 20 min at 55°C, followed by alkylation with 40mM iodoacetamide / 50 mM Tris pH 8.1 for 20 min at room temperature. Overnight digestion was performed with trypsin (Promega Corporation, Madison WI) in 25 mM Tris pH 8.1 at 37°C. Peptides were extracted from the gel bands, first with 2% formic acid followed by 70% acetonitrile / 30% water / 0.1% formic acid.
**Mass spectrometry**-LC-MS/MS analysis of the peptides was performed on a ThermoFinnigan LCQ Deca ion trap mass spectrometer (ThermoFinnigan San Jose, CA). The LCQ Deca ion trap was coupled with an Applied Biosystems 140D pump, with a 1:100 split into a New Objective ProteoPep C18 PicoFrit column (75um x 5.0 cm) mounted on the New Objective PicoView source (New Objective, Woburn, MA). Peptides were loaded onto a 100um x 2.0 cm C18 trap and then eluted and chromatographed with a gradient of 4% acetonitrile / 0.1% formic acid to 40% acetonitrile / 0.1% formic acid in 50 minutes. The LCQ was set to run in data dependant triple play mode consisting of full scan (400-1900 amu), zoom scan on most abundant ion, followed by MS/MS mode on that ion. Once a precursor ion was fragmented, it was placed on an exclusion list for 5 minutes, to avoid repeating tandem mass spectrometry analysis of the same precursor ion. The MS/MS raw data were converted to DTA files using Bioworks 3.0 and correlated to theoretical fragmentation patterns using the SEQUEST search algorithm with tryptic peptide sequences from the NCBI database downloaded in February of 2003.

**Statistics**-Statistical analyses were performed by Student’s *t* test. Values of *P*<0.05 were considered significant and are presented in the *Results* section.

**Results**

**Androgens protect LNCaP cells from apoptosis induced by FKHR**-Ectopic expression of PTEN in LNCaP cells induces apoptosis, which can be inhibited by androgen treatment (40,41). Downstream of PTEN, members of the FOXO subfamily of forkhead transcription factors are critical effectors of cell death, since overexpression of active FKHR mediates death in LNCaP cells (23). To examine whether androgens affect FKHR-induced death in these cells, we conducted cell viability analyses. In cells without androgen treatment, fewer cells remained
viable after being transfected with a constitutively active form of FKHR, FKHR(AAA), in comparison to cells transfected with the empty vector (Fig. 1A and 1B, left panel). However, the killing effect of FKHR(AAA) was inhibited by the treatment of cells with 1 nM of the synthetic androgen R1881 (Fig. 1B, right panel). There was only a slight decease in cell viability when cells were transfected with wild-type FKHR, FKHR(WT) (Fig. 1C, left panel). This confirms a previous report that wild-type FKHR is only partially active because of its phosphorylation state (23). This slight difference was attenuated following androgen treatment (Fig. 1C, right panel). Co-transfection of FKHR(WT) along with PTEN resulted in a marked decrease in cell viability (Fig. 1D, left panel), which is consistent with the decreased phosphorylation of FKHR and thereby activation of FKHR in cells in the presence of PTEN (23). This effect of PTEN was inhibited by androgen treatment (Fig. 1D, right panel). As shown in Fig. 1E, PTEN-induced loss of cell viability was also inhibited markedly by androgens.

Next we measured apoptosis by a nuclear condensation and fragmentation assay (40). The constitutively active FKHR induced more than 15% apoptosis in LNCaP cells, and this was largely inhibited by androgens (Fig. 2). The death of LNCaP cells induced by FKHR(WT) alone or plus PTEN was markedly abrogated by androgen treatment. Androgens also inhibited PTEN-induced cell death (Fig. 2). Importantly, the androgen-mediated effects were reversed completely by co-treatment of cells with an androgen antagonist, bicalutamide, indicating that this is an androgen receptor-dependent event. Therefore, androgens inhibit cell death that is either induced by activated FKHR or potentiated by PTEN. Moreover, the androgen receptor appears to mediate these effects.
**Transactivation of FKHR is diminished by androgen action**—Akt remains constitutively activated in PTEN mutated LNCaP cells (7,21,22,45). This results in FKHR phosphorylation and disruption of its transactivation ability. However, the entire process can be reversed in LNCaP cells by forced expression of PTEN (23). Therefore, we were interested to determine whether PTEN-stimulated transactivation of FKHR is regulated by androgens. LNCaP cells were co-transfected transiently with PTEN and a luciferase reporter 3xIRS, which contains three copies of an FKHR-response element from the promoter of the *IGFBP-1* gene. Ectopic expression of PTEN resulted in an approximately 8-fold increase in the transcriptional activity of endogenous FKHR protein in cells without R1881 treatment (Fig. 3A, white columns #2 versus #1). However, this effect of PTEN was diminished by androgen treatment (Fig. 3A, black column #2 versus white column #2). Co-expression of PTEN and FKHR in LNCaP cells resulted in a robust increase in activity of the reporter gene (Fig. 3A, white columns #3 and #4) in comparison to the level activated by endogenous FKHR. However, this increase was also inhibited by the treatment of cells with 1 nM of R1881 (Fig. 3A, black columns #3 and #4). These results suggest that androgens inhibit transcriptional activities of both the endogenous and transfected FKHR protein. A similar inhibitory effect of androgens on transactivation of FKHR was observed in LNCaP cells treated with LY294002, a chemical inhibitor of PI3K (Fig. 3B). Together, these findings suggest that androgens inhibit FKHR transactivation through a common mechanism or at different points within the PI3K/PTEN/Akt/FKHR cascade.

To determine the level of inhibition of FKHR by androgens, LNCaP cells were transfected with a constitutively active FKHR(AAA). FKHR(AAA) transfection of androgen-untreated cells resulted in an increase in transcriptional activity (Fig. 3C). In contrast, androgen treatment markedly inhibited transcription from the 3xIRS reporter (Fig. 3C). A similar inhibitory effect of...
androgens on FKHR transactivation was observed with a luciferase vector, which contains an
FKHR-response element from the promoter of the FasL gene (Fig. 3D). Thus, these results
suggest that the inhibitory effects of androgens occur at the level of FKHR.

The effect of androgens on nuclear localization of the FKHR protein—Nuclear localization is
required for the normal function of forkhead transcription factors (23,26,34). Active FKHR is
retained in the nucleus, whereas, inactive FKHR remains in the cytoplasm. Thus, we were
interested in determining whether androgens inhibit transcriptional activity of the FKHR protein
by blocking its nuclear localization. Since expression levels of endogenous FKHR are quite low,
we utilized transfected FKHR. As shown in Fig. 4A and 4B, transfected wild-type FKHR was
retained in the cytoplasm of LNCaP cells treated either with or without androgens. Most of the
transfected FKHR was localized in the nucleus following ectopic expression of PTEN (Fig. 4C),
as has been demonstrated previously (23). This nuclear localization was not affected by
androgen treatment (Fig. 4D). A similar result was obtained by transfecting the active
FKHR(AAA) (data not shown). Androgenic effects were evident by increased expression of the
AR protein in the nucleus (Fig. 4B and 4D). It has been well established that androgens induce
the stability of the AR. Therefore, there appears to be no effect of androgens on nuclear
localization of the transfected FKHR protein in LNCaP cells in the presence of PTEN, and the
inhibitory effect of androgens on transactivation of FKHR does not appear to be mediated by
blocking its nuclear localization.

The effect of androgens on FKHR protein and mRNA—In order to elucidate the mechanism by
which androgens inhibit the function of FKHR, we examined the effect of androgens on
expression of FKHR protein in LNCaP cells. Under normal culture conditions (Fig. 5A, 5B, and
5C, the first lane in the top panel), there existed two immunoreactive bands of FKHR, one major
band of 70 kDa (the expected molecular mass of intact FKHR) and a faster migrating species of
approximately 60 kDa. Importantly, the amount of the 70kDa band decreased and the amount of
the 60-kDa band increased following androgen treatment (Fig. 5A, lane 1 versus lane 2, and 5B).

To determine whether the effect of androgens on FKHR protein is mediated by androgen
receptor, we treated LNCaP cells with the antiandrogen bicalutamide. Androgen-induced
reduction of the intact FKHR and accumulation of the 60-kDa form of FKHR were abolished in
the cells treated with bicalutamide (Fig. 5A, top panel). Evidence that bicalutamide did inhibit
transactivation of the AR was demonstrated by the blockage of expression of endogenous
prostate-specific antigen (PSA), a gene that is transcriptionally regulated by the androgen
receptor (Fig. 5A, panel 3).

The androgenic effect was further examined in BPH-1, an immortalized prostatic cell line which
does not express endogenous androgen receptor (43). The similar dynamic changes in FKHR
proteins were induced by ectopic expression of the androgen receptor in BPH-1 cells (Fig. 5E).
Taken together, these data indicate that androgens affect expression of FKHR protein through
the androgen receptor.

Time course studies showed that the effect of androgens on FKHR protein was detected at 48h or
later following androgen administration (Fig. 5C), suggesting that this is not a direct
transcriptional response. Also, the 60-kDa form of the FKHR protein was inhibited by treatment
of cells with cycloheximide, an inhibitor of new protein synthesis (Fig. 5D). Thus, the effect of
androgens on FKHR protein appears to require new protein synthesis. This supports the
conclusion that androgens are inducing the expression of a protein(s), which in turn affects changes in FKHR.

In order to determine whether androgen-induced appearance of the 60 kDa species of FKHR originates from alternative splicing of pre-exist exons, we performed RT-PCR for the mRNA isolated from LNCaP cells treated with androgens. The human FKHR gene contains three exons (46). Therefore, forward and reverse primers were designed from sequences adjacent to the 5’ and 3’ ends of the exon 2 (Fig. 6A, top). Only one PCR product was observed (the expected 1.5 kb band), and no additional bands were amplified from the LNCaP cells treated with androgens (Fig. 6A, bottom). When reverse transcriptase was omitted from the reaction mixture, no amplification was observed, suggesting that the observed PCR product was amplified from the synthesized cDNA. Also, no extra mRNA species was detected by Northern blot analysis by using the entire ORF sequence as a probe (Fig. 6B), suggesting that unpredictable gene arrangement is not involved. Therefore, these results suggest that the effect of androgens on FKHR may occur through a post-translational mechanism that likely affects the FKHR protein itself.

The effect of protease inhibitors on FKHR protein - We sought to determine whether proteases play a role in the androgen-mediated effects on the FKHR proteins. LNCaP cells were treated with a panel of protease inhibitors. After treatment with R1881, LNCaP cells were exposed to MG132, a broad-spectrum inhibitor of proteasomes. In the absence of MG132, androgens induced an accumulation of the endogenous 60-kDa FKHR (Fig. 7A). However, the process was inhibited by the exposure of cells to MG132 (Fig. 7A). Similar results were obtained in studies with the transfected FLAG-tagged FKHR protein (Fig. 7B, left panel). Since MG132 is not
specific for proteasomes (47,48), we treated LNCaP cells with a highly specific proteasome inhibitor lactacystin (49). No inhibitory effect of lactacystin was observed (Fig. 7B, right panel), suggesting that proteasomes are not involved in formation of the 60-kDa FKHR. These findings are similar to the protease-mediated cleavage of β-secretase that is sensitive to MG132, but resistance to lactacystin (50). Thus, a mechanism other than proteasome degradation appears to underlie the proteolysis of the FKHR protein.

Next we treated LNCaP cells with the lysosomal acidic cysteine protease inhibitors chloroquine, ammonium chloride, and leupeptin (48,51,52). Treatment of LNCaP cells with chloroquine inhibited accumulation of endogenous 60-kDa FKHR induced by androgens (Fig. 7C). Chloroquine also inhibited androgen-induced formation of the 60-kDa FLAG-tagged FKHR in a dose-dependent manner (Fig. 7D, left panel). Similar results were obtained by treating cells with ammonium chloride and leupeptin (Fig. 7D, middle and right panels). Together, these data suggest that lysosomal acidic cysteine proteases are involved in the androgen-induced cleavage of the FKHR protein.

Calpain also belongs to the superfamily of cysteine proteases. It has been documented that both MG132 and leupeptin have inhibitory effects on calpain (51,53,54). Therefore, we sought to determine whether calpains are involved in androgen-augmented proteolysis of FKHR by exposure of LNCaP cells to ALLnL and EGTA, two inhibitors of calpain (55,56). No inhibitory effect of ALLnL was found on androgen-induced accumulation of either the endogenous or FLAG-tagged 60-kDa FKHR (Fig. 7E and Fig. 7F, left panel). ALLnL was functional since cyclin B, a calpain target protein (57), was stabilized in the cells treated with ALLnL (data not shown). Likewise, no effect of EGTA on the androgen-induced appearance of the 60-kDa
FLAG-tagged FKHR band was detected (Fig 7F, middle panel). Thus, these data suggest that calpain proteases are not involved in the proteolytic cleavage of FKHR. In addition, cells treated with phenylmethylsulfonyl fluoride (PMSF), a serine protease inhibitor (51), exhibited no inhibitory effect on FKHR cleavage (Fig. 7F, right panel), thus ruling out serine proteases as mediators of this effect.

R537G mutation abolishes the effect of androgens on proteolysis and transactivation of FKHR-In order to determine the protease cleavage site in FKHR protein, we performed mass spectrometry (MS) analyses. FLAG-tagged FKHR was expressed in LNCaP cells treated with androgens. FLAG-tagged FKHR proteins were purified using anti-FLAG affinity resins. Purified proteins were eluted and separated by SDS-PAGE. Two FKHR products (70 kDa and 60 kDa, respectively as determined by Western blot analysis in a sister gel using the anti-FKHR antibody) were detected in the eluted sample (Fig. 8A), in addition to a major band of the heavy chain of immunoglobulin at ~50 kDa. These bands were excised separately and digested with trypsin. The peptides were analyzed by LC-MS/MS. The spectra were compared with FKHR peptide sequences using the SEQUEST database-searching program. Tryptic peptides identified by tandem-MS from the 70 kDa and 60 kDa FKHR bands are underlined in Fig. 8B and 8C, respectively. The SEQUEST cross correlation scores ($X_{corr}$) for these peptides are higher than 2.9 (range 2.91 - 5.93) with corresponding $\Delta C_n$ values > 0.1 (range 0.11 - 0.59). Importantly, the two tryptic peptides, AA560-589 and AA598-619 that were observed for the 70 kDa protein were very abundant and resulted in SEQUEST $X_{corr}$ values of 5.74 and 4.05 and $\Delta C_n$ values of 0.43 and 0.37, respectively. These two tryptic peptides were not observed for the 60-kDa FKHR protein, thus providing strong evidence that the breakdown protein is a result from cleavage at the C-terminus. Additionally, a comparison of the peptide sequences from non-treated and androgen-
treated extracts revealed common regions of coverage, except for the last about 120 amino acids at the C-terminal region. These results are consistent with a breakdown product of 60 kDa. It is unlikely that the end point (K515) of the peptide identified by MS in the C-terminal of the 60 kDa product is a natural cleavage site of FKHR because K515 was also detected by MS in a peptide in the full-length protein (Fig. 8B and 8C). Indeed, the mass spectrometry results of the intact and breakdown proteins suggest that the protease cleavage site of FKHR exists between K515 and T560.

Because the candidate protease might be an acidic cysteine protease, we were interested to determine whether mutations in the basic amino acids in the potential cleavage region (see top panel of Fig. 9A) would generate cleavage resistance. As shown in Fig. 9A, conversion of Arg 537 to a Gly in FKHR largely abolished androgen-induced accumulation of the 60 kDa product. However, no effect of mutations at the basic amino acid residues R554 or K559 was obtained on protease cleavage of the FKHR protein. Importantly, mutating the amino acid adjacent to R537 (P538G) had no effect on androgen-induced FKHR cleavage. These results support the specificity of the R537G mutation in resistance to FKHR cleavage. Therefore, given the basic nature of R537, the mutagenesis data suggest that this amino acid might be a cleavage site of the androgen-regulated acidic cysteine protease. It is worth mentioning that despite the R537G mutation, a trace degree of cleavage was still observed (Fig. 9A), suggesting either incomplete resistance to proteolysis due to a single-point mutation or the presence of minor cleavage sites near R537.

To assess the effect of the R537G mutation on the biological function of FKHR, we compared the transcriptional activities and cell death induced by the FKHR(AAA)-R537G with that of a
constitutively active FKHR(AAA) in cells treated with androgens. As a positive control, androgen treatment of LNCaP cells resulted in a significant decrease in transactivation of transfected FKHR(AAA) (Fig. 9B) and cell death (Fig. 9C). However, the androgenic effects on transcriptional activity and apoptosis were abrogated by the R537G mutation in FKHR (Fig. 9B, 9C). Therefore, the R537G mutation not only establishes resistance of FKHR to androgen-induced protease cleavage, but also diminishes the inhibitory effect of androgens on its biological function. Interestingly, the transcriptional activity of the FKHR(AAA)-R537G was slightly lower than FKHR(AAA) (Fig. 9B), the reason for which is unclear.

**Cellular localization and transcriptional activity of the C-terminal truncated FKHR proteins**—As suggested by results of mass spectrometry (Fig. 8) and mutagenesis (Fig. 9), R537 appears to be a potential cleavage site of FKHR induced by androgens. In an attempt to gain insight into the function of the breakdown product of FKHR, we generated two mutants, WT(Δ) and AAA(Δ), in which amino acids from the C-terminal end (after the residue R537) were truncated in FKHR(WT) and FKHR(AAA), respectively. We examined the cellular localization of these truncated FKHR proteins in LNCaP cells. As shown in Fig. 10A, transfected WT(Δ) was localized in the cytoplasm in the absence of PTEN, but was localized in the nucleus following PTEN transfection (Fig. 10B). In contrast, AAA(Δ) was localized in the nucleus in the absence of PTEN (Fig. 10C). This is similar to the cellular localization of FKHR(AAA) (23). The cellular localization of these mutants was not affected by androgen treatment (Fig. 10D-F). These results suggest that lack of the C-terminal fragment (AA538-655) does not affect nuclear localization of the protein.
To test whether truncation of the C-terminal end at R537 would affect the transcriptional activity of FKHR, we measured the reporter gene activity in LNCaP cells transfected with AAA(Δ). Little or no activity was obtained in cells transfected with AAA(Δ) (Fig. 11A), suggesting that lack of the C-terminal end after R537 leads to loss of transcriptional activity of FKHR. Restoration of PTEN into LNCaP cells induced transactivation of endogenous FKHR (Fig. 11B). This activity was diminished by ectopic expression of WT(Δ) in a dose-dependent manner (Fig. 11B). Ectopic expression of the truncated protein, AAA(Δ), had a similar inhibitory effect on transfected full-length FKHR(AAA) (Fig. 11C). As a control, transfection of the constitutively active FKHR(AAA) resulted in a dramatic increase in transcription of the reporter gene (Fig. 11A and 11C). Therefore, these data suggest that lack of the C-terminal domain results in loss of transcriptional activity of truncated protein, but gain of an inhibitory activity of the intact FKHR protein.

**Discussion**

Androgens have been linked to a number of survival pathways. For example, they protect prostate cancer cells from death induced by etoposide (58). They also inhibit death of prostate cancer cells mediated by tumor necrosis factor α (TNFα) or Fas activation (59). Recently, results from our laboratory and others have demonstrated that androgens inhibit apoptosis of the prostate cancer cell line LNCaP induced by PTEN or the PI3K inhibitors LY294002 and wortmannin (40,41,59-61). Logical targets for these effects include Akt and PTEN. However, neither Akt activity nor PTEN function is affected by androgens (40,41,59). Thus it has been suggested that androgens exert their anti-apoptotic functions by modulating survival pathways independent of Akt (60) or targets downstream of Akt (41). Here, we demonstrate that androgens inhibit death of LNCaP prostate cancer cells mediated by FKHR, a cell death effector
downstream of Akt. Furthermore, we provide evidence that androgens induce proteolysis of the FKHR protein and loss of its transcriptional activity. Thus this study defines a novel mechanism of androgenic regulation of FKHR function in prostatic cells.

A previous study has demonstrated that synthetic oligonucleotides of the androgen response element (ARE) induce apoptosis in LNCaP cells (62). This suggests that ARE-dependent transcription may play an essential role in prostate cancer cell survival. The protective effect of androgens on wortmannin-induced death of LNCaP cells appears to be mediated by a similar mechanism. However, it appears to be indirect, since no androgenic effect was detected when dihydrotestosterone was added simultaneously or after wortmannin was applied to cells (61). Likewise, in our current study, neither the inhibitory effect of androgens on FKHR-induced cell death nor the androgen-induced proteolysis of FKHR protein was detected until 48 h of androgen treatment. Moreover, the androgenic effect was abolished by blockage of new protein synthesis. Therefore, it appears that the inhibitory effect of androgens on FKHR-induced apoptotic death of LNCaP cells is mediated primarily through an indirect mechanism.

FKHR is a nuclear transcription factor (29,38). The DNA binding domain of the human FKHR is located between amino acid residues 158 and 258, and transactivation domain is at the C-terminal end (AA\textsubscript{596-655}) (63). The current study demonstrates that the breakdown product of FKHR is missing approximately 120 amino acids of the C-terminal end. Therefore, it is not surprising that the active AAA(\Delta) mutant, with a C-terminal truncation starting at the potential protease cleavage site R537, exhibits no transcriptional activity. This mutant protein still contains the DNA binding domain and is retained in the nucleus, but it lacks the transactivation domain. However, expression of this mutant protein inhibited transactivation of both endogenous
and transfected intact FKHR. Thus, the truncated protein may have a squelching effect on transactivation of the intact protein since forkhead transcription factors bind as monomers to their target sequence (64).

Although our current data suggest that androgens inhibit FKHR activity mainly through a proteolytic mechanism, we also found that the protease-resistant mutation did not completely abolish androgen-induced decrease in transcriptional activity of the active FKHR(AAA). This observation raises a question as to whether other mechanism(s) may contribute in part to the androgen-mediated inhibition of FKHR in LNCaP cells. While this manuscript was in preparation, a study conducted in DU145 cells, a prostate cancer cell line with expression of endogenous PTEN but no AR, suggests that androgens may inhibit FKHR transactivation by a protein-protein interaction between AR and FKHR (65). Interestingly, no protein-protein interaction was detected between these two proteins by an in vitro assay, although FKHR can interact with the estrogen receptor under the same condition (66). We demonstrated in Fig. 4D that in the presence of PTEN, both FKHR and AR proteins exist in the nucleus in androgen-treated LNCaP cells. Therefore, it is possible that AR may also form a complex with FKHR in LNCaP cells. It is worth pointing out that in the absence of PTEN, the AR is retained in the nucleus, whereas, FKHR exists in cytoplasm of LNCaP cells. Therefore, the LNCaP cell line would be an ideal model to explore whether the interaction between AR and FKHR is a PTEN-dependent, or FKHR unphosphorylation-dependent event.

In summary, our data demonstrate for the first time that androgens induce proteolysis of the FKHR protein. This is a novel mechanism by which androgens inhibit FKHR-induced death of prostate cancer cells. Our observation that androgens abrogate transactivation of FKHR
potentiated by PTEN provides evidence that androgen-mediated proteolysis of FKHR is at least one of the mechanisms by which androgens antagonize PTEN function. Further characterization of the androgen-regulated cysteine protease machinery in prostate cancer cells is warranted. This will allow a better understand of the anti-apoptotic effects of androgens on prostate cancer cells and may lead to new therapeutic strategies for the treatment of this disease.

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Abbreviations

FKHR, forkhead transcription factor in rhabdomyosarcoma; PCa, prostate cancer; PTEN, phosphatase and tensin homolog deleted on chromosome ten; PI3 kinase, phosphoinositide 3-kinase; IGFBP1, insulin-like growth factor binding protein 1; FasL, Fas Ligand.

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Figure Legends

Figure 1. Effect of androgens on the loss of viability of LNCaP cells caused by activation of FKHR. After treated with 1 nM of R1881 or ethanol (ETOH) for 48 h, LNCaP cells were cotransfected with pEGFP plasmid along with the plasmids for control vector pcDNA3 (A), constitutively active FKHR, FKHR(AAA) (B), wild-type FKHR, FKHR(WT) (C), FKHR(WT) plus PTEN (D), or PTEN alone (E). Androgen treatments were resumed 12 h after transfection. Viable cells were photographed by confocal microscopy with transmit light for whole cell population or 488 nm for transfected cells 48 h after transfection. Magnification x 100. Data are representative of three independent experiments.

Figure 2. Effect of androgens on apoptosis of LNCaP cells induced by FKHR and PTEN. At 48 h after the treatment with 1 nM of R1881, R1881 plus 5 μM of bicalutamide (Bi), or the vehicle ethanol (ETOH), LNCaP cells were transfected with pEGFP and plasmids encoding the proteins as indicated above. Media were changed 12 h after transfection to remove cell debris from the electroporation effect, and cells were treated with ETOH, R1881, or R1881 plus bicalutamide for 48 h. Dead cells from the transfected population were scored as described in Experimental Procedures. Data represent the mean value and standard error from three independent experiments. *P<0.01 compared the R1881 treatment to the vehicle (ETOH). **P<0.01 compared the treatment with R1881 plus bicalutamide to R1881.

Figure 3. Effect of androgens on the transcriptional activation of FKHR. A, effects of androgens on the transcriptional activity of FKHR activated by PTEN expression. LNCaP cells, pretreated with 1 nM of R1881 or ETOH for 48 h, were transiently cotransfected with the 3xIRS luciferase reporter plasmid along with the plasmids encoding the proteins as indicated. At 36 h after
transfection, luciferase activity was measured as described in Experimental Procedures. Changes in activity was determined by normalizing the measured light units by the measured renilla luciferase activity, empty backbone reporter construct, and then normalizing to the activity of these report constructs when transfected with empty expression vector. B, effects of androgens on the transcriptional activation of FKHR by PI3K inhibitor LY294002 (LY). LNCaP cells were treated with R1881 or ETOH, and transfected with wild-type FKHR as described as in (A). At 12 h after transfection, cells were treated with 10 µM of LY294002 in combination with 1 nM of R1881 or ETOH for an additional 24 h. Luciferase measurement, and data analysis were performed as described in (A). C and D, effects of androgens on the transactivation of the constitutively active FKHR, FKHR(AAA). After pretreated with 1 nM of R1881 or ethanol (ETOH) for 48 h, LNCaP cells were transiently cotransfected with the 3xIRS-luciferase reporter plasmid (C), or the FasL promoter luciferase reporter plasmid (D) along with FLAG-FKHR(AAA) plasmid. 12 h after transfection, R1881 or ETOH was refreshed. At 36 h after transfection, luciferase activity was measured as described in Experimental Procedures. Data analysis was performed as described in (A). Data shown in this Figure represent the mean value and standard error from three independent experiments. *P<0.05 compared the R1881 treatment to the vehicle (ETOH).

**Figure 4.** Lack of effect of androgens on FKHR cellular localization. Confocal microscopy of the transfected FKHR and the endogenous androgen receptor in LNCaP cells. Cells were co-transfected with FLAG-tagged FKHR with a control vector pcDNA3 (A and B) or an expression vector for PTEN (C and D). LNCaP cells treated with 1 nM of R1881 (B and D) or ETOH (A and C) for 48 h were transfected with plasmids as indicated. At 24 h after transfection, cells were treated with R1881 or ETOH. After 24-h treatment, cells were double stained with anti-FLAG
and anti-AR antibodies for the FKHR and AR expression. Cell nuclei were counterstained with DAPI (blue). Data are representative from three independent experiments.

**Figure 5.** Influence of androgens on the size of the FKHR protein. A, effect of the androgen receptor agonist R1881 and antagonist bicalutamide (Bi) on FKHR protein. LNCaP cells were treated with or without 5 µM of bicalutamide for 30 min before cells were exposed to 1 nM of R1881 or the vehicle ethanol (ETOH). At 72 h after the treatment, expression of FKHR protein (top panel) was analyzed by Western blot. Northern blot analysis of PSA mRNA was conducted to monitor the efficacy of these compounds in parallel experiments (panel 3). Erk2 (panel 2) and GAPDH (bottom panel) were used as loading controls. B, dose-dependent effect. LNCaP cells were treated with different doses of R1881 (1 – 10 nM) or the vehicle ethanol for three days. 80 µg of proteins from each sample was analyzed for expression of the proteins for FKHR by Western blot analysis. Erk2 was used as a loading control. C, time-dependent effect. LNCaP cells were treated with or without 1 nM of R1881 for different periods of time (24 h-72 h). Protein samples were collected at each time point and analyzed for expression of FKHR protein. D, effect of new protein synthesis inhibitor cycloheximide on FKHR protein. LNCaP cells were treated with or without 1 nM of R1881. After 24 h, cells were treated with 25 µg/ml of cycloheximide for 24 h. Protein samples were collected and analyzed for the status of FKHR expression. E, effect of androgens and androgen receptor on FKHR in BPH-1 cells. BPH-1 cells (5x10^5) were transfected with pcDNA-AR or a backbone empty vector. At 12 h after transfection, cells were treated with 1 nM of R1881 for 72 h. 100 µg of proteins from each sample was analyzed for expression of the proteins for FKHR by Western blot analysis. Erk2 was used as a loading control.
Figure 6. Lack of androgenic effects on FKHR mRNA. A, analysis of androgenic effect on FKHR mRNA by RT-PCR. Above, a schematic representation of the genomic DNA and mRNA for the human FKHR gene. The FKHR gene contains three exons (I, II, and III). The shaded areas represent the region encoding the FKHR protein. ‘F’ and ‘R’ with the arrows represent the forward and reverse primers used for the RT-PCR experiments (bottom panel), respectively. LNCaP cells were treated with ETOH (R0), 1 nM (R1) and 10 nM (R10) of R1881 for 72 h. 1 ug of total RNA from each sample was used for cDNA synthesis in the presence or absence of reverse transcriptase (RT). RT-PCR products were analyzed with 1.2 % agar gel and stained with ethidium bromide. 1 kb DNA ladder (Promega) was used as a marker (M). B, analysis of androgenic effect on FKHR mRNA by Northern blot. LNCaP cells were treated with different doses of R1881 (1 – 10 nM) or the vehicle ethanol for three days. Total RNA was isolated by using TRIZOL. 15 µg of cellular RNA from each sample was analyzed for expression of FKHR mRNA by Northern blot analysis. GAPDH was used as a loading control.

Figure 7. The effect of protease inhibitors on the FKHR proteins. A, effect of MG132 on the endogenous FKHR protein. LNCaP cells pretreated with R1881 or ETOH were treated with 50 µM of MG132 or the vehicle DMSO for 24 h. Samples analyzed for the effects of MG132 on endogenous FKHR protein. B, effect of MG132 (left panel) and lactacystin (Lact, right panel) on Transfected FLAG-tagged FKHR. LNCaP cells pretreated with 1 nM of R1881 for 48 h were transfected b with FLAG-tagged FKHR. 12 h after plating, cells were co-treated with 1 nM of R1881 and 50 µM of MG132 or 20 µM of lactacystin. Protein samples were collected at each time point as indicated from the cells treated with MG132 or 24 h after cells were treated with lactacystin, and analyzed for the expression status of FLAG-tagged FKHR protein. C, effect of chloroquine on endogenous FKHR protein. LNCaP cells pretreated with R1881 or EOTH were
treated with 100 µM of chloroquine (CHQ) or the vehicle DMSO for 24 h. Samples were analyzed for the effects of chloroquine on endogenous FKHR protein. D, effect of chloroquine (CHQ, left panel), ammonium chloride (AC, middle panel), and leupeptin (Leup, right panel) on transfeced FLAG-tagged FKHR. LNCaP cells treated with for 48 h were transfected with FLAG-tagged FKHR. 12 h after plating, cells were co-treated with 1 nM of R1881 along with different doses of chloroquine, ammonium chloride, or leupeptin. Protein samples were collected 24 h after treatment, and analyzed for the expression status of FLAG-tagged FKHR protein. E, effect of ALLnL on the endogenous FKHR protein. LNCaP cells were treated with 100 µM of ALLnL or the vehicle DMSO for 24 h. Protein samples were collected and analyzed for the effects of ALLnL on FKHR protein. F, effect of ALLnL (100 µM, left panel) and EGTA (0.5 mM, middle panel) and the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF, 2mM, right panel) on ectopically expressed FLAG-tagged FKHR. LNCaP cells were transfected as described in (B). 12 h post transfection, cells were co-treated with 1 nM R1881 and each individual protease inhibitor for an additional 24 h. Protein samples were analyzed by Western blotting.

**Figure 8.** Purification and mass spectrometric analysis of FLAG-tagged FKHR proteins. A, purification of FLAG-tagged FKHR proteins. LNCaP cells treated with androgens were transfected with an expression vector for wild-type FLAG-tagged FKHR. Whole-cell lysate was prepared and FKHR proteins were purified with anti-FLAG affinity resins. Purified proteins and input controls were separated by 4-12% SDS-PAGE gels and stained with GelCode blue. The multiple color proteins (Invitrogen) were used as markers. B and C, tryptic peptides identified upon in-gel digestion of the 70 kDa and 60 kDa FKHR proteins, respectively. Tryptic peptides identified by tandem-MS with SEQUEST cross correlation scores, $X_{corr} > 2.9$ (range 2.91 - 5.93) with corresponding $\Delta C_n$ values > 0.1 (range 0.11 - 0.59) are underlined.
**Figure 9.** R537G mutation in FKHR rescues androgen-induced both protein proteolysis and inhibition on FKHR function. 

_A_ A, effect of mutations in FKHR on androgen-induced proteolysis. LNCaP cells treated with androgens for 48 h were transfected with plasmids as indicated. At 24 h after transfection, cells were treated androgens for 24 h. Whole-cell lysates were prepared and analyzed by Western blotting with an anti-FLAG antibody. Above, the residues surrounding the mutated amino acids (underlined) are listed. 

_**B**_ B, inhibition of R537G mutation on androgen-induced decrease in transcriptional activity of FKHR(AAA). LNCaP cells were treated with 1 nM of R1881 or ETOH for 48 h and transfected with plasmids as indicated. 12 h after transfection, R1881 or ETOH was refreshed. At 36 h after transfection, luciferase activity was measured as described in Experimental Procedures. Data represent the mean value and standard error from three independent experiments. *P<0.01 compared the R1881 treatment to the vehicle (ETOH).

_**C**_ C, Comparison of the effect of androgens on apoptosis of LNCaP cells induced by either FKHR(AAA) or FKHR(AAA)-R537G. At 48 h after the treatment with 1 nM of R1881 or the vehicle ethanol (ETOH), LNCaP cells were transfected with pEGFP and plasmids encoding the proteins as indicated above. Media were changed 12 h after transfection to remove cell debris from the electroporation effect, and cells were re-treated with ETOH or R1881 36 h. Dead cells from the transfected population were scored as described in *Experimental Procedures*. Data represent the mean value and standard error from three independent experiments. *P<0.01 compared the R1881 treatment to the vehicle (ETOH).

**Figure 10.** Effects of androgens on cellular localization of WT(Δ) and AAA(Δ). Confocal microscopy of the transfected truncated FKHR and the endogenous androgen receptor in LNCaP cells. Cells were co-transfected with WT(Δ) with a control vector pcDNA3 (A and D) or an
expression vector for PTEN (B and E) or AAA(Δ) alone (C and F). LNCaP cells treated with 1 nM of R1881 (D, E, and F) or ETOH (A, B and C) for 48 h were transfected with plasmids as indicated. At 24 h after transfection, cells were treated with R1881 or ETOH. After 24-h treatment, cells were double stained with anti-FLAG and anti-AR antibodies for the truncated FKHR and AR expression. Cell nuclei were counterstained with DAPI (blue). Data are representative from three independent experiments.

**Figure 11.** Effects of the truncated FKHR WT(Δ) and AAA(Δ) on transcriptional activity of intact protein. A, transactivation of AAA(Δ). LNCaP cells were transiently cotransfected with 3xIRS along with the plasmids encoding the proteins as indicated. At 36 h after transfection, luciferase activity was measured as described in Experimental Procedures. B, effects of expression of WT(Δ) on transcriptional activation of endogenous FKHR. LNCaP cells were transfected with 3xIRS and the plasmids as indicated. At 36 h after transfection, luciferase measurement, and data analysis were performed as described in (A). C, effects of expression of AAA(Δ) on transcriptional activation of transfected active FKHR(AAA). LNCaP cells were transiently cotransfected with 3xIRS 3xIRS and the plasmids as indicated. At 36 h after transfection, luciferase measurement, and data analysis were performed as described in (A). Data in this Figure represent the mean value and standard error from three independent experiments. Statistical significance was determined by Student’s t test (*P<0.01; **P<0.05).
Figure 4

A

B

C

D

Anti-FLAG  Anti-AR  DAPI  Merge

pcDNA3 + ETOH

pcDNA3 + R1881

PTEN + ETOH

PTEN + R1881
Figure 9

A

M516 - MNPSSHTHPHAQOTSAVNGRPLPHTVSMMPHTSQNRLTOYK - K559

70 kDa

60 kDa

ID: FLAG

B

Luciferase Fold Activity
(3xRS-Luc)

ETOH

R1881

FKHR (AAA)

FKHR(AAA) R537G

C

Percentage of Apoptotic Cells (%)

ETOH

R1881

pcDNA3

FKHR (AAA)

FKHR(AAA) R537G
Figure 10

A

B

C

D

E

F

WT(Δ) + pcDNA3

WT(Δ) + PTEN

AAA(Δ)

WT(Δ) + pcDNA3

WT(Δ) + PTEN

AAA(Δ)

Anti-FLAG  DAPI  Anti-AR  Merge

ETOH

R1881
Figure 11

A

![Graph A showing luciferase fold stimulation (3xRS-Luc) with FKHR (μg) 0, 0.5, and 1.]

B

![Graph B showing luciferase fold stimulation (3xRS-Luc) with PTEN (μg) 0.5, 0.5, and 0.5, and WT(Δ) (μg) 0, 0.1, and 0.2.]

C

![Graph C showing luciferase fold stimulation (3xRS-Luc) with FKHR(ΔAA) (μg) 1, 1, and 1, and AAA(Δ) (μg) 0, 0.5, and 1.]
Androgens negatively regulate forkhead transcription factor FKHR (FOXO1) through a proteolytic mechanism in prostate cancer cells

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