Role of FoxO Factors as the Nuclear Mediator for PTEN-AR Antagonism in Prostate Cancer Cells

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Role of FoxO Factors as the Nuclear Mediator for
PTEN-AR Antagonism in Prostate Cancer Cells

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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Date of Approval:
July 9, 2008

Keywords: akt, androgen, n/c interaction, src, nuclear receptor,fkhr

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Dedication

To my parents Yiyun Ma and Shuixian Li
Acknowledgements

Thanks to Dr. Wenlong Bai, my advisor, for his considerate mentorship, insight and support.

Thanks to committee members: Drs. Cameron, Nicosia, Tsibris and Wu for their invaluable guidance, suggestion, and encouragement.

My appreciation also goes to Dr. Zafar Nawaz for willing to be my outside chair.

Thanks to all the faculty, staff and students in the Department of Pathology and Cell Biology, Office of Graduate Affairs for helping me along the way.

Thanks to Dr. Tsibris for valuable suggestions on my proposal, paper and dissertation.

Thanks to my friends and colleagues for sharing my joy and sadness in my study life.

This dissertation and all my success are dedicated to my dear parents: Yiyun Ma and Shuixian Li and my brothers for their life-long loving, unselfish giving and unlimited support.
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Role of FoxO Factors as the Nuclear Mediator for PTEN-AR Antagonism in Prostate Cancer Cells

Qiuping Ma

ABSTRACT

FoxO proteins are transcriptional factors acting downstream of the tumor suppressor PTEN. Their activity is negatively regulated by AKT-mediated phosphorylation. Our previous studies showed a mutual suppression between PTEN and the androgen receptor (AR) in regulating growth and apoptosis in prostate cancer (PCa) cells. We hypothesize that nuclear FoxO proteins are involved in mediating this mutual antagonism. In this dissertation, we report that PTEN inhibits AR activity through FoxO1 and provide evidence for the involvement of FoxO factors in the androgen-mediated suppression of PTEN-induced apoptosis. Our studies identify a novel mechanism for AR inhibition by FoxO1 and demonstrated the participation of FoxO1 in AR inhibition by PTEN. Ectopic expression of active FoxO1 decrease the transcriptional activity of the AR as well as androgen-induced cell proliferation and production of prostate-specific antigen in PCa cells. FoxO1 knock down by RNA interference increased the transcriptional activity of the AR in PTEN intact cells and relieved its inhibition by ectopic PTEN in PTEN
null cells. Mutational analysis revealed that FoxO1 region 150-655, which contains the fork head box and C-terminal activation domain, was required for AR inhibition.

Mammalian two-hybrid assays demonstrated that the inhibition of AR activity by PTEN through FoxO1 involved the interference of androgen-induce interaction of the N- and C-termini of the AR and the recruitment of the p160 coactivators to the AR N-terminus. In addition to the inhibition of AR by FoxO1, we also demonstrated that PTEN-induced apoptosis is mediated through FoxO factors and that AR inhibited FoxO1 activity by yet-to-be identified downstream target gene. Mutation of AR DNA binding domain partially relieved the inhibition of FoxO1 transcriptional activity by androgens. Inhibition of new protein synthesis abolished the AR-mediated decrease in the mRNA level of FoxO1 target gene. Overall, these studies reveal novel mechanisms for the mutual inhibition of AR and FoxO1 activity and establish FoxO proteins as important nuclear factors that mediate the mutual antagonism between AR and PTEN tumor suppressor in PCa cells.
INTRODUCTION

1. Nuclear Receptor Superfamily

   Effects of androgens are mediated through the Androgen Receptor (AR) that is a ligand regulated sequence specific transcription factor belonging to the nuclear receptor superfamily. The nuclear receptors are characterized by a central DNA-binding domain (DBD), which targets the receptor to specific DNA sequences known as hormone response elements. The DBD is composed of two highly conserved zinc fingers that set the nuclear receptors apart from other DNA-binding proteins. The C-terminal half of the receptor encompasses the ligand-binding domain (LBD), which possesses the essential property of hormone recognition and ensures both specificity and selectivity of the physiologic response (1) (Fig.1)

1.1. Classification

   The nuclear receptor superfamily can be broadly divided into four classes based on their dimerization and DNA-binding properties (1). Class I receptors include the known steroid hormone receptors, which contain highly conserved DBDs binding to DNA half-sites organized as inverted repeats, moderately conserved LBDs, and divergent
Class I: Steroid Receptors

- AR: androgen
- GR: glucocorticoid
- PR: progesterone
- MR: mineralcorticoid
- ER: estrogen

Class II: RXR Heterodimers

- VDR: 1,25-(OH)_{2}-VD3
- TR\alpha,\beta: thyroid hormone
- RAR\alpha,\beta,\gamma: all-trans RA
- PPAR\alpha,\beta,\gamma: eicosinoids
- EcR: ecdysone

Class III: Dimeric Orphan Receptors

- RXR(usp): 9-cis RA
- COUP/ARP: ?
- HNF-4: ?

Class IV: Monomeric Steroid Receptors

- NGFI-B: ?
- NGFI-B (CEB-1): ?
- ELP/SF-1 (Ftz-F1): ?

Figure 1. Nuclear Receptors Share Common Structure/Function Domains.
Figure 1. Nuclear Receptors Share Common Structure/Function Domains.

A typical nuclear receptor contains a variable N-terminal region (A/B) containing transcriptional activation function 1 (AF-1), a central conserved DBD (C), a variable hinge region (D), a conserved LBD (E) containing activation function 2 (AF-2), and a variable C-terminal region (F), the function of which is poorly understood. Nuclear receptors can be grouped into four classes according to their ligand binding, DNA binding, and dimerization properties: steroid receptors, RXR heterodimers, homodimeric orphan receptors, and monomeric orphan receptors. Shown are representing receptors for each group with the corresponding ligands. Question marks refer to orphan receptors for which ligands are not known. Adapted from Mangelsdorf, D. J. et al.(1)
N-terminal domains NTD (1-4). Class II receptors heterodimerize with Retinoid X receptor (RXR) and characteristically bind to direct repeats (although some bind to symmetrical repeats as well). Class III receptors bind primarily to direct repeats as homodimers. Class IV receptors typically bind to extended core sites as monomers. Most of the orphan receptors fall into class III and IV categories (1)

1.2. Androgens and the Androgen Receptor

1.2.1. Androgens

Testosterone is a Carbon 19 steroid that, together with the more potent 5α-reduced metabolite dihydrotestosterone (DHT), is responsible for male development in utero as well as secondary sexual characteristics, male reproductive function and fertility at puberty and in adult life. The prostate gland depends on androgens for its development and for the maintenance of its integrity. Androgens are also mitogens that stimulate Prostate Cancer (PCa) growth (see below).

1.2.2. Androgen Receptor

1.2.2.1. AR Gene’s Chromosomal Location and Structure

In 1988, human AR complementary DNA was cloned demonstrating that the AR gene is located on chromosome Xq11-12 (5, 6). The gene is oriented with the 5’ end toward the centromere and spans ~90 kb of DNA containing eight exons that code for a ~ 2,757-base pair (bp) open reading frame within a 10.6-kb mRNA (7, 8). The AR genomic organization is conserved throughout mammalian evolution from rodents to man. Fig.2
Figure 2. Genomic Organization and Protein Domains of the AR.
Figure 2. Genomic Organization and Protein Domains of the AR.

The genome spans more than 80 kb that includes the exonic organization shown in the second panel. Location of three codon repeat regions in the first exon that codes for the NTD is shown in the third panel. The diagram of the protein structure demonstrates how the exon organization translates into discrete functional regions of the receptor.

N-terminal A/B region (NTD) is encoded by a single large exon (exon 1). It contains the major activation function (AF-1) and is involved in contacting the transcriptional machinery and transcription regulation. A most highly conserved DBD, the central 60-100 amino acids, encoded by exon 2 and 3, containing two zinc fingers, are responsible for targeting the receptor to specific sequences in the genome associated with target genes.

A short highly conserved hinge region, containing a bipartite nuclear localization signal (NLS, amino acids 608-625), links the LBD and DBD. A highly conserved LBD, which contains a weaker activation function (AF-2), is encoded by exons 4-8. Adapted from Quigley et al. (9).
illustrates the organization of the AR gene and the domain structure of the protein. The NTD is encoded by a single large exon (exon 1). A most highly conserved DBD, the central 60-100 amino acids, encoded by exon 2 and 3. A short highly conserved hinge region, containing a bipartite nuclear localization signal (NLS, amino acids 608-625), links the LBD and DBD. A highly conserved LBD, which contains a weaker activation function (AF-2) encoded by exons 4-8.

1.2.2.2. AR Protein Structure and Activity

1.2.2.2.1. N-terminal Domain

The AR NTD is characterized by a number of amino acid repeat sequences, including poly-glutamine (Q), poly-glycine (G) and poly-proline (P). Most noteworthy of these is a CAG triplet repeat that begins at codon 58 and extends for an average of 21 ± 2 repeats (10). The CAG triplet codes for the amino acid glutamine. The length of the CAG repeat unit can affect AR activity and influence PCa risk (see below). Expansion of the AR CAG repeat to a length greater than 40 results in Kennedy’s disease or spinal and bulbar muscular atrophy, a neurodegenerative condition associated with selective neuronal cell death in brainstem and spinal cord (10, 11). This change leads to a reduction in the ability of the full-length AR or the isolated NTD to activate transcription.

Two transactivating functions (AFs) have been characterized in the AR, AF1 in the NTD and AF2 in the LBD. The NTD is the primary effector region that is largely responsible for transactivation whereas AF2 in the LBD appears to be a weak
transactivation domain. Deletion of the LBD from the AR results in a residual N-terminal fragment with constitutive activity nearly equal to the transcriptional activity of the ligand-bound, full-length protein (12-15). This finding is in contrast to what occurs with the closely related estrogen receptor (ER), in which AF2 is the major activation domain and AF1 has variable independent activity (16). The situation in the AR is still more complex, in that two discrete, overlapping activation domains exist in NTD and their usage is context dependent (17). The precise residues and mechanisms that contribute to the AF1 activity of the AR have not been conclusively established. Studies have defined amino acids 142-485 as the AF1 domain. A core region Tau1 (transcription activation unit 1) located between residues 101 and 360 contributes more than 50% of the activity. In contrast, a smaller part of the NTD (amino acids 360-528) is sufficient for the constitutively active AR to induce transcription, whereas Tau5 (amino acid 370-494) contributes more than 50% of activity. However, the size and location of the active TAUs in the human AR NTD are variable, being dependent on the promoter context and presence or absence of the LBD (18, 19).

1.2.2.2.2. DNA Bind Domain

Recognition and binding of DNA is achieved by the DBD (amino acid 537-626), which comprises two Zn-binding motifs, with four cysteine residues coordinating each Zn ion. A consensus DNA-binding site, 5’-AGGCTCTnnnA/TGTTCT/C-3’, is identified as androgen response element (ARE) for DNA binding. Interestingly, this sequence can be
read as an imperfect palindrome or as a direct repeat. In addition, the presence of multiple AREs in a promoter region causes tandem promoter binding and enhancement of AR specificity and action (20).

1.2.2.2.3. Ligand Binding Domain

Whereas ER and thyroid hormone receptor (TR) LBDs form dimers in crystal structure, the crystal structure of the ligand-bound AR LBD is monomeric (21). Since there is evidence to suggest that ligand-bound, full-length AR dimerizes in vivo, it is likely that the N-terminal region of AR is important for protein dimerization. In contrast, ligand-induced conformational changes within the LBD are important for the AR N- and C-terminal interaction (N/C interaction) (22, 23). Helices 4, 5, and 10 are the primary AR contact regions for ligand binding. Ligand binding that induces folding of helix 12 to overlie the pocket discloses a groove that binds a region of the NTD. Coactivator molecules can also bind to this groove, but the predominant site for coactivator binding to AR is in the NTD (21, 24, 25) (see below).

1.2.2.3. Molecular Mechanisms of AR Action

The process of ligand-induced transformation of the AR is not completely understood, the ligand-free AR exists predominantly in the cytoplasm, where it is sequestered by binding to chaperone molecules such as heat shock proteins (26). Upon ligand binding: 1) heat shock proteins are dissociated; 2) the AR translocates to the nucleus influenced by the hinge region; 3) the AR conformation changes; 4) the AR
forms homodimers; 5) post-translational modifications of the AR occur, such as phosphorylation and acetylation; 6) the AR binds to AREs on the DNA; 7) interaction with specific target proteins, such as TFIIF or Trimethylamine n-oxide (TMAO), results in folding of the AF1 domain and an increase in α-helix content. The folding of helix 12 results in the generation of a surface that facilitates further protein-protein interactions. In order to regulate transcription, two general steps must be accomplished: 1) the remodeling of chromatin structure to open up regulatory regions and the promoter and 2) the recruitment of the general transcription machinery to the promoter to enhance transcription initiation and/or elongation. The AR can potentially regulate both these steps, leading to an increase in the level of target gene mRNA.

1.2.2.4. Regulation of AR Function

1.2.2.4.1. Phosphorylation, Acetylation and Sumoylation

AR is phosphorylated at a number of sites in response to agonist binding that results in nuclear localization, but usually not in response to antagonists (27, 28). The majority of the identified phosphorylation sites map to the NTD and the AF1 region (29), suggesting that this modification may modulate receptor-dependent transactivation directly. Possible mechanisms could involve alteration in protein-protein interactions and/or in protein structure and stability.

AR acetylation is induced by the ligand DHT and by histone deacetylase (HDAC) inhibitors in living cells. Fu et al. reported that acetylation of the AR enhances binding of
the p300 coactivator protein(30). The AR acetylation promotes survival and growth of PCa cells in soft agar and in nude mice and trichostatin A (TSA) augments the transcriptional activity of androgens-ligated AR (31). The HDAC binding protein Smad3 inhibits the activity of the androgens-ligated AR (32, 33). HDAC1 bound to the AR in vivo, and HDAC1 binding to the AR is dissociated by the ligand DHT.

Recent reports indicate that the AR could be regulated by SUMO (named for small ubiquitin-like modifier protein) modification. AR is sumoylated in vivo at lysine residues 386 and 520. Mutation of both sumoylation sites results in enhancing AR transcriptional potential (34), indicating that SUMO modification plays a negatively regulatory role in modulating AR activity. SUMO-specific proteases, SENP1, profoundly enhances AR-dependent transcription (35).

1.2.2.4.2. Coactivator Binding

Transcriptional coactivators are recruited to the promotor through protein-protein interaction with the receptor (36-39). Most known coactivators are complex proteins that harbor multiple activation domains and receptor-interacting domains (40, 41). The best studied group of coactivators is the p160 steroid receptor coactivator family with three family members SRC1 (steroid receptor coactivator 1), AIB1 (amplified in breast cancer-1), TIF2/GRIP1 (the transcription intermediary factor 2, or the human orthologue of glucocorticoid receptor-interacting protein 1). Upon recruitment to the promoters by nuclear receptors, the coactivators affect transcription by modifying the chromatin
structure either by themselves or through the recruitment of cofactors involving histone acetyltransferases (HATs) such as CBP/p300, ATP-dependent chromatin remodeling complexes such as SWI/SNF or PBAF, the mediator complex (Mediator/TRAP/DRIP) and histone methyltransferase such as coactivator-associated arginine methyltransferase 1 (CARM1) (42, 43). The ubiquitin-proteasome pathway enzymes have also been reported to be recruited to the steroid nuclear receptor target gene promoters during transcription. One such enzymes is E6-associated protein (E6-AP), which possesses two independent separable functions; a coactivation function and an ubiquitin-protein ligase activity (44).

The three-dimensional structures of the LBDs of the steroid hormone receptors fold into 12 helices that form a ligand-binding pocket. Helices 3, 4, 5, and 12 are the sites of AF2 activity that was discovered by mutational analysis to be important for p160 coactivators binding (24). Coactivator molecules contain a consensus motif LXXLL (L is leucine and X is any amino acid) in a region called the nuclear receptor (NR) box, which binds to the groove in steroid hormone receptors (45, 46). Many of these proteins belong to the steroid receptor coactivator family and bind to the AF2 region in the C terminus of ligand-bound receptors.

Whereas TR, for example, interact with coactivators via contact points in the LBD, the AR interacts with the p160 coactivators via two surfaces, one in the LBD domain and one in the NTD. The LBD interacts with the NR box, whereas the NTD has a high affinity
for a specific glutamine-rich region in the p160 coactivators (for example, 1053-1123 in SRC1). Both Tau1 and Tau5 domains are implicated in the recruitment of p160 coactivators (17, 47). p160 coactivators in which the LXXLL motifs are mutated retain most of their coactivator activity for the full-length AR, although they are no longer functional for the isolated LBD. These data suggest that in the native AR the efficient recruitment of coactivators occurs primarily through the NTD (14). The binding site for p160 coactivators includes amino acids 360-494, although additional regions of the AR NTD may contribute to this interaction. In addition to common p160 coactivators, AR is also modulated by AR-selective coactivators such as ARA54, ARA70 (48-50).

Even though the mechanism of interaction between AR and coactivators may differ from that of other steroid hormone receptors, coactivators are still essential for AR action in vivo. This is underscored by the observation that the androgen insensitivity syndrome occurred in an individual with a mutation in a coactivator gene and a normal AR gene (51). Overexpression of these coactivators increases AR transactivation at physiological concentrations of adrenal androgen. AR coactivators enhance transactivation of AR several fold and therefore potentially increase the risk of PCa.

1.2.2.4.3. AR N- and C-terminal Interaction

Interaction between the NTD and LBD of the ligand-bound receptor, namely N/C interaction, is essential for AR activity (52-57). The importance of N/C interaction is underscored by the fact that complete androgen insensitivity syndrome can be caused by
missense mutations in helix 12 and in other regions of the LBD that interact with the NTD (9). This interaction facilitates binding of p160 coactivators to AR in a manner independent of the LXXLL NR boxes. Moreover, the N/C interaction stabilizes receptor-ligand interactions and is required for ligand-dependent activation of the receptor. In fact, two pentapeptide regions of the AR NTD, $^{23}\text{FQNLF}^{27}$ (FXXLF) and $^{433}\text{WHTLF}^{437}$ (WXXLF), mediate binding of the N terminus to the C-terminal region of AR. $^{23}\text{FQNLF}^{27}$ interacts with the AF2 groove formed by helices 3, 4, 5, and 12 and competes favorably with LXXLL-containing coactivator proteins for ligand-dependent binding to the LBD (58). The pentapeptide $^{433}\text{WHTLF}^{437}$ also binds to the C-terminal region but at a site outside the AF2 groove. Substitution of glutamic acid by glutamine at position 888 (E888Q) in the AF2 activation domain (AD) core region in the LBD, markedly decreased the interaction with the NTD. This mutation neither influenced hormone binding nor LBD homodimerization, suggesting a role of the AF2 AD core region in the functional interaction between the NTD and the LBD.

1.2.2.5. Androgen-Regulated Genes

Results from DNA microarrays (59-61) have led to the identification of a large number of androgen regulated genes which can be classified into several functional groups, including metabolism, protein chaperoning and trafficking, protein synthesis, secretions, cell cycle and apoptosis, structural and extracellular matrix proteins, and proteins with no known functions. The specific identified androgen-responsive genes
include Prostate Specific Antigen (PSA) (62) (see below), hKLK2 (human prostate-specific kallikrein) (63), which has been proposed as potential biomarkers for PCa (64); NKX3.1 (65), which is a potential tumor suppressor gene for PCa (66-68); probasin (69), prostatein C3 (70), and cyclin D1 (71).

PSA is a secreted protein that is abundantly expressed by prostate epithelial cells with serum levels correlating with tumor burden. Serum PSA levels are routinely used by clinicians to monitor treatment responses, prognosis and progression of patients with PCa. A rising titer of serum PSA after an initial response to androgen deprivation is the earliest indication of tumor progression and is correlated with reduced survival. Once serum PSA levels are elevated in the absence of androgens, the average survival time is 2 years.

2. **PI3K-PTEN-AKT Pathway**

2.1. **PI3K Activity and Regulation**

Phosphatidylinositol (PtdIns) plays a crucial role in signal transduction as the precursor of several second-messenger molecules. Although multiple forms of phosphoinositide 3-kinases (PI3Ks) exist in higher eukaryotes, the class Ia enzymes are primarily responsible for production of D-3 phosphoinositides in response to growth factors (72). The class I family of PI3K is activated downstream of receptor tyrosine kinases (RTK) or G protein-coupled receptors (GPCR). Class Ia enzymes are heterodimers of regulatory and catalytic subunits. The regulatory subunit called p85
proteins maintains the p110 catalytic subunit in a low-activity state in quiescent cells and mediates its activation by direct interaction with phosphotyrosine residues of activated growth factor receptors or adaptor proteins. The activated PI3K converts the plasma membrane lipid phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2 or PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PI(3,4,5)P3 or PIP3). Signaling proteins with pleckstrin-homology (PH) domains accumulate at sites of PI3K activation by directly binding to PIP3. Of particular interest are the protein serine-threonine kinases AKT [also called protein kinase B (PKB)] and phosphoinositide-dependent kinase 1 (PDK1) (Fig.3). Association with PIP3 at the membrane brings these proteins into proximity and facilitates phosphorylation of AKT by PDK1. This phosphorylation stimulates the catalytic activity of AKT. Recently, many activating mutations in the PI3KCA gene (coding for the p110α catalytic subunit of PI3K) have been described to be present in human tumors. Activation of PI3K and AKT are reported to occur in breast (75-77), ovarian (78, 79) and other cancers (80-83).

The termination of PI3K signaling by degradation of PI(3,4,5)P3 can be mediated by at least two different types of phosphatases. The Src-homology 2 (SH2)-containing phosphatases (SHIP1 and SHIP2) dephosphorylate the 5 position of the inositol ring to produce PI(3,4)P2. Although this dephosphorylation impairs some signaling downstream of PI3K, PI(3,4)P2 can also mediate PI3K-dependent responses and may mediate events
Figure 3. Signalling Pathways of PI3K-PTEN-AKT-FoxO.
Figure 3. Signalling Pathways of PI3K-PTEN-AKT-FoxO.

In the cytoplasm, PI3K is activated downstream of RTK and GPCR to covert PIP2 to PIP3, leading to AKT activation. PTEN phosphatase antagonizes PI3K by converting PIP3 to PIP2. Localized in the nucleus in the absence of insulin or growth factors, FoxO factors cause cell cycle arrest, stress resistance, and cell death by upregulating a series of key target genes. In the presence of insulin or growth factors, the PI3K–AKT pathway is activated. AKT translocates to the nucleus where it directly phosphorylates FoxO transcription factors on three conserved residues. Phosphorylated FoxO factors bind to 14-3-3 proteins, which result in the export of FoxO factors from the nucleus into the the cytoplasm and degradation. The sequestration of FoxO into the cytoplasm inhibits FoxO-dependent transcription and allows cell proliferation, stress sensitivity, and cell survival. Note that this figure does not include all FoxO target genes. p21 and p27: CDK inhibitor; GADD45: growth arrest and DNA damage-inducible protein 45; Cyclin G2: a cyclin that associates with active protein phosphatase 2A and promotes a G1/S-phase cell cycle arrest, CDK2 inhibition and the formation of aberrant nuclei; MnSOD: manganese superoxide dismutase; Catalase: an enzyme that catalyzes the decomposition of hydrogen peroxide into water and oxygen; Atrogin-1: a muscle-specific F-box protein highly expressed during muscle atrophy; FasL: Fas ligand; Bim: pro-apoptotic BH3-only Bcl-2 family member; Puma: p53-upregulated modulator of apoptosis; BTG-1: B-cell translocation gene 1; DDB1: damage-specific DNA-binding
protein 1; PEPCK: phosphoenolpyruvate carboxykinase; G6Pase:
glucose-6-phosphatase; SCPx: Sterol carrier protein X-related thiolase. Partly adapted
from van der Horst, A.(73) and Baker, S. J. (74).
independent of those stimulated by PI(3,4,5)P3. Another phosphatase is PTEN, which dephosphorylates the 3 position of PI(3,4,5)P3 to produce PI(4,5)P2 (See below).

2.2. PTEN

The discovery of PTEN/MMAC1 for “phosphatase and tensin homolog deleted on chromosome 10’ or “mutated in multiple advanced cancers” were first reported by two groups in 1997 (84, 85). The PTEN gene resides within chromosome region 10q23.

Germline mutations of PTEN are found in three related human autosomal dominant disorders, Cowden disease (CD) (86), Lhermitte-Duclos disease (LDD) and Bannayan-Zonana syndrome (BZS) (87), characterized by tumor susceptibility and developmental defects.

Somatic deletions or mutations of PTEN have been identified in a large fraction (12-60%) of a wide variety of tumors, tumor cell lines and xenographs (84, 85). Loss of PTEN is more often associated with advanced stage tumors, such as glioblastoma (88), melanoma (89) and PCa (90, 91).

Evidence that PTEN functions as a tumor suppressor genes include: 1) homozygous disruption of PTEN results in early embryonic lethality; 2) heterozygous PTEN mice display hyperplastic-dysplastic changes in the prostate, skin and colon, which are also seen in CD, LDD and BZS. 3) PTEN inactivation enhanced the ability of embryonic stem (ES) cells to generate tumours in nude and syngeneic mice, due to increased anchorage-independent growth (92); 4) PTEN-deficient immortalized mouse
embryonic fibroblasts exhibit decreased sensitivity to cell death in response to a number
of apoptotic stimuli, accompanied by constitutively elevated activity and phosphorylation
of AKT, a crucial regulator of cell survival (93).

PTEN encodes a dual and protein phosphatase whose main in vivo substrate is
PIP3. PTEN suppresses tumor growth by negatively regulating this signal transduction
pathway (94). Loss of PTEN function, either in murine ES cells or in human cancer cell
lines, results in accumulation of PIP3 mimicking the effect of PI3K activation and
triggering the activation of its downstream effectors, PDK1, AKT/PKB and Rac1/Cdc42.

2.3. AKT

AKT is a serine-threonine kinase downstream of PTEN/PI3K that has three family
members: AKT1, AKT2 and AKT3, which are encoded by three different genes (95). They
are ubiquitously expressed, but their levels are variable depending upon the tissue type.
The N-terminus of AKT contains a PH domain that binds phospholipids, a central kinase
domain, and a regulatory serine phosphorylation site in the C-terminus. AKT activity is
regulated by PI(3,4,5)P3, which recruits AKT to the cell membrane, permitting its
activation by PDK1(96). Activated PDK1 phosphorylates AKT at T308, activating its
serine-threonine kinase activity (100-fold over the basal). Once phosphorylated in T308,
further activation occurs by PDK2 (the complex rictor-mTOR or DNA-PK) by
phosphorylation at S473. AKT activation stimulates cell cycle progression, survival,
metabolism and migration through phosphorylation of many physiology substrates
Most of the known protein targets of AKT become inhibited by the phosphorylation event to keep them in inactive states or to promote their degradation. This includes FoxO1, the apoptosis-inducing protein BAD, glycogen synthase kinase 3 (GSK3).

### 2.4. The FoxO Family of Forkhead Transcription Factors

#### 2.4.1. The Fox Family

The winged helix/forkhead class of transcription factors is characterized by a conserved 100-amino-acid, monomeric DBD termed the 'forkhead box' (101). The Forkhead family is present in all eukaryotes. In humans, the Forkhead family is comprised of 39 distinct members, which have been divided into 19 subgroups (Fox for 'Forkhead Box' A to S). Fox transcriptional regulators play a wide range of roles during development, ranging from organogenesis (FoxC) to language acquisition (FoxP) (102).

#### 2.4.1.1. FoxO Subgroup

Among the Forkhead family, the FoxO subgroup contains four members (FoxO1/FKHR, FoxO3/FKHRL1, and FoxO4/AFX and FoxO6). FoxO1, FoxO3, and FoxO4 mRNAs are expressed to varying degrees in all tissues in mammals (103-105). FoxO1 mRNA is particularly abundant in adipose tissues, FoxO3 mRNA is highly expressed in the brain, and FoxO4 mRNA is abundant in the heart. FoxO6 mRNA is predominantly expressed in the developing brain, indicating that FoxO6 may play an important role in the nervous system.
2.4.1.1.1. Structure

FoxO proteins consist of four domains: a highly conserved forkhead DBD, a nuclear localization signal (NLS) located just downstream of DBD, a nuclear export sequences (NES) and a C-terminal transactivation domain. The first 1-150 residues in the NTD of FoxO1 was reported to contain a transactivation function (106). The regions with the highest sequence conservation include the N-terminal region surrounding first AkT/PKB phosphorylation site, the forkhead DBD, the region containing NLS and the part of the C-terminal domain (Fig.4).

2.4.1.1.2. Regulation of FoxO Functions through Multiple Mechanisms

2.4.1.1.2.1. Post-Translational Modification of FoxO Proteins

FoxO functions are regulated through post-translational modifications induced by two different but interconnected circuits: the regulation by growth factor (insulin) signalling mainly through PI3K–AKT/PKB and a second pathway that is activated by oxidative-stress signalling. In contrast to insulin signalling, oxidative stress induces nuclear translocation of FoxO, which correlates with acetylation, monoubiquitylation (72), JNK-mediated phosphorylation (72) and β-catenin binding (107). Although these events correlate with nuclear translocation, the mechanisms that mediate FoxO nuclear translocation remain largely unknown.
2.4.1.1.2.1.1. Phosphorylation

The FoxO family of transcription factors (except FoxO6) is one of the major direct substrates of the protein kinase AKT in response to cellular stimulation by growth factors or insulin. Phosphorylation of FoxO factors by AKT triggers the rapid relocalization of FoxO proteins from the nucleus to the cytoplasm by binding to 14-3-3 proteins (108, 109). AKT phosphorylates FoxO1, FoxO3, and FoxO4 at three key regulatory sites that are conserved from Caenorhabditis elegans to mammals and are part of a perfect consensus sequence for AKT phosphorylation (RXRXX(S/T)) (110). Thus, FoxO transcription factors integrate a broad range of external stimuli via phosphorylation of three conserved residues by AKT. FoxO6 lacks the third AKT regulatory site and, although it is phosphorylated at the first two sites and inactivated, it does not translocate from the nucleus to the cytoplasm like other FoxO factors. Additional regulatory pathways can also influence FoxO activity such as SGK, a protein kinase related to AKT and recognizes the same consensus sites has been shown to phosphorylate FoxO factors, casein kinase 1 (CK-1), dual-specificity tyrosine-phosphorylated and regulated kinase 1A (DYRK1A), IKKβ, and the Ras–Ral pathway (111).

In contrast, phosphorylation of FoxO factors by the MAPK family member, JNK, mammalian sterile 20-like kinase-1 (MST1) in response to stress stimuli, results in the movement of FoxO factors from the cytoplasm to the nucleus.
Figure 4. Schematic of the Domains in FoxO1.

AF1, AF2: transactivation function 1,2; FK: forkhead domain; NLS, nuclear localization signal; NES, nuclear export sequence. LXXLL domain: nuclear receptor interaction domain. Amino acids are for human FoxO1. The posttranslational phosphorylation modifications site numbers for human FoxO family members are also shown. S: serine; T: threonine.
2.4.1.1.2.1.2. Acetylation

Oxidative stress enhances the histone acetyltransferases (HATs)–FoxO interaction, which correlates with the observed increased in FoxO acetylation (112-114). Mammalian FoxOs that are acetylated by HATs are deacetylated \textit{in vitro} and \textit{in vivo} by the mammalian homologue of SIR-2.1, SIRT1 (106, 112-119). In addition to SIRT1, other HDACs may regulate FoxO activity because the treatment of cells with TSA induces FoxO acetylation (109-111) and affects FoxO localization (120). The precise role of acetylation and SIRT1 in FoxO regulation are unclear. The effect of deacetylation is target gene specific. The expression of proapoptotic genes is inhibited, while expression of genes that regulate cell cycle arrest (\(p27^{kip}\)) and resistance to oxidative stress (\(MnSOD\)) is increased (112).

2.4.1.1.2.1.3. Ubiquitination

Monoubiquitylation strongly increases FoxO transcriptional activity and therefore monoubiquitylated FoxO might represent the fully active form of FoxO. The degradation of FoxO transcription factors is mediated by the ubiquitin–proteasome pathway (111, 121-124). For FoxO1 and FoxO3, ubiquitin degradation requires AKT activation and phosphorylation of the AKT regulatory sites. Data from our lab suggest that FoxO1 needs to be present in the cytoplasm to be successfully polyubiquitinated by an E3 ubiquitin ligase and subsequently degraded. The S-phase kinase-associated protein 2 (p45)
(SKP2) ubiquitin ligase is required for FoxO1 proteolysis (122, 124). IKKβ phosphorylation of FoxO3 at Ser 644 results in ubiquitination and degradation of FoxO3.

2.4.1.1.2.2. FoxO Interacting Proteins

The observation that FoxO mutant in which DNA-binding was abolished was still able to effectively regulate a specific subset of target genes suggest that FoxO likely regulates these gene through interaction with other transcription factors (125).

Indeed it has become apparent that FoxO proteins are able to associate with a wide variety of diverse transcription factor partners resulting in a far broader spectrum of receptor gene regulations (Table 1) (126).

2.4.1.1.3. FoxO Functions in Mammalian Cells

In cell culture-based systems, FoxO1, FoxO3, and FoxO4 behave similarly in biochemical studies, appear to regulate common target genes, and bind to the same target DNA sequence (104, 105, 108, 111, 124). Yet, mouse FoxO knockouts have revealed unique roles for the FoxOs, such as the requirement for FoxO3 in ovarian primordial follicle activation (127, 128) and FoxO1 in vasculogenesis (128, 129). However, while the three FoxOs serve some discrete functions, they likely have significant redundancies, as they are broadly expressed during embryonic development and in adult tissues (105). This family of transcription factors, depending on the promoter context and extracellular conditions, may activate or repress transcription, leading to various functions including metabolism, muscle atrophy, cell cycle repair and detoxification, cell cycle
Table 1. FoxO Factors Binding Partners

<table>
<thead>
<tr>
<th>Partner</th>
<th>Citation</th>
<th>Effect on FoxO ( or Partner)</th>
</tr>
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<tbody>
<tr>
<td>CBP/P300</td>
<td>Fukuoka M et al., 2003</td>
<td>-</td>
</tr>
<tr>
<td>SirT1</td>
<td>Brunet A et al., 2004 ; Motta MC et al., 2004</td>
<td>+/-</td>
</tr>
<tr>
<td>14-3-3</td>
<td>Brunet A et al., 1999</td>
<td>-</td>
</tr>
<tr>
<td>USP7/HAUSP</td>
<td>Van der horse A et al., 2006</td>
<td>-</td>
</tr>
<tr>
<td>B-catenin</td>
<td>Almeida M et al., 2007; Essers MA et al., 2005</td>
<td>+</td>
</tr>
<tr>
<td>IKK</td>
<td>Hu MC et al., 2004</td>
<td>-</td>
</tr>
<tr>
<td>MST1</td>
<td>Lehtinen MK et al., 2006</td>
<td>+</td>
</tr>
<tr>
<td>Myocardin</td>
<td>Liu ZP et al., 2005</td>
<td>(+)</td>
</tr>
<tr>
<td>JNK</td>
<td>Oh SW et al., 2005</td>
<td>+</td>
</tr>
<tr>
<td>PGC1</td>
<td>Puigserver P et al., 2003</td>
<td>+</td>
</tr>
<tr>
<td>FoxG1</td>
<td>Seoane et al., 2004</td>
<td>-</td>
</tr>
<tr>
<td>SMAD</td>
<td>Seoane, J et al., 2004</td>
<td>+</td>
</tr>
<tr>
<td>Notch</td>
<td>Kitamura T et al., 2007</td>
<td>(+)</td>
</tr>
<tr>
<td>FHL2</td>
<td>Yang, Y et al., 2004</td>
<td>+</td>
</tr>
<tr>
<td>RUNX3</td>
<td>Yamamura, Y et al., 2006</td>
<td>+</td>
</tr>
<tr>
<td>PR</td>
<td>Kim et al., 2005</td>
<td>+/-</td>
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<tr>
<td>PPAR α</td>
<td>Qu et al., 2007</td>
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<tr>
<td>PPAR γ</td>
<td>Dowell P et al., 2003</td>
<td>- (−)</td>
</tr>
<tr>
<td>ERα</td>
<td>Schuur, ER et al., 2001; Zhao HH et al., 2001</td>
<td>- (+/−)</td>
</tr>
<tr>
<td>AR</td>
<td>Li et al., 2003; Fan et al., 2007</td>
<td>- (−)</td>
</tr>
</tbody>
</table>
Table 1. FoxO Factors Binding Partners

‘-’, inhibition of FoxO, ‘+’ activation of FoxO; (-) or (+), inhibition or activation of partner protein. Myocardin, a transcriptional coactivator of SM gene; BTG-1, a argine methyl transferase; USP7, Ubiquitin-specific Protease; HUPSA, herpevirus-associated ubiquitin-specific protease; CBP, CREB-binding protein; IKK, IkappaB kinase; MST1, Mammalian STE20-like protein kinase 1; JNK, c-Jun N-terminal kinase; PPARγ, proliferator-activated receptorγ; PGC-1α, PPARγ coactivator-1; SMAD, Small Mothers Against Decapentaplegic; FHL2, Four-and-a-half LIM 2; RUNX3, Runt-related transcription factor 3; PR, progesterone
arrest, cell differentiation, apoptosis, tumor suppression, and stem cell maintenance (130) (Fig. 3).

2.4.1.1.4. FoxO and Cancer

Several lines of evidence indicate that FoxO factors are likely to play a significant role in tumorigenesis: 1) FoxO factors are found at chromosomal translocations in human tumors (rhabdomyosascomas for FoxO1, and acute myeloid leukemias for FoxO3 and FoxO4) (131, 132); 2) FoxO3 is dysregulated in breast cancer (111). The presence of cytoplasmic FoxO3 in breast cancer highly correlates with poor survival; 3) Sustained inhibition of FoxO function is emerging as a critical event in tumourigenesis by PTEN inactivation through mutation or allelic loss (133); 4) FoxO proteins functionally or physically interact with tumor suppressors such as p53 and SMAD (134) or oncogenes such as β-catenin (107) or Myc (135); 5) Expression of active forms of FoxO factors reduces tumorigenicity in nude mouse paradigms induced by IKKβ expression (111) or HER2 oncogene (136); 6) Cells that are deficient for PTEN induce tumorigenesis in nude mice. These tumors are decreased by the expression of a constitutively active form of FoxO1 (125); 7) FoxO1, FoxO3 or FoxO4 nullizygous mice do not have a clear reported increase in tumour-incidence. However, FoxOs probably function redundantly because conditional deletion of Foxo1, Foxo3 and Foxo4 simultaneously results in the development of thymic lymphomas and haemangiomas (137). This confirms that there is
functional redundancy among these closely related transcription factors. It is somewhat surprising that the tumor phenotype of these mice is restricted to only certain tissues.

3. Prostate Cancer

3.1. AR and PCa Risk

PCa continues to be the most common cancer diagnosis and the second leading cause of cancer deaths in American men (138). PCas that have spread beyond the gland are typically treated with hormone ablation therapy aimed at inducing castrate levels of testosterone or blocking testosterone signaling at the AR. Although surgical castration is effective, the majority of men choose medical castration with a leutenizing hormone releasing hormone (LHRH) agonist that is often combined with an oral anti-androgen. Castration induces apoptosis in the majority of PCa cells, which translates clinically to improvement in cancer related symptoms and lowering of AR regulated genes including PSA. Clinically PCa progression has been defined in terms of the patient's response to or failure from hormone therapy. The majority of prostate cancers (85%) will have an initial favorable response to hormone therapy. However, over time molecular and cellular changes occur, which allow PCa cells to grow despite a physiologically low serum testosterone level. Eventually, these PCas will stop responding to all currently prescribed hormone therapy. At this stage the cancers are described as androgen-independent (AI) or hormone refractory. Chemotherapy will have favorable palliative response in 50-75% of men with AI PCa but most will die from their disease within 1-2 years (139).
Androgens are natural PCa promoter. Studies of eunuchoid individuals have suggested that prostates remain small and hypotrophy that PCa does not develop (140). Moreover, animal models of prostate carcinogenesis require the presence of functioning testes or exogenous androgens to support the development of PCa (141, 142). Transgenic mice engineered for elevated AR expression in the prostate have high turnover of prostatic epithelial cells and develop prostatic intraepithelial neoplasia later in life (143).

Different studies have shown that shorter CAG repeat length in the AR NTD is associated with the occurrence of more aggressive PCa (144), earlier age of onset (145, 146), and likelihood of recurrence (147). Intact AR signaling is felt to be necessary for the development of PCa. AR gene amplification has been reported in 25-30% of AI PCa (148-150). Of note the AR amplification was not found in any untreated PCa samples suggesting that AR amplification is involved in the development of AI. AR amplification highlights the strong selective pressure for continued AR signaling as tumors evolve over the course of therapy. The true incidence of AR point mutations in PCa is not known but the literature suggests that they can be found in 20-40% of the cancers. The majority of AR point mutations in PCa have clustered in three areas of the LBD. Mutations in these areas flank the ligand-binding pocket and alter this pocket relative to the wild-type AR to allow binding of ligands other than testosterone or dihydrotestosterone (151) during the
development of AI PCa. The AR is believed to remain an important mediator of growth in AI PCa.

3.2. PTEN-AKT-FoxO1 in PCa

Homozygous deletions, frameshift, or nonsense mutations in PTEN in 100% (4/4) of PCa cell lines (84). PTEN mutations have been identified in 10–15% of all prostate tumors (91, 152, 153) and the loss of heterozygosity (LOH) involving 10q23 is common (49%) in primary prostate carcinomas (154) and occurs in ~60% of advanced PCas (84, 91). Loss of PTEN protein is correlated with pathological markers of poor prognosis in PCa since total absence of PTEN expression correlated with the Gleason score and correlated more significantly with a Gleason score of 7 or higher and with advanced pathological stage (155). The conditional PTEN-/- mouse model develops all stages of progressive PCa including invasion, metastasis and AI proliferation (156).

PCa cell lines that have been obtained from metastatic lesions (e.g. LNCaP, PC3) or that are strictly AI (e.g. 22RV-1, C4-2) harbour highly active PI3K/AKT signalling (157-159). Phosphorylated AKT is also highly expressed in PCas with high Gleason grade (160) and is an excellent predictor of clinical PSA failure (161). Chemical inhibitors targeting the catalytic unit of PI3K, such as LY294002 and Wortmanin, induce a potent apoptotic response in most PCa cell lines including LNCaP, LAPC4 and LAPC9 (162). These results collectively demonstrate the significance of upstream PI3K/AKT signalling activity in providing critical signals for progressive PCa.
Amplification of AR occurs in the PTEN-/ conditional mouse model (H Wu, lab unpublished observations). In vitro observations by our laboratory have shown a mutual antagonism between PTEN and AR in regulating growth and apoptosis in PCa cells (163). We have also reported an AR-dependent repression of the AKT downstream FoxO1 and FoxO3a function by androgens (164). The repression was believed to be through protein-protein interaction between C-terminal residues 350-655 of FoxO1 and the NTD and LBD of AR. The activated AR blocks FoxO’s DNA binding activity and impairs its ability to induce Fas ligand expression and PCa apoptosis and cell cycle arrest. Tindall’s group has reported another mechanism by which androgens induce proteolytic cleavage of FoxO1 at residue Arg537 leading to an inhibitory effect on the transcriptional activity of the intact FoxO protein (165).
HYPOTHESIS AND OBJECTIVES

Previous work from our laboratory showed mutual antagonism effects of AR and PTEN on growth and apoptosis of PCa cells (163). We have also reported an AR-dependent repression of FoxO1 and FoxO3a function by androgens (164). These findings led to the hypothesis that FoxO transcription factors mediate the mutual antagonism between AR and PTEN in PCa cells. In order to verify this hypothesis, the study has two specific aims: Aim1. To clarify whether PTEN inhibits the AR activity through FoxO factors. To pursue this aim, we will: 1. Determine whether FoxO factors inhibit AR activity; 2. Examine the mechanism underlying the AR inhibition by FoxO factors and 3. Test whether FoxO factors participate in the PTEN-induced AR inhibition. Aim2. To investigate whether PTEN induces apoptosis in PCa cells through FoxO and whether androgens suppress PTEN-induced apoptosis by inhibiting FoxO activity. To pursue this aim, we will: 1. Test whether PTEN induced-apoptosis requires FoxO factors and 2. Determine whether AR suppresses FoxO activity, and explore other potential mechanisms for the AR repression of FoxO1.
MATERIALS AND METHODS

1. Plasmids

AREe1bLuc (163), pCMVhAR (163), GalLuc (163), Gal-VP16 (163), pSG5L-HA-PTEN:WT (163), pSG5L-HA-PTEN:G129R (163), pCMVβ (164), FoxO siRNA (166)
PBLuc (167), pT81Luc (167), VP16rAR (5-338) (168), PMLBD (168), GalARN [pAR4G in (18)], ARΔLBD [pAR5 in (18)], PBINDAIB1 (169), Flag-FoxO1 [Flag-FKHR:WT in (164)], Flag-FoxO1ca [FLAG-FKHR:TSS in (164)], 3xIRS (insulin response sequence) (164)
have already been described, VP16 and PM vectors are from Clontech (Mountain View, CA). pCMV5-HA-AKT:T308D,S473D (AKTDD) (170) was a gift from Dr. Alessi, D.R., and pCMV5-HA-AKT:T308A,S473A (AKTAA) (unpublished plasmid) from Dr. Cheng J. ARcagwt, ARcag0, ARcaglong are gifts from Dr. DeFranco D (171).

HA-FoxO1ca was constructed by inserting the BamH1/XbaI fragment from Flag-FKHR:TSS (164) into pcDNA3-HA (Invitrogen, Carlsbad, CA). To construct HA-FoxO1ca(1-150), FoxO1:TSS cDNA fragment expressing N-terminal 150 amino acids with BamHI and XbaI sites at the 5’ and 3’ ends of the DNA fragment, respectively, was amplified by PCR. The amplified FoxO1(1-150) fragment was cloned into the BamHI and
XbaI sites of pcDNA3.1 HA vector (Invitrogen). HA-FoxO1ca(150-270),
HA-FoxO1ca(1-270), HA-FoxO1ca(150-655), HA-FoxO1ca(150-537),
HA-FoxO1ca(256-655) were constructed similarly. The sequences of the upstream and
downstream primers are shown in Table 2. pCDNA3.1-Flag-FoxO1caNRmut and
HA-FoxO1caH215R were generated by site-directed mutagenesis using a QuikChange
site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by direct
sequencing. Flag-FKHR:TSS and HA-FoxO1 were used as the templates for the
mutagenesis. The sequences of the upstream and downstream primers to mutate
Leu-462, Leu-465 and His-215 of HA-FoxO1ca are shown in Table 2. pCMVhARmut was
generated by site-directed mutagenesis using pCMVhAR as the template for the
mutagenesis. The sequences of primers are also shown in Table 2.

2. Cell Culture, Transient Transfections and Reporter Assays

PC3, COS-7 and DU145 cells were maintained in Dulbecco's modified Eagle's
medium (Gibco-BRL, Gaithersburg, MD USA) containing 10% fetal bovine serum (FBS)
(Hyclone, Logan, Utah). LNCaP cells were maintained in RPMI 1640 (Gibco- BRL)
containing 10% FBS. RWPE-1 cells were maintained in Keratinocyte- Serum Free
medium supplemented with 5 ng/ml human recombinant EGF and 0.05 mg/ml bovine
pituitary extract (Gibco-BRL).

For reporter and mammalian two-hybrid assays, cells were plated in 6-well plates
in medium supplemented with 5% dextran charcoal-stripped fetal calf serum (sFBS)
### Table 2: The Primer Sequences for the Plasmids:

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Uptream Sequence</th>
<th>Downstream Sequence</th>
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<tr>
<td>FoxO1ca(1-150)</td>
<td>5'-CGGGGGTCACCGGATCCATGGCCGAGGC-3'</td>
<td>5'-GGAGAAGAGCTGGATCCATGGACAACAAC-3'</td>
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<tr>
<td>FoxO1ca(150-270)</td>
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<td>5'-GagonGTCAGAAGTCGGCCACTCCTTCAAGAGTCC-3'</td>
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<tr>
<td>FoxO1ca(1-270)</td>
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<td>5'-GagonGTCAGAAGTCGGCCACTCCTTCAAGAGTCC-3'</td>
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<tr>
<td>FoxO1ca(150-655)</td>
<td>5'-CTCGCGGGGCGAGGGATCCAAGAGGAGCTCG-3'</td>
<td>5'-GagonGTCAGAAGTCGGCCACTCCTTCAAGAGTCC-3'</td>
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<tr>
<td>FoxO1ca(150-537)</td>
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<td>5'-GagonGTCAGAAGTCGGCCACTCCTTCAAGAGTCC-3'</td>
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<tr>
<td>FoxO1caH215R</td>
<td>5'-GGACTCTTTGAGGAGCTGGCCGACTTCTGACTCTC-3'</td>
<td>5'-GTAGGGACAGATTACGGAGATTTCAATTCCTCCATAC-3'</td>
</tr>
<tr>
<td>pCMVhARmut</td>
<td>5'-GGACTCTTTGAGGAGCTGGCCGACTTCTGACTCTC-3'</td>
<td>5'-GTAGGGACAGATTACGGAGATTTCAATTCCTCCATAC-3'</td>
</tr>
</tbody>
</table>
(Hyclone). 24 h later, cells were transfected by Lipofectamine-plus following the protocol from Invitrogen. Transfected cells were treated with DHT (Sigma, St. Louis, Mo.) or R1881 (NEN Life Science Products, Boston) in medium containing 1% sFBS for 24 h and washed with phosphate-buffered saline (PBS). Cell lysate was prepared as described (163) and luciferase activity determined using the assay systems from Promega Corporation (Madison, WI) following the company’s protocol. β-gal activity was determined as previously described (163). Results represent at least three independent experiments performed in duplicate and error bars stand for standard deviations.

3. **Immunoblotting Analysis**

To determine the expression of various proteins, cells transfected in parallel to the cells prepared for reporter assays or infected with adenovirus were lysed in modified RIPA buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM Na3VO4, 1 mM NaF and protease inhibitor mixture (Calbiochem, San Diego, California). The lysate was separated on an 8% or 10% SDS-PAGE and transferred to nitrocellulose membrane. The membrane was probed with antibodies for AR (Santa Cruz Biotechnolog, Inc., Santa Cruz, CA), FOXO1 (Santa Cruz, H-128), PTEN (Santa Cruz), HA (Berkeley Antibody Laboratories, HA11), Flag (M2, Sigma), β-actin (Sigma), PSA antibody (Santa Cruz), and GFP (Cambridge, MA) and visualized with ECL as described (163).
4. **Adenovirus Infection and Colorimetric MTT Assay**

Recombinant adenovirus expressing GFP-FoxO1TSSA (GFP-FoxO1ca) and GFP control vector were gifts from Dr. J. Milbrandt (172). The recombinant virus were purified on a cesium chloride gradient, titrated, aliquoted, and stored at -80°C until further use. For gene delivery, purified adenovirus was added to LNCaP cells at a multiplicity of infection (MOI) of 100 in RPMI medium containing 5% sFBS for 24 h. Infected cells were washed with medium and cultured for 24 h in the same medium containing ethanol (EOH) or 10 nM R1881 before cell extracts were prepared for immunoblotting analyses.

For MTT assays, LNCaP cells were plated in RPMI 1640 containing 10% FBS at 5 × 10⁴ cells/well in 96-well plate and infected with recombinant adenovirus as described above. The cells were treated with EOH or R1881 and MTT assays performed as previously described (163). The absorbance at 595 nm (OD₅₉₅) was determined with a MRX microplate reader (DYNEX Technologies, Chantilly, VA). For each data point, eight samples were analyzed in parallel. The analyses were repeated two times. Statistical analysis was performed using the independent sample t test.

5. **Immunofluorescence**

Transfected DU145 cells were cultured in 1% sFBS and double-stained with rabbit anti-Flag and mouse anti-AR antibodies. FoxO1 and AR expression were detected by goat anti-rabbit IgG conjugated with Alexa Fluor 594 (red) and fluorescein isothiocyanate (FITC; green)-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR), respectively.
Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue) at a concentration of 1.5 mg/ml in the VECTASHIELD Mounting Medium (Vector laboratories, Burlingame, CA). Fluorescent microscopic images were obtained with a Nikon Diaphot microscope using a Photometric PXL cooled CCD camera. The microscope was equipped with the appropriate filters for three-color imaging of cells and with a motorized stage for obtaining z-series images.

6. **Northern Blot Analysis**

To determine the mRNA level of 3xIRS1uc, DU145 cells transfected with the indicate plamids were incubated with 10nM R1881 and 25 μM cycloheximide (Sigma) for 6 h. Total cellular RNA was isolated using the Trizol (Invitrogen) following manufacture’s instruction. Sample containing 20 μg total RNA was mixed with the sample buffer (Ambion, Austin, TX), denatured via heating at 65°C for 10 min, resolved on 1% agarose/formaldehyde gels, and transferred to a Hybond nylon membrane. The membrane was pre-hybridized at 65°C for 4 h with 3xIRS1uc-specific probe of 540 bp was generated from pGL2 plasmid with EcoRI and XbaI digestion and Labeled with 32P using random-primed DNA labeling kit (Ambion). Hybridizations were carried out overnight at 65°C with 32P-labeled 3xIRS1uc probe. Furthermore, filters were exposed to autoradiographic film for 24 h. To test for the uniform loading of the samples, blots were stripped and reprobed using a cDNA probe for the human GAPDH (Ambion).
7. Reverse Transcriptase-Polymerase Chain Reaction Analysis

Cells were transfected and treated as for Northern Blot analysis. Total RNA was also isolated as described above. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was performed using 100 ng total RNA and the SuperScript One-step RT-PCR kit (Invitrogen) in a DNA Engine thermal cycler (MJ Research Inc., Waltham, MA). To avoid amplification of contaminating genomic DNA in RT-PCR, upstream and downstream primers were placed in different exons. The sequences of the 3xIRS-specific primers used were as follows: sense, 5’-CCAGGGATTTCAGTCGATGTAC-3’; and antisense, 5’- GTCTCCAGAATGTAGCCATCC-3’. RT-PCR was performed at 50°C for 30 min, 94°C for 2 min, 29 cycles at 94°C for 15 sec, at 55°C for 30 sec, at 68°C for 1 min and at 72°C for 5 min. The integrity of the RNA used for RT-PCR was confirmed using GAPDH synthesis as a positive control. The amplified RT-PCR products were separated on a 1% agarose gel, stained with ethidium bromide for visualization, and photographed under ultraviolet illumination.

8. Apoptotic Assay

Transfected cells were washed with PBS and fixed in 2% paraformaldehyde/PBS at room temperature for 10 min. For the demonstration of apoptotic cells, fixed cells were stained at room temperature for 15 min with DAPI. Green and blue fluorescence were observed with a Leitz Orthoplan 2 Microscope. The apoptotic index of GFP positive cells was determined by scoring 400 GFP-positive cells for chromatin condensation and
apoptotic body formation. Chi Square test of Independence was used to calculate the statistical p-value.
RESULTS

1. FoxO Factors Inhibit AR Transcriptional Activity

1.1. Inhibition of AR activity by FoxO1

To investigate the involvement of FoxO1 in the suppression of AR activity by PTEN, we first tested whether FoxO1 inhibits AR transcriptional activity. In PTEN intact COS-7 cells, wild-type FoxO1 decreased ectopic AR activity on a synthetic ARE-based reporter in a dose-dependent manner (Fig. 5A) without affecting the levels of AR protein (Fig. 5B). In PC3 cells that contain low level of nuclear FoxO factors due to loss of PTEN and the ensuing increase in the kinase activity of AKT, a nuclear FoxO1, FoxO1ca, in which all three AKT phosphorylation sites were mutated to alanines (164), decreased androgen-induced AR activity whereas wild-type FoxO1 had little effect (Fig. 5C). Parallel immunoblotting analyses showed that the wild-type FoxO1 was expressed to a much higher level than FoxO1ca (Fig. 5D), showing the low effect of the wild-type FoxO1 on AR was not due to its insufficient expression. Furthermore, in the absence of AR, FoxO1ca did not decrease basal reporter activity, demonstrating that the inhibition was specific to AR (Fig. 5E).
Figure 5. Dose-Dependent Inhibition of AR Activity by FoxO1.
Figure 5. Dose-Dependent Inhibition of AR Activity by FoxO1.

A, COS-7 cells were transfected with 0.5 µg AREe1bluc, 50 ng pCMVβ, 50 ng pCMVhAR, and the indicated amounts of Flag-FoxO1 and were treated for 24 h with 10 nM R1881. Reporter activity was determined and normalized to cognate β-galactosidase (β-gal) activity. B, Lysates of COS-7 cells transfected as in panel A were immunoblotted with antibodies against AR, Flag or β-actin. C, PC3 cells were transfected with 0.5 µg AREe1bluc, 50 ng pCMVβ, 50 ng pCMVhAR, 1 µg Flag-FoxO1 or HA-FoxO1ca. Transfected cells were treated for 24 h with 10 nM R1881. Reporter activity was determined as in Figure 5A. D, Lysates of PC3 cells transfected as in panel C were immunoblotted with the AR or FoxO1 antibody. Equal amounts of protein were loaded per lane. E, PC3 cells were transfected with the indicated amounts of HA-FoxO1ca with or without AR and reporter activity was determined after treatment with 10 nM R1881. F, LNCaP cells were transfected with 0.5 µg PBluc, 0.1 µg pCMVβ and the indicated amounts of HA-FOXO1ca. Transfected cells were treated with 100 nM DHT and the activity of endogenous AR was determined and normalized with β-gal.
To determine whether FoxO1 also represses the activity of endogenous AR on the promoter of natural androgen target genes, we transfected FoxO1ca into PTEN null, AR positive LNCaP cells (173) and examined its effect on the activity of endogenous AR on the PBluc reporter in which the expression of firefly luciferase gene is under the control of the rat probasin gene promoter containing the proximal ARE (174). As shown in Fig. 5F, the transcriptional activity of the endogenous AR was stimulated by its natural ligand DHT, and FoxO1ca inhibited the activity in a dose-dependent manner. The data suggest that the inhibition by active FoxO1 is not limited to ectopic AR or synthetic AREs.

1.2. Inhibition of AR Activity by FoxO3a and FoxO4

To determine whether the other FoxO family members also repress the activity of AR, the effect of FoxO3a or FoxO4 on AR activity was determined in COS-7 cells. As shown in Fig. 6, wild-type FoxO3a and FoxO4 inhibited the androgen-induced AR activity nearly to the same degree as wild type FoxO1. These data suggest that the effect of FoxO factors on AR transcriptional activity is similar.

1.3. Involvement of Endogenous FoxO Factors in AR Inhibition

Next, we investigated whether endogenous FoxO1 in PCa cells is involved in the negative regulation of AR activity. For this, we employed a siRNA plasmid (166) that was effective in knocking down FoxO1, FoxO3a, and FoxO4 in mammalian cells (Fig.7A). In PTEN intact RWPE-1 cells, the FoxO siRNA decreased the expression of endogenous FoxO1 and increased AR activity in a dose-dependent manner without altering the level
Figure 6. Foxo3a and Foxo4 Also Inhibit AR Activity.

A, COS-7 cells were transfected with Flag-FoxO1 or Flag-FoxO4, together with AREe1bluc, pCMVβ, pCMVhAR. Transfected cells were treated for 24 h with 10 nM R1881 and luciferase activity was determined. Lysates of the parallel experiment were subjected to immunoblotting with antibodies against AR, β-actin and Flag. B, COS-7 cells were transfected with HA-FoxO1 or HA-FoxO3a, and measured as A. FoxOs were detected with HA11 anti-HA antibody.
Figure 7. Knockdown of FoxO Factors Enhances the AR Activity.

**A**, 293T cells (upper panel) or PC3 cells (lower panel) were transfected with 0.4 µg FoxO siRNA or U6 control. 72 h later, cell lysates were immunoblotted with antibodies against FoxO1, FoxO3a, FoxO4 or β-actin.  

**B**, RWPE-1 cells were transfected with 0.5 µg AREe1bLuc, 0.1 µg pCMVβ, 0.1 µg pCMVhAR together with the indicated amounts of FoxO siRNA or U6 control. 48 h later, cells were treated with EOH as vehicle control or 10 nM R1881 for 24 h. Reporter activity was determined as in Figure 5A. Cell extracts from parallel transfections were immunoblotted with antibodies against AR, FoxO1, or β-actin as indicated.
of AR (Fig. 7B). These experiments show that endogenous FoxO factors decrease the transcriptional activity of AR. It is important to note that RWPE-1 is a non-neoplastic human prostate epithelial cell line immortalized by the human papilloma virus, HPV-18. This cell line was described as AR positive and androgen-sensitive for growth (175). In our hands, however, the cell expresses low basal level of AR protein and is androgen insensitive. Androgen-induced transcriptional activity was only detectable when ectopic AR was expressed (data not shown).

2. Defining FoxO1 Domains Involved in AR Repression

FoxO1 is a 655 amino acid protein with a short N-terminus, a highly conserved forkhead box necessary for DNA binding, a LXXLL motif and a proline-, acidic residue-, and serine/threonine rich activation domain (AD) (176). To define the FoxO1 domain involved in AR repression, we generated a set of plasmids expressing different regions of FoxO1ca (Fig. 8A) and tested their ability to inhibit the activity of the AR when ectopically expressed in PC3 cells on pT81Luc, a reporter gene in which the expression of the firefly luciferase gene is under the control of the complex ARE in the distal region of the rat probasin gene (167). In these assays, FoxO1ca(150-655) decreased AR activity to a similar degree as full length FoxO1ca whereas FoxO1ca(1-150), FoxO1ca(150-270), FoxO1ca(1-270), and FoxO1ca(150-537) did not decrease AR activity (Fig. 8B). FoxO1ca(256-655) increased the AR activity. Parallel immunoblotting analyses showed that the FoxO1ca fragments were expressed to a level either comparable with or higher
than that of full length FoxO1ca, showing that the lack of inhibition by the FoxO1 fragments was not due to insufficient expression (Fig. 8B). Similar results were obtained with endogenous AR on PBluc reporter in LNCaP cells (Fig. 8C). These analyses demonstrate that the inhibition of AR activity by FoxO1 requires the forkhead box and the C-terminal activation domain whereas the N-terminal 150 amino acids are nonessential and C-terminal alone may increase the AR activity.

p160 coactivators proteins interact with nuclear receptors via short motifs, nuclear receptor or NR box, consisting of the amino acid sequence LXXLL, where X can be any amino acid. The presence of a putative NR box sequence \textsuperscript{462}LKELL\textsuperscript{466}, close to the C-terminal AD of FoxO1 prompted us to test whether this motif is involved in the AR repression by FoxO1. We generated a mutant FoxO1, FoxO1caNR in which the LKELL motif was mutated to WKEWL, and assessed its ability to inhibit AR in PC3 cell. As shown in Fig. 8D, the mutant FoxO1 inhibited the AR to the same degree as FoxO1ca. Parallel immunoblotting analyses showed that the FoxO1caNR was expressed to a comparable level as FoxO1ca. These analyses show that the putative NR box is not involved in the inhibition of the AR by FoxO1ca.

Since the FoxO1 inhibition requires the DNA binding domain, it is possible that a FoxO1 target gene mediates the AR inhibition. Although there are multiple ways for FoxO factors to regulate gene expression (125), the primary mechanism is through the binding of forkhead box to insulin response sequence (IRS). To test whether the AR inhibition
Figure 8. Mapping FoxO1 Domains Required for AR Inhibition.
Figure 8. Mapping FoxO1 Domains Required for AR Inhibition.

A, A diagram of human FoxO1 fragments. Different fragments corresponding to the indicated amino acids were amplified by PCR, and inserted into pCDNA3.1-HA vector to make a subset of FoxO1ca subclones. FK: forkhead box; AD: activation domain. B, Upper panel: PC3 cells were transfected with 0.5 µg PT81luc, 50 ng pCMVβ, 50 ng AR, and different amounts of the FoxO1ca fragments. Transfected cells were treated and reporter activity was determined as in Figure 5A. Lower panels: Lysates of PC3 cells transfected with the plasmids, exactly as in the upper panel, were immunoblotted with anti-AR or HA11 antibody. C, FoxO1ca fragments were transfected into LNCaP cells together with PBLuc and treated with 10 nM R1881. The activity of endogenous AR was measured as in Figure 5F. D and E, FoxO1ca (CA), FoxO1ca with mutations in the NR box (CaNRmut) or FoxO1ca with mutation in the forkhead box (CaH215R) was transfected together with AR and AREe1bLuc into PC3 cells as in Figure 5C. Reporter assays and immunoblotting analyses were similarly performed for the indicated proteins.
depends on the ability of FoxO1 to bind IRS elements, we generated a FoxO1 mutant, FoxO1CaH215R, in which a conserved histidine in helix 3 of the forkhead box was mutated to arginine, which was shown to abrogate the ability of FoxO1 to bind IRS (125) and tested its ability to inhibit AR activity. As shown in Fig. 8E, FoxO1CaH215R inhibited AR activity to the same degree as FoxO1ca. These analyses show that the forkhead box and the AD in the C-terminus are required for AR inhibition but the inhibition occurs independently of the putative NR box and the ability of FoxO1 to bind IRS.

3. Effect of FoxO1 on Nuclear Localization of AR

Nuclear localization is required for the normal function of AR upon androgen treatment. Thus, we tested whether FoxO1 inhibits AR transcriptional activity by blocking AR nuclear localization. The intracellular distribution of ectopic AR alone or AR coexpression with FoxO1 in PTEN-intact, AR-negative DU145 cells was analyzed by immunofluorescence. As expected, the AR predominantly showed fluorescence in the cytosol with very weak nuclear fluorescence in the absence of the ligand (Fig. 9), whereas androgens induced nuclear translocation. The AR distribution was not altered by FoxO1ca, suggesting that inhibition of AR by FoxO1 is not due to the blockage of AR nuclear localization induced by androgens.

4. Inhibition of AR N/C Interaction by FoxO1

Our previous studies have shown that FoxO1 interacts with both the NTD and LBD of the AR (164), raising the possibility that FoxO1 may inhibit AR activity by
Figure 9. Lack of an Effect of FoxO1 on AR Cellular Localization.

The immunofluorescence microscopic pictures of the transfected AR and FoxO1ca or vector control in DU145 cells are shown. DU145 cells were transfected with 0.2 µg pCMVhAR and 0.3 µg Flag-FoxO1ca or a vector control pcDNA3. After a 24 h treatment with 10 nM R1881 or EOH, cells were fixed and double stained with anti-Flag and anti-AR antibodies for the FoxO1ca (red) and AR (green). Cell nuclei were counterstained with DAPI (blue).
disrupting the N/C interaction. Accordingly, we tested the effect of FoxO1 on AR N/C interaction in a mammalian two-hybrid assay. As shown in Fig. 10A, cotransfection of AR LBD fused to Gal4DBD (PMLBD) and AR NTD fused to the VP16 (VPARNT) allowed a strong androgen induced activation of the cotransfected Gal4 reporter gene in PC3 cells, which was inhibited slightly by wild type FoxO1 and strongly by FoxO1ca in a dose-dependent manner (Fig. 10A). Wild type FoxO1 or FoxO1ca did not decrease the activity of the Gal4 reporter in cells expressing VPARNT or PMLBD alone or in cells expressing both but treated with vehicle (Fig. 10A). These data suggest that one mechanism by which active FoxO1 inhibits the transcriptional activity of AR is to disrupt the androgen induced N/C interaction. Consistent with this idea, the ability of FoxO1 to inhibit AR N/C interaction depended on the protein region 150-655 containing both the forkhead box and the C-terminal AD (Figs. 10B, 10C, 10D), which both were shown earlier to be required to inhibit AR activity. Fig. 10E is the normalized data combining Figs. 10B, 10C, 10D.

The fact that FoxO1 interacted with both NTD and LBD regions of AR (164) raises the possibility that FoxO1 may inhibit N/C interaction through its action on either the NTD or the LBD. We found that the activity of PMLBD on Gal4 reporter induced by androgens was not decreased by FoxO1ca (Fig. 10A), implying that FoxO1 is likely to work through its effect on the NTD to inhibit AR. Consistently, the transcriptional activity of the NTD fused to either the Gal4 DBD (Fig. 11A) or its own DBD (Fig. 11B) was inhibited by FoxO1ca.
A

```
0.2 0.2 0.2 0.2 0.01 0.05 0.2 μg
```

B

```
0
```

C

```
0
```

---

**Legend**

- **VP16**
- **PM**
- **PMLBD**
- **VPARNT**
- **FoxO1ca**
- **FoxO1ca(1-150)**
- **FoxO1ca(150-270)**

---

**N/C Interaction (RLU \times 10^{-4})**

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**Factors**

- **Vector**
- **FoxO1WT**
- **FoxO1ca**

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**Concentration**

- **EOH**
- **R1881**
Figure 10. FoxO1 Suppresses AR N/C Interaction in Mammalian Two-hybrid Assays.
Figure 10. FoxO1 Suppresses AR N/C Interaction in Mammalian Two-hybrid Assays.

A, PC3 cells were transfected with 0.3 µg GalLuc, 0.1 µg pCMVβ, 0.1 µg VP16, PM, PMLBD, or VPARNT together with the indicated amounts of wild type FoxO1 or FoxO1ca and treated with EOH or R1881 for 24 h. Luciferase activity was normalized with β-gal. B, C, D, PC3 cells were transfected as in panel A but with FoxO1ca fragments as indicated. Cells were treated with R1881 and reporter activity was determined. E, Luciferase activity of cells transfected with PMLBD, VPARNT and indicated FoxO1ca fragments from B, C, D were normalized by that of the cells transfected with PMLBD and VPARNT and vector and plotted in one figure. The N/C interaction value of cells transfected with PMLBD and VPARNT and vector was set as 100%.
Figure 11. FoxO1 Inhibits AR NTD Activity.

A, PC3 cells were transfected with 0.3 µg GalARNT, 0.3 µg Galluc, 0.1 µg pCMVβ, and 0.5 µg FoxO1ca (+) or vector (-) as indicated. Luciferase activity was determined 24 h later and normalized with β-gal. B, PC3 cells were transfected with 0.3 µg ARΔLBD, 0.5 µg AREe1bluc, 0.1 µg pCMVβ, and 0.5 µg FoxO1ca (+) or vector (-) as indicated. The reporter activity was determined 24 h later and normalized with β-gal.
5. FoxO1 Negates the Positive Effect of p160 Coactivators on AR Transcriptional Activity by Blocking p160 Coactivator Interaction with AR NTD

5.1 FoxO1 Represses the Enhancement of AR Transcriptional Activity by p160 Coactivators

It is known that the strong activation function in AR NTD is the predominant interface for the binding of p160 coactivators in the presence of androgens (14, 41). If FoxO1 acts through NTD, it is likely to exert a negative effect on the ability of p160 coactivators to increase AR activity. When AIB1 was transfected into LNCaP cells, it increased the transcriptional activity of the endogenous AR induced by DHT as expected (Fig. 12A). Cotransfection of FoxO1ca negated the positive effect of AIB1 in a dose-dependent manner (Fig. 12A). In addition to AIB1, the other two p160 coactivators SRC1 and TIF2 gave similar results (Figs. 12B and 12C), suggesting that the inhibition is commonly applicable to p160 coactivators members.

5.2 FoxO1 Inhibits p160 Coactivator Interaction with AR NTD

We next asked whether the interaction of the AR NTD with p160 coactivators is interrupted by FoxO1. As expected, AIB1 interacted with AR NTD in mammalian two-hybrid assay (Fig 13). The interaction was suppressed by FoxO1ca (Fig. 13A) and FoxO1ca (150-655) (Fig. 13C and Fig. 13D) to a similar degree. Conversely, FoxO1 siRNA enhanced the binding of ARNT and AIB1 (Fig. 13B). In agreement with the results from AR transcriptional assays (Fig. 8B and 8C) and the N/C interaction studies, other fragments besides FoxO1ca(150-655) did not inhibit the interaction between AIB1 and...
Figure 12. FoxO1 Represses Enhancement of AR Transcriptional Activity by p160 Coactivators.

A, LNCaP cells were transfected with 0.5 µg of PBluc, 0.1 µg of pCMVβ, 0.1 µg of AIB1 and 0, 0.01, 0.2 and 0.5 µg of FoxO1ca as indicated and treated with EOH or DHT. Endogenous AR activity was measured and normalized. B, COS-7 cells were transfected with AR and SRC1, together with FoxO1ca or vector control. AREe1bLuc luciferase activity and the expression level of indicated proteins were measured. C, COS-7 cells were transfected with AR and TIF2, together with FoxO1ca or vector control. AREe1bLuc luciferase activity was measured.
Figure 13. FoxO1 Blocks AIB1 Recruitment to AR NTD.
Figure 13. FoxO1 Inhibits AIB1 Recruitment to AR NTD.

A, COS-7 cells were transfected with 0.3 µg GalLuc, 0.1 µg pCMVβ, 0.1 µg VP16, PM, PBINDAIB1, VPARNT and FoxO1ca as indicated. Reporter activity was determined. B, The experiment was performed as in panel A except the replacement of FoxO1ca with FoxO siRNA. C, COS-7 cells were transfected as in panel A but with different FoxO1ca fragments. Reporter activity was determined. D, Luciferase activity of cells transfected with VPARNT and PBINDAIB1 and indicated FoxO1ca fragments from panel C was normalized with that of the cells transfected with VPARNT and PBINDAIB1 to measure the effect of FoxO1 fragments on AR-AIB1 interaction.
AR NTD (Fig. 13C and Fig. 13D). These data suggest that suppression of p160 coactivator’s binding to AR NTD, as well as AR N/C interaction, represents possible mechanisms for AR inhibition by FoxO1.

We envisioned two possible ways by which FoxO1 attenuates the interaction between AR NTD and coactivators. One would involve competition of FoxO1 and AIB1 for the AR NTD. Alternatively, FoxO1 may “squelch” the limited coactivators from AR, since both AR and FoxO1 are transcription factors. Mammalian two-hybrid assays showed an interaction of the AR NTD with FoxO1ca(150-655) but not with FoxO1(1-150) (Fig. 14A). Similar assays showed an interaction between FoxO1ca and AR NTD but not AIB1 (Fig. 14B). The increase in GalLuc reporter activity was not due to the enhancement of GalFoxO1ca(150-655) transactivation by AR NTD, because ARΔLBD that contains the NTD but is not fused to VP16 did not increase the reporter activity. These observations support a competition mechanism instead of the “squelching” mechanism of action.

6. Inhibition of AR Activity by FoxO1 Is Not Affected by CAG Repeats

The AR NTD gene contains a polymorphic CAG repeat sequence. The CAG repeat length has been reported to affect AR activity. We then examined the activity of AR containing different numbers of CAG repeats in PC3 cells. As shown in Fig 15, FoxO1 inhibited the activity of an AR expressing vector without cag repeats (ARcag0) or with long cag repeats (ARcaglong) at the same degree as that of wild-type AR in the same
Figure 14. FoxO1 Interacts with AR NTD But Not Coactivators.

**A,** COS-7 cells were transfected with 0.3 µg GalLuc, 0.1 µg pCMVβ, 0.1 µg VP16, PBIND, PBINDAIB1, VPARNT or VPFoxO1ca as indicated. Reporter activity was determined. **B,** COS-7 cells were transfected with 0.3 µg GalLuc, 0.1 µg pCMVβ, 0.1 µg VP16, PM, PBINDAIB1, VPARNT or FoxO1ca as indicated. Reporter activity was determined.
Figure 15. AR CAG Repeats Is Not Associated with AR Inhibition by FoxO1.

PC3 cells were transfected with indicated amount of FoxO1 and AR vectors containing different numbers of CAG repeats together with ARE-luc, pCMVβ. ARcagwt: wild type AR with normal cag repeats; ARcag0: AR without cag repeats; ARcaglong: AR with long cag repeats. Reporter activity was examined after being treated with R1881 for 24 h.
backbone vector (ARcagwt). These data suggest that FoxO1 suppresses AR activity regardless of its number of the CAG repeat.

7. **Enhancement of AR Activity by Constitutively Active AKT is Decreased by FoxO1ca**

   The FoxO family of transcription factors is one of the major direct substrates of the protein kinase AKT in response to cellular stimulation by growth factors or insulin and inactivated by AKT. PTEN is a negative regulator of AKT. This information prompted us to test whether AKT regulates AR activity and whether the regulation is modulated by FoxOs. As shown in Fig 16, a constitutive active AKT, AKTDD, of which the two phosphorylation sites T308 and S473 are mutated to aspartic acid, increased AR activity in PTEN-intact RWPE-1 cells relative to an AKTAA control, of which T308 and S473 are mutated to alanine, which was suppressed by the expression of FoxO1ca.

8. **FoxO Factors Mediate the AR Suppression by PTEN**

   To test the participation of FoxO1 in the suppression of AR activity by PTEN, the effect of active PTEN on AR activity was assayed in the presence or absence of FoxO siRNA. Ectopic expression of the active PTEN in PC3 cells decreased AR activity as relative to phosphatase-inactive PTEN. Cotransfection of FoxO siRNA relieved the PTEN suppression in a dose-dependent manner. This siRNA decreased the expression of endogenous FoxO1 without altering the level of AR, PTEN and β-actin (Fig. 17). These experiments show that endogenous FoxO factors mediate the PTEN inhibition.
Figure 16. Enhancement of AR Activity by Constitutive Active AKT Is Decreased by FoxO1ca.

A, RWPE-1 cells were transfected with 0.5µg ARE-luc, 0.1µg pCMVβ, 0.1µg pCMVhAR, together with 0.3 µg AKTDD or AKTAA. 48 h post-transfection, the cells were treated with R1881 for another 24 h. Reporter activity was determined as previous. B, RWPE-1 cells were transfected with 0.3 µg AKTDD and/or with FoxO1ca, cells were treated and reporter activity was determined as in A.
Figure 17. FoxO Knockdown Relieves the AR Inhibition by PTEN in Prostate Cells.

PC3 cells were transfected with 0.5 µg of AREe1bLuc, 0.1 µg of pCMVβ, 0.1 µg of pCMVhAR, 0.05 µg of pSG5L-HA-PTEN:WT (WTPTEN) or pSG5L-HA-PTEN:G129R (MTPTEN) together with the indicated amounts of FoxO siRNA or U6 control. 48 h later, cells were treated with EOH as vehicle control or 10 nM R1881 for 24 h. Luciferase activity was determined and normalized to cognate β-galactosidase (β-gal) activity. Duplicate samples were analyzed in parallel for each data point and the error bars represent standard deviations. The data were reproduced twice. Cell extracts from parallel transfections were subjected to immunoblotting with antibodies against AR, FoxO1, HA or β-actin as indicated.
9. **FoxO Mediates the Negative Effect of PTEN on AR N/C Interaction and AIB1 Recruitment.**

So far, we have demonstrated that FoxO mediated the negative effect on AR transcriptional activity and interfered with AR N/C interaction as well as the interaction of the AIB1 coactivator to AR NTD. The next question is whether PTEN inhibits AR activity through these mechanisms. As shown in Fig. 18A, wild-type PTEN significantly decreased AR N/C interaction induced by androgen treatment in PC3 cells as compared to phosphatase-inactive PTEN. More importantly, FoxO siRNA negated the majority of the suppressive effect exerted by PTEN. Similar to the AR N/C interaction, the ability of AIB1 to increase AR transcriptional activity (Fig. 18B) and the interaction between AIB1 and AR NTD (Fig. 18C) were suppressed by wild-type PTEN, which were partially relieved by FoxO siRNA. At the same dose, PTEN did not decrease basal GalLuc activity or the transcriptional activity of PBINDAIB1. These data show that PTEN inhibits AR N/C interaction and AIB1 interaction through FoxO factors.

10. **FoxO1 Suppresses Androgen-Induced Proliferation and PSA Expression in PCa Cells.**

If FoxO1 mediates the inhibition of AR activity and interferes with AR N/C interaction and coactivator recruitment, restoring the expression of active FoxO1 in PTEN null cells in which endogenous FoxO factors are mainly dislocated to cytosol should exert a negative effect on the biological activity of endogenous AR. Indeed, when PTEN-null LNCaP cells were infected, as a control, with a recombinant adenovirus expressing GFP (Ad-GFP), PSA expression was significantly induced by treatment with R1881. In a
Figure 18. Participation of Endogenous FoxO Factors in PTEN-Induced Suppression of AR N/C Interaction and Coactivators Recruitment.
Figure 18. Participation of Endogenous FoxO Factors in PTEN-Induced Suppression of AR N/C Interaction and Coactivators Recruitment.

A, PC3 cells were transfected with 0.3 µg GalLuc, 0.1 µg pCMVβ, 0.1 µg VP16, PM, PMLBD, or VPARNT, and 0.05 µg WTPTEN or MTPTEN together with the indicated amounts of FoxO siRNA or U6 control and treated with 10 nM R1881. AR N/C interaction was determined by measuring the reporter activity. B, PC3 cells were transfected with 0.1 µg pCMVAR, 0.1 µg AIB1 and 0.05 µg MTPTEN or WTPTEN together with FoxO siRNA or U6 control. Cells were then treated with 10 nM R1881 and AR activity was determined. C, COS-7 cells were transfected as in panel A except that PMLBD was replaced with PBINDAIB1. The interaction between ARNT and AIB1 was determined as in panel A for AR N/C interaction.
Figure 19. Suppression of Biological Activities of the AR in PCa Cells by Nuclear FoxO1.

A, LNCaP cells were infected for 24 h with 100 MOI of Ad-GFP-FoxO1ca or Ad-GFP as control and treated with EOH or 10 nM R1881 for an additional 24 h. Proteins were detected by immunoblotting with the cognate antibody. B, LNCaP cells were infected and treated for the indicated times as in panel A. Cell growth was determined by MTT colorimetric assays. Eight samples were analyzed for each data point and the data were reproduced three times. Statistical analyses were performed with standard student t test. Differences in androgen-induced cell growth between cells infected with control and FoxO1ca virus reached statistical significance (P<0.05).
parallel experiment, infection with a recombinant adenovirus expressing both GFP and FoxO1ca (Ad-GFP-FoxO1ca) significantly decreased androgen induction of PSA expression (Fig. 19A). This inhibition was not due to a decrease in the level of AR protein expression (Fig. 19A). The comparable expression of the GFP proteins in all the lanes shows that the same number of viral particles was used in the studies. Similar to PSA expression, in LNCaP cells infected with control GFP virus, treatment with R1881 induced an increase in cell numbers as measured in MTT assays. The induction was significantly suppressed ($p=0.0017$) in cells infected with Ad-GFP-FoxO1ca (Fig. 19B). These analyses demonstrate that in PCa cells, the repression of AR transcriptional activity by FoxO1 can translate into the impaired biological actions of androgens through endogenous AR.

**11. Working Model for PTEN Inhibition on AR through FoxO1**

So far, our studies support the mechanism (Fig. 20) that PTEN acts through nuclear FoxO factors to disrupt AR N/C interaction and coactivator recruitment to AR NTD, resulting in decreased transcriptional activity of the AR and the suppression of androgen action.

**12. FoxO1 Inhibition Mediated through AR Is Diminished by Mutated AR DBD**

As discussed in the introduction, two mechanisms have been reported for the AR inhibition of FoxO1 activity. We are interested in studying whether the inhibition also involves an AR downstream target gene. To test this idea, we first constructed an ARmut
Figure 20.  A Working Model Depicting the Role of FoxO1 in PTEN-induced AR Inhibition in PCa Cells and Its Suppression by AKT.

The model predicts that a function of nuclear FoxO factors in prostatic cells is to suppress AR N/C interaction and limit AR activation by p160 coactivators and that the negative and positive effects of the coactivators and FoxO factors provide a balance mechanism that keeps AR action in check. During prostatic tumorigenesis, the lost of PTEN results in cytoplasmic localization of FoxO factors owing to increased AKT activity that phosphorylates FoxO factors. This permits unopposed AR to drive prostate epithelial proliferation to a stage that is out of control. The model also predicts that increased expression or activity of AIB1 or AKT, two known prostatic oncogenes, or decreased expression or nuclear activity of FoxO factors themselves will have a similar consequence as the loss of PTEN.
expression vector, in which two cysteine residues of AR DBD were mutated. We then compared in DU145 cells the ability to suppress FoxO1 activity of wild-type AR and ARmut using the FoxO luciferase reporter 3xIRS\textsubscript{Luc}, which contains three copies of a FoxO1 response element from the promoter of the insulin-like growth factor-binding protein-1 gene. As this ARmut abolished the AR transcriptional activity on ARE\textsubscript{e1bLuc} reporter (Fig. 21A), this expression of the mutant AR at the same protein level as wild-type AR partially relieved the wild-type AR inhibition of FoxO1 (Fig. 21B). Similar data were obtained when replacing FoxO1 with FoxO1\textsubscript{ca}.

13. **The Effect of AR on Nuclear Localization of the FoxO1 Protein**

FoxO1 activation requires its localization to the nucleus, whereas cytoplasmic FoxO1 is inactive. Thus, we determined whether AR inhibits transcriptional activity of the FoxO1 protein by blocking its nuclear localization using immunofluorescence staining. Since the level of endogenous FoxO1 expression is quite low, we tested the AR effect on transfected FoxO1. As shown in Fig. 22, transfected FoxO1\textsubscript{ca} was retained in the nucleus when cotransfected with wild-type AR treated either with or without androgens. The nuclear localization was not affected if cotransfected with ARmut. Therefore, abolishing AR transcriptional activity does not appear to affect the nuclear localization of the transfected FoxO1\textsubscript{ca} protein in DU145 cells.
Figure 21. Mutation of AR DBD Partially Relieved the Inhibition on FoxO1 Activity.
Figure 21. Mutation of AR DBD Partially Relieved the Inhibition on FoxO1 Activity.

A, DU145 cells were transfected with 0.5 µg AREe1bluc, 50 ng pCMVβ, 0.2 µg pCMVhAR or pCMVhARmut. Cells were treated and reporter activity was measured as in Figure 5A. B, Upper panel: DU145 cells were transfected with 0.5 µg AREe1bluc, 50 ng pCMVβ, 0.1 µg Flag-FoxO1 together with indicate amounts of pCMVhAR or pCMVhARmut. Transfected cells were treated with 10 mM R1881. Reporter activity was determined. Lysates of DU145 cells transfected with the plasmids, exactly as in the upper panel, were immunoblotted with anti-AR, M2, or anti-β-actin antibody.
Figure 22. Lack of an Effect of the AR and the ARmut on FoxO1 Cellular Localization.

Immunoflorescence microscopic pictures of the transfected FoxO1 and AR or ARmut in DU145 cells were shown. DU145 cells were cotransfected with 0.3 µg Flag-FoxO1ca and 0.2 µg pCMVhAR or pCMVhARmut. After a 24 h treatment with 10 nM R1881 or EOH, cells were double-stained with anti-Flag and anti-AR antibodies for the FoxO1ca (red) and AR/ARmut (green). Cell nuclei were counterstained with DAPI (blue).
14. Decreased mRNA level of FoxO1 reporter gene by Androgens Is Alleviated by Inhibiting New Protein Synthesis

To test whether an androgen target gene participates in the inhibition of FoxO1, we investigated the effect of activated AR on FoxO1 target gene transcription in the presence or absence of a new protein synthesis inhibitor. We carried out Northern blot to measure the mRNA level of ecotopic 3xIRS1uc, of which the luciferase transcription is induced by FoxO1. As shown in Fig. 23, activated AR decreased the mRNA level of 3xIRS1uc (lane 2 compared to lane 1). However, cycloheximide (CHX), an inhibitor of protein synthesis, significantly alleviated the decrease. In addition, this observation was confirmed by RT-PCR (Fig. 23B). These data suggest that AR inhibition of the transcriptional activity of FoxO1 requires the synthesis of a new protein, presumably an androgen target gene.

15. FoxO Knockdown Inhibits PTEN-Induced Apoptosis

Because the expression of PTEN or its downstream FoxO in LNCaP cells induces apoptosis and androgens oppose the functions of both PTEN and FoxO, we next investigated whether the knockdown of FoxO would make a difference in PTEN induced apoptosis. LNCaP cells were transfected with a GFP expression vector together with wild-type or mutant PTEN, along with FoxO siRNA or U6 control vector. As shown in Fig. 24, apoptotic index, as determined by counting apoptotic cells in 400 green cells per sample, was 37% for cells transfected with WTPTEN and 10% for MTPEN control without androgen treatment. However, although the FoxO siRNA did not completely abolish the PTEN-induced apoptosis, it decreased the WTPTEN-induced apoptotic index to 29% in
Figure 23. Decreased Transcription of FoxO1 Reporter Gene by Androgens Is Alleviated by A Protein Synthesis Inhibitor.

A, DU145 cells were transfected with 3 µg 3xIRSluc, 2 µg pCMVhAR and 2 µg Flag-FoxO1ca. 24 h later, cells were treated with EOH as vehicle control or 10 nM R1881 in the absence or presence of 50 ng/µl cyclohemimide (CHX) for 24 h. Total RNA was isolated, and 20 µg RNA was subjected to Northern blot analysis to detect the mRNA level of 3xIRSluc and GAPDH control. B, 0.1 µg RNA from A was subjected to RT-PCR and the mRNA levels of both 3xIRSluc and GAPDH control were tested.
**Figure 24. FoxO Knockdown Suppresses PTEN-Induced Apoptosis.**

LNCaP cells were transfected with 0.3 µg GFP, 0.2 µg MTPTEN or WTPTEN and 0.8 µg FoxO1 siRNA or U6 control as indicated. 48 h later, cells were treated with EOH or 10 nM R1881 for 24 h. Apoptotic index of GFP-positive cells was determined by scoring 400 GFP-positive cells for chromatin condensation and nuclear fragmentation. Duplicate samples were analyzed and the graph represents two independent experiments.
the absence of androgens. These data suggest that FoxO factors play a major role in
the PTEN function. Moreover, in the presence of androgens, activated AR did not protect
apoptosis induced by WTPTEN in the absence of FoxOs, suggesting that the
AR-mediated protection of cells from apoptosis induced by PTEN is mediated through
FoxOs.

16. Woring Model for AR Inhibition of FoxO1

Literatures have reported that AR inhibits FoxOa activity by proteolytic cleavage of
FoxO1 at residue Arg537, which generates a dominant negative FoxO1(165), or
blockage of FoxO's DNA binding activity by protein-protein interaction between AR and
FoxO1 (164). Our studies support the mechanism (Fig. 25) that activated AR produces a
yet-to-be identified androgen target gene to inhibit FoxO activity.
Figure 25. A Working Model Depicting the Mechanisms of FoxO1 Suppression by Androgens.

The model predicts that activated AR suppresses FoxO activity by at least three possible mechanisms: 1, proteolytic cleavage of FoxO1 at residue Arg537, which generates a dominant negative FoxO1 leading to an inhibition of the activity of the intact FoxO1; 2, blockage of FoxO’s DNA binding activity by protein-protein interaction between AR and FoxO1; 3, synthesis of a yet-to-be identified androgen target gene X to inhibit FoxO activity.
DISCUSSION

As discussed earlier, the loss of PTEN tumor suppressor is an important event during human prostatic tumorigenesis. Our previous studies identified PTEN protein as an AR suppressor that opposes androgen actions in prostate cells through down regulation of AKT (163). The present studies provide multiple lines of evidence to support the mechanism (Fig. 20) that PTEN acts through nuclear FoxO factors to disrupt AR N/C interaction and coactivator recruitment to AR NTD, resulting in decreased transcriptional activity of the AR and suppression of androgen action. First, FoxO1 siRNA relieved the inhibition of AR activity by ectopic PTEN in PTEN-null cells and increased AR activity in PTEN-intact cells. Second, the constitutively nuclear FoxO1 inhibited AR activity in a dose-dependent manner in PTEN-null cells whereas wild-type FoxO1 had little effect. Along with our observation that wild-type FoxO1, FoxO3a and FoxO4 inhibited AR activity in PTEN-intact cells, these data suggest that it is the nuclear FoxO factors that inhibit AR activity. Third, nuclear FoxO1 inhibited AR N/C interaction and AIB1 recruitment to AR NTD. PTEN exerted a similar effect, which was relieved by FoxO siRNA, suggesting that endogenous FoxO factors inhibit N/C interaction and AIB1 binding in a manner sensitive
to PTEN status. Finally, adenovirus-mediated delivery of nuclear FoxO1 into PTEN-null LNCaP cells suppressed androgen-induced PSA production and cell growth, indicating that the inhibition of the transcriptional activity is likely translatable into suppression of the biological actions of androgens by PTEN and FoxO factors. AI PCa cells often display overexpression of coactivator molecules important for AR signaling (177). The model shown in Fig. 20 implies that increased expression or activity of AIB1 or AKT, two known prostatic oncogenes, or decreased expression or nuclear activity of FoxO factors themselves will have a similar consequence on androgen actions in prostate cells as the loss of PTEN. They all expose prostate cells to increased growth stimulation by androgens.

During our investigation, two research groups reported the inhibition of AR activity by FoxO1 (178, 179). Dong et al. showed the inhibition without describing the underlying mechanism (178). Fan et al. showed that FoxO1 inhibited AR nuclear localization, subnuclear distribution and DNA binding and yet detected FoxO1 on the promoter of PSA gene together with the AR (179). In our investigation, we found that FoxO1 did not inhibit the AR nuclear localization (Fig. 9). Different from our previous finding that FoxO1 interacted with both AR NTD and the LBD (164), Fan et al. confirmed that androgen induced interaction with LBD but reported that FoxO1 did not interact with AR NTD, leading to their assumption that the AR inhibition by FoxO1 was due to interaction with AR LBD. It is important to point out that, in their two-hybrid assays, Fan et al. used AR
NTD fused to Gal4 DBD as one of the hybrid molecules to show a lack of FoxO1 interaction with AR NTD. Because AR NTD fused to Gal4 DBD strongly activated GalLuc reporter by itself (Fig. 11A), it is difficult to measure with this construct protein-protein interactions in mammalian two-hybrid assays. More importantly, the two-hybrid assays based on AR NTD fused to Gal4 DBD could not separate two opposing activities of FoxO1: the binding that increases the Gal4 reporter activity and the inhibitory effect of FoxO1 on the transcriptional activity associated with AR NTD, which decreases the reporter activity. Using FoxO1ca (150-655) fused to Gal4 DBD and AR NTD fused to VP16 activation domain, we detected a measurable interaction between FoxO1 and AR NTD. Furthermore, we found that transcriptional activity of AR NTD with its own DBD or Gal4 DBD was inhibited by FoxO1ca whereas the activity of AR LBD fused to Gal4 was neither inhibited by FoxO1ca nor increased by FoxO siRNA (Fig. 11B and data not shown).

Unlike other members of the nuclear hormone receptor family which usually contain a strong AF-2 in their LBD, the recruitment of p160 coactivators to AR is mediated primarily by the AF-1 domain in the NTD (14, 41). The AF-1 is known to exhibit strong constitutive activity and deletion of the LBD creates a molecule that activates androgen target genes to the same extent as the full length receptor in the presence of ligand(12, 14). Consistent with this scheme, our analyses showed that FoxO1 inhibited AIB1 recruitment to the AR NTD. The observations that FoxO1 did not bind to AIB1, that both
FoxO1 and AIB1 bound to AR NTD (Fig. 14 and Fig. 13), and that FoxO1 inhibited the binding of AIB1 to NTD (Fig. 13) support a competition model instead of the “squelching” mechanism of action. Our mapping analyses showed that the FoxO1 fragment deleted of the forkhead box lost whereas the DNA-binding deficient FoxO1 mutant retained the ability to inhibit AR (Fig. 8E), suggesting that the AR inhibition requires the forkhead box but not the ability of FoxO1 to bind DNA. This is consistent with the competition mechanism. Besides the forkhead box, the ability of FoxO1 to inhibit AR also requires the C-terminal AD since FoxO1ca(150-537), which contains an intact forkhead box but lacks the AD, inhibited neither AR activity on PT81Luc in PC3 cells (Fig. 8B) nor the interaction of AR NTD with SRCs (data not shown). Clearly, neither the N-terminal 1-150 amino acids of FoxO1 nor the putative NR box is required for AR inhibition.

The AR N/C interaction makes a major contribution to AR transcriptional activity. It influences receptor dimerization and slows down the dissociation of ligand from LBD as well as AR degradation. Mutations that disrupt AR N/C interactions have been linked to androgen-insensitive syndrome (180, 181). Besides the competition with coactivator recruitment to NTD, our data suggest the disruption of AR N/C interaction as an additional mechanism underlying the AR inhibition by the PTEN-FoxO axis. It is important to note that coactivator recruitment and N/C interaction are two closely related events. p160 coactivators interact with both the NTD and the LBD to bridge the N/C interaction (54, 182, 183) and the efficient recruitment of certain coactivators to the NTD of native AR appears
also to require N/C interaction (14). Consistent with the association of the two events, FoxO domains necessary for inhibition of N/C interaction were also required for the interference with AIB1 binding. Nevertheless, AR N/C interaction was found not to be always required for the transcriptional activity of the AR as ligands that do not support the interaction still activate AR when used at sufficiently high concentrations and peptides that block the N/C interaction do not necessarily inhibit AR transcriptional activity (184, 185). Mutation in the NTD (I182A/L183A) interfered with the AR N/C interaction and dramatically impaired the activity of full length AR but had little effect on the intrinsic transcriptional activity associated with the NTD (14). In Fig. 11, we showed that FoxO1 inhibited the activity of AR NTD, which is clearly free of N/C interaction. The data suggest that the effect of FoxO1 on coactivator recruitment does not depend on N/C interaction in the context of truncated AR. It is important to find out whether the effect of FoxO1 on N/C interaction and coactivator recruitment to NTD depend on each other in the context of full length AR. Because PTEN inhibits AKT activity, the function of all AKT substrates is expected to be regulated by PTEN. However, FoxO factors are arguably the best AKT substrates whose role in mediating PTEN action has been consistently demonstrated by both genetic and biochemical analyses, which were proved by their role in PTEN-induced apoptosis in LNCaP cells (Fig. 24). Genetic analyses in C. elegans have shown that Daf-16, the sole worm FoxO factor, acts downstream of PTEN to regulate stress response and longevity (186, 187). Similarly, genetic analysis in mice has placed FoxO3a
downstream of PTEN in controlling the premature follicular activation (127). Our conclusion that nuclear FoxO proteins serve as the mediator for the suppressive effect of PTEN on the AR is consistent with observations that AKT is involved in AR inhibition by PTEN (163) does not directly increase AR activity through receptor phosphorylation at putative AKT sites (29, 188). This conclusion is also consistent with the demonstrated synergy between AR and AKT in PCa progression (189). Based on our initial observation that PTEN inhibited AR activity, we predicted that PTEN deletion would induce prostate tumorigenesis through unopposed action of the AR and contribute to resistance of PCa to androgen ablation therapy (190). This prediction was supported by prostate specific genetic deletion of PTEN in mice (156) and cell lines (191). The current study argues that the depletion of FoxO factors would have a similar effect on AI growth of PCa cells.

At least two mechanisms have been described for the AR-mediated inhibition of FoxO1 activity by androgens. One is through the blockage of FoxO’s DNA binding activity by protein-protein interaction between AR and FoxO1. The other is through the proteolytic cleavage of FoxO1 at residue Arg537, which generated a dominant negative FoxO1 leading to an inhibition of the activity of the intact FoxO1(165) by androgens. Our further investigation using AR DBD-deficient mutant and protein synthesis inhibitor suggest that AR-mediated inhibition of FoxO1 activity may also involve one of its target proteins. Although we have not yet identified this target protein, it might be participating in the acidic proteolytic cleavage of FoxO1 or form a complex with FoxO1 to block FoxO’s DNA
binding activity. It is possible that the protein synthesis inhibitor also suppresses new AR protein synthesis and thus relieves the AR inhibition of FoxO. Half-life of AR protein is approximately 6 h in the presence of 10 nM R1881(192). Treated with CHX for 6 h relieved the AR inhibition of FoxO more than two-fold, suggesting other proteins whose half-life less than 6 h might be involved in the AR inhibition of FoxO activity. Fig. 23 should be repeated after treated with CHX for different time points, and AR protein levels should be tested in parallel experiments to rule out the possibility that the data shown in Fig. 23 is only due to suppression of new AR protein synthesis.

Our current studies establish FoxO proteins as important nuclear factors that mediate the mutual antagonism between AR and PTEN tumor suppressor in PCa cells (Fig. 26). On the one hand, the inhibition of AR activity by PTEN through FoxO1 involved the interference of androgen-induce AR N/C interaction and the recruitment of the p160 coactivators to the AR N-terminus. On the other hand, the observations that PTEN fulfilled its biological function such as inducing apoptosis partly through FoxOs and previous information about AR inhibition of PTEN suggest that the suppression of FoxO factors by activated AR might contribute to the AR-mediated inhibition of PTEN action. In addition to the two mechanisms for AR inhibition on FoxO1 reported in the literature, we also suggest that AR-mediated inhibition of FoxO1 activity involves an unknown downstream target protein as a new mechanism.
Fig. 26. Model for Roles of Foxo Factors in the Mutual Antagonism between AR and PTEN. (See page 89 for details).
FoxO factors have been shown to be deregulated in several tumor types including PCa. The multiple FoxO functions associated with tumor protection including cell cycle arrest, cell death, resistance to oxidative stress and DNA repair, argue strongly for FoxO factors as new therapeutic targets. Much of cancer-related drug discovery has been focused on inhibitors of oncoproteins activated in tumor cells such as Gleevac, the inhibitor of the leukemia-associated BRC/ABL fusion gene product, and Herceptin for HER2 positive breast cancer and the various EGFR inhibitors. Alternatively, developing chemical molecules that act to restore the function of a defective tumor suppressor gene is a more specific strategy. Unlike PTEN expression, which is frequently lost in advanced PCa, multiple FoxO factors are redundantly expressed in prostate cells (137). Therefore, restoring the expression of FoxO factors in the nucleus through targeted inhibition of PI3K-AKT pathway would offer a possible approach to oppose androgen-AR action and to sensitize PCa to androgen ablation therapy.
SUMMARY AND THE PERSPECTIVES FOR FUTURE STUDIES

PCa is the most common cancer and the second leading cause of cancer deaths in American men. However, the mechanisms underlying the relapse and conversion of the tumor cells to an androgen refractory status after androgen ablation therapy remain unclear. Our studies on how the FoxO transcriptional factors participate in the antagonistic crosstalk between AR and PTEN tumor suppressor in PCa growth and progression reveal FoxOs as a novel molecular target for intervention to treat advanced PCa or improve the response of PCa patients to androgen ablation therapy.

PTEN mutations or deletions have been frequently identified in PCa. PTEN and AR play important roles in prostatic tumorigenesis by exerting opposite effects on the homeostasis of prostatic epithelium. These information lead to our previous work that showed a mutual repression between PTEN and AR in the growth and the apoptosis of PCa cells (163). The FoxO family is an AKT substrate and is inactivated by AKT after cellular stimulation by growth factors or insulin. Mainly though its negative effect on AKT, PTEN acts through FoxO factors to cancer cell growth (193). We have reported an AR-dependent repression of FoxO1 and FoxO3a function by androgens (164). These
findings led to the hypothesis that FoxO transcription factors mediate the mutual antagonism between AR and PTEN tumor suppressor in PCa cells.

Based on the data presented in this study, here we first demonstrated repression of the AR activity by FoxOs. Active FoxO1 decreased the AR transcriptional activity on reporters under the control of synthetic or natural AREs. FoxOs knockdown increased AR activity in PTEN-intact cells. The transcriptional inhibition also translated to the inhibition of AR biological function in terms of androgen-induced cell proliferation and production of the AR target protein PSA in PCa cells. More importantly, the data provide evidence to support that PTEN acts through nuclear FoxO factors to suppress AR as FoxO1 siRNA relieved AR inhibition by ectopic PTEN in PTEN null cells.

Several investigations have been made to understand the mechanisms for this AR inhibition by FoxOs and PTEN. Mutational analysis revealed that FoxO1 fragment 150-655, which contains the forkhead box and C-terminal activation domain, was required for AR inhibition. Neither the N-terminal 1-150 amino acids of FoxO1 nor the putative NR box was required for AR inhibition. The inhibition of AR neither was due to the nuclear exclusion by FoxO1 nor was associated with posttranscriptional modification such as acetylation. Mammalian two-hybrid assays demonstrated that the inhibition of AR activity by PTEN through FoxO1 involved the interference of androgen-induce interaction of the N- and C-termini of the AR and the recruitment of the p160 coactivators to the AR N-terminus.
We and another lab (165) have reported the AR inhibition on FoxO1 by two mechanisms. Our current data suggest that PTEN exerts its biological function at least partly through FoxOs since FoxO siRNA attenuated the PTEN-induced apoptosis. This indicates that AR-mediated suppression of PTEN function might be through the inhibition on FoxOs activity by androgens. We also reveal a new mechanism for the FoxO inhibition by AR. The observations that AR DBD-deficient mutant partially relieved AR inhibition on FoxO1 transcriptional activity and inhibition of new protein synthesis abolished the AR-related decrease in the mRNA level of FoxO1 target gene suggest that AR inhibits FoxO1 activity by producing a downstream target protein. Future study will try to find out this specific AR target gene.

There are several goals of future studies. We used mammalian two-hybrid assay to examine the mechanisms for the FoxO inhibition of AR. It would better to corroborate with other techniques such as the pull down assay to show that AR N/C interaction or interaction between AR and AIB1 is suppressed by FoxO factors. Furthermore, our findings are basically presented on the artificial but not endogenous AR target promoters. ChiP analysis should be performed to verify the finding that FoxO1 inhibits AIB1 recruitment to the endogenous AR target promoters, such as PSA gene promoter to support that the results are physiologically related. We have shown the suppression of biological activities of endogenous AR by FoxO1 in terms of cell growth and androgen-induced PSA production. It would be important to test whether knockdown of
FoxO1 will relieve the PTEN inhibition of AR biological activities. We have not yet identified the androgen target gene which is involved in the AR inhibition of FoxO1. One approach is to purify the FoxO protein upon androgen treatment in the cells with endogenous AR and to analyze the FoxO interaction proteins by Mass Spectrometry to find out the relative AR regulated gene.

As mentioned in the introduction, in cell culture-based systems, FoxO1, FoxO3, and FoxO4 behave similarly. Yet, mouse FoxO knockouts have revealed unique roles for the FoxOs, while the three FoxOs likely have significant redundancies. The differences in phenotypes of FoxO-null mice may be due to different patterns of expression of each FoxO isoform, but may also reflect specific regulations, protein partners, or target genes of these isoforms. Our work is cell line-based. The use of PCa mouse models will likely provide important clues as to whether in vivo loss of PTEN is a direct cause for promoting AR-specific gene activation. And specifically, what are the alterations of AR-specific gene activation and PCa status when loss of the nuclear function of each of the FoxO factor in the mouse model. Future studies should also investigate the correlation among wild type PTEN, AR and nuclear FoxO expression levels in human patient samples, as well as how this correlation relates to the stage or grade of PCa.

The wide range of benign to malignant phenotypes mediated by PI3K-PTEN-AKT signaling is consistent with the existence of diverse downstream effectors differentially utilized in conferring neoplastic phenotypes in distinct cell lineages. Among such potential
effectors are AKT phosphorylation targets TSC2, GSK3, and the FoxOs (108, 194, 195). Recently, much attention has been focused on TSC2 and mTOR signaling as mounting pharmacological evidence suggests that mTOR is the prime effector of the PI3K-AKT pathway. Indeed, the potent anti-neoplastic impact of pharmacologic mTOR inhibition raises questions as to the relevance of other AKT targets, particularly the FoxOs, in the development of cancer. However, the demonstration that FoxO inactivation is sufficient to drive development of hemangiomas suggests that misregulation of the FoxOs is a prime mechanism by which PTEN loss leads to the formation of hemangiomas in these patients, and also suggest that FoxO proteins exert their tumor-suppressive capability in the presence of additional mutations. In support of this view, Bouchard et al. identified Akt-mediated phosphorylation of FoxO proteins as the critical PI3K signaling component that substitutes for oncogenic Ras in Myc-induced proliferation and focus formation in vitro (135). Our cell-based analysis strongly suggests that FoxO factors play prominent roles in the cancer-relevant activities of the PTEN-PI3K-AKT network and AR in PCa cells, providing the rationale for future drug discovery to restore the nuclear FoxO activity in combination with androgen ablation to suppress AR signaling and prostate tumorigenesis. The approaches to restore nuclear FoxO activity include developing PTEN enzyme analogues, or PI3K inhibitors such as wortmannin and LY294002, or AKT inhibitors, or FoxO mutant proteins whose subcellular localization is restricted to the nucleus. In summary, these studies established a mutual inhibition of AR and FoxO1 activity and
establish FoxO proteins as important nuclear factors that mediate the mutual antagonism between AR and PTEN tumor suppressor in PCa cells. Our studies provide rationale for future investigation of restoring the nuclear expression of FoxO factors through targeted inhibition of PI3K-AKT pathway as a possible approach to oppose androgen-AR action and to sensitize PCa to androgen ablation therapy.
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