Insulin-like Growth Factor 1/Insulin Signaling Activates Androgen Signaling through Direct Interactions of Foxo1 with Androgen Receptor*\(^5\)

WuQiang Fan\(^1\), Toshihiko Yanase\(^1\), Tidetaka Morinaga\(^1\), Taijiro Okabe\(^1\), Masatoshi Nomura\(^5\), Hiroaki Daitoku\(^5\), Akiyoshi Fukamizu\(^1\), Shigeki Kato\(^1\), Ryoichi Takayanagi\(^1\), and Hajime Nawata\(^3\)

From the \(^1\)Department of Medicine and Bioregulatory Science, Graduate School of Medical Science, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, \(^2\)Center for Tsukuba Advanced Research Alliance, Graduate School of Life and Environmental Sciences, University of Tsukuba, Tsukuba, Ibaraki 305-8577, \(^3\)Institute of Molecular and Cellular Biosciences, Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo 113-0032, and \(^4\)Graduate School of Medical Science, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

The androgen-androgen receptor (AR) system plays vital roles in a wide array of biological processes, including prostate cancer development and progression. Several growth factors, such as insulin-like growth factor 1 (IGF1), can induce AR activation, whereas insulin resistance and hyperinsulinemia are correlated with an elevated incidence of prostate cancer. Here we report that Foxo1, a downstream molecule that becomes phosphorylated and inactivated by phosphatidylinositol 3-kinase/Akt kinase in response to IGF1 or insulin, suppresses ligand-mediated AR transactivation. Foxo1 reduces androgen-induced AR target gene expressions and suppresses the in vitro growth of prostate cancer cells. These inhibitory effects of Foxo1 are attenuated by IGF1 but are enhanced when it is rendered Akt-nonphosphorylatable. Foxo1 interacts directly with the C terminus of AR in a ligand-dependent manner and disrupts ligand-induced AR subnuclear compartmentalization. Foxo1 is recruited by liganded AR to the chromatin of AR target gene promoters, where it interferes with AR-DNA interactions.

Androgen receptor (AR)\(^2\) is a member of a nuclear receptor superfamily and functions as a ligand-dependent transcription factor. The androgen-AR system mediates male sexual differentiation in the uterus, sperm production at puberty, prostate development in the adult, and primary prostate cancer (PC) growth in patients with PC (1). PC is the most common malignancy in men worldwide and the second leading cause of cancer-related mortality in the United States (2). The fact that over 70% of PCs rely on androgen stimulation for growth sets the basis for androgen ablation therapy, which is initially effective but invariably results in treatment resistance after a period of time (3). The disease is then referred to as androgen-independent PC and progresses to a fatal outcome. Recent loss-of-function studies have revealed that AR still plays a key role in hormone-refractory progression of PC (4, 5). An adaptation of AR signaling in order to function under low or absent androgen levels may occur (6). Among the various suggested mechanisms by which AR may be reactivated in a low androgen environment (7), signal by growth factors, especially insulin-like growth factor 1 (IGF1), is reportedly of significant importance (8–11). High IGF1 serum levels are correlated with an increased risk of PC (8, 9), whereas IGF1 enhances AR transactivation under very low or absent androgen levels (12, 13) and promotes PC cell proliferation (10). Recent studies have also revealed that high serum insulin levels are associated with an increased incidence of PC (14, 15), although there is a lack of mechanistic studies implicating insulin signaling in the regulation of AR function.

Foxo1, also known as FKHR, together with other two homologs, FKHR1 and AFX, belong to the Foxo subfamily of the forkhead transcription factor family, which includes a large array of transcription factors characterized by the presence of a

* This work was supported by a grant-in-aid for the “Mechanism of Sex Differentiation” and a grant from the 21st Century COE Program from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^1\) The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–9.

\(^2\) To whom correspondence should be addressed. Tel.: 81-92-642-5280; Fax: 81-92-642-5287; E-mail: yanase@intmed3.med.kyushu-u.ac.jp.
conserved 110-amino acid winged helix DNA-binding domain (16). Foxo subfamily members play important roles in cell cycle regulation and apoptosis, as well as in metabolic homeostasis (17).

Phosphatidylinositol 3-kinase (PI3K)/Akt signaling, which is activated by both liganded IGF1 receptor (IGF1R) and insulin receptor, phosphorylates each of the Foxo proteins at three different Ser/Thr residues (17). The phosphorylated Foxo proteins become inactive and are exported from the nucleus. Subsequently, they become sequestered in the cytoplasm, where they interact with 14-3-3 protein.

In this study, we observe that Foxo1, which is endogenously expressed in PC cells, can interact with AR via the C terminus of the receptor in a ligand-dependent manner and suppress ligand-induced AR transactivation. Foxo1 impaired AR signaling by interfering with ligand-induced AR nuclear translocation and subnuclear compartmentalization as well as receptor-target gene promoter interactions. Furthermore, IGF1/insulin-PI3K/Akt pathway-induced phosphorylation of Foxo1 ameliorated the suppression. Intriguingly, liganded AR stimulated IGF1R expression, suggesting the presence of local positive feedback between IGF1 and AR signaling in PC cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—The human PC cell lines LNCaP, DU145, ALVA41, and PC3 were maintained as described previously (18). HEK293 and COS7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 10 units/liter penicillin, and 10 μg/ml streptomycin in 75-cm² flasks in a humidified 5% CO₂ incubator at 37 °C. The following items were obtained commercially: 5(PTEN) (mutated in multiple advanced cancers 1) (GenBank™ accession number NM_000314) was obtained from OriGene Technologies, Inc. (Rockville, MD). A full-length cDNA encoding human phosphatase and tensin homolog (PTEN) gene, in a ligand-bound AR were investigated using relative luciferase activity assays. COS7 cells were cotransfected with pCMV-hAR and an artificial luciferase reporter for AR (pGL3-MMTV) together with increasing doses of a Foxo1 expression vector. As shown in Fig. 1a, Foxo1 inhibited agonist-induced transcription from the MMTV promoter in a dose-dependent manner. Similarly, the transactivation of endogenous AR monitored by the MMTV promoter in LNCaP human PC cells was suppressed by Foxo1 in a dose-dependent manner (supplemental Fig. 1a). Foxo1 also inhibited liganded AR-mediated expression of the promoter of a native AR target gene, PSA, in both COS7 (supplemental Fig. 1b) and DU145 (supplemental Fig. 1c) human PC cells. Thus, the inhibitory effect of Foxo1 on AR, either exogenous or endogenous, is not limited to one cell line and/or promoter. Endogenous Foxo1 mRNA was readily detectable by RT-PCR in all four PC cell lines.
Foxo1 Represses AR

LNCaP cells are typical androgen-responsive PC cells, and their proliferation is largely dependent on the availability of androgen as well as functional AR. To study the effects of Foxo1 on LNCaP cell growth, the two cell lines stably expressing either FLAG-Foxo1 or the control FLAG tag were grown in medium containing charcoal-stripped serum supplemented with either DHT (10⁻⁹ M) or vehicle (ethanol), and a time course experiment was performed. Among the cells grown in the presence of DHT, the number of FLAG-Foxo1-expressing cells was decreased compared with control cells, even after 1 day of treatment, and then continued to decrease in a time-dependent manner (Fig. 1d). The basal proliferation in the presence of ethanol was also reduced to some extent in the stable FLAG-Foxo1-expressing cells, but more prominent inhibition was observed in cells treated with DHT. Thus, Foxo1 overexpression reduced the proliferation of LNCaP cells.

IGF1 Ameliorates Foxo1 Suppression over AR Transactivation—Foxo1 protein is a target of Akt kinase and negatively regulated by phosphorylation in an insulin- and/or IGF1-dependent manner, with resultant nuclear exportation and cytoplasmic sequestration. Therefore, either IGF1 or insulin is expected to attenuate the inhibitory effects of Foxo1 on AR. Initially, we observed that a constitutively active mutant, Foxo1-3A (nonphosphorylatable mutant with all three Akt target residues mutated to alanine, specifically T24A, S253A, and S316A), was more potent than Foxo1 at suppressing DHT-induced AR transactivation (Fig. 2) in LNCaP cells, in which the impaired PI3K/Akt signaling was rescued by cotransfection of PTEN (LNCaP cells lack functional PTEN, and their Akt is constitutively active (27, 28)). In these PTEN-expressing LNCaP cells, IGF1 significantly augmented liganded AR-mediated PSA promoter activities. Of importance, the IGF1-augmented DHT-induced AR transactivation was sharply suppressed by DHT. Thus, Foxo1 overexpression delayed the proliferation of LNCaP cells.

To further explore the relevance of the inhibitory effects of Foxo1 on AR observed in the promoter assays, the androgen-mediated endogenous PSA expression levels were studied in LNCaP cells stably expressing either FLAG-Foxo1 or the control FLAG tag. As shown in Fig. 1, b and c, DHT-stimulated PSA expression, which was dose-dependent in terms of both the mRNA level (assayed by real time PCR) and the amount of protein secreted into the medium (quantified by EIA), was significantly lower in cells overexpressing Foxo1, indicating that Foxo1 down-regulates the expressions of endogenous androgen-responsive genes. Lentivirus was applied as an additional method for overexpressing Foxo1 and LNCaP cells, and similar results were obtained (supplemental Fig. 1e).

Foxo1 also acted on other steroid hormone receptors (supplemental Fig. 2). Specifically, it enhanced the transcription mediated by liganded PR-A, PR-B, and GRα, exhibited no dramatic effect on ERα, but suppressed ERβ function in a similar manner to that observed for AR. Thus, the inhibitory effects are relatively specific for AR and ERβ.

FIGURE 1. Foxo1 represses ligand-induced AR transactivation and delays LNCaP cell proliferation. a, COS7 cells growing in 24-well plates were transiently cotransfected with a DNA mixture consisting of 300 ng of pGL3-MMTV, 5 ng of pRL-CMV, 30 ng of pCMV-hAR, and increasing amounts (0–120 ng/ml) of FLAG-Foxo1. The empty pcDNA-FLAG vector was cotransfected to equalize the total amount of DNA (in terms of the molar amount) and control for artifacts of the vector DNA. The cells were then treated with 10⁻⁷ M DHT or the solvent (ethanol) dissolved in serum-free medium for 24 h, before being analyzed by luciferase assays. b, and c, LNCaP cells stably expressing FLAG-Foxo1 or the control FLAG tag were grown in the presence of 10⁻⁷ M DHT or the solvent (ethanol), and cell proliferation was dynamically evaluated using a CellTiter 96 (nonradioactive cell proliferation kit. Data are presented as the mean ± S.D. (a, c, and d) or mean ± S.E. (b). Letters above the bars show statistical groups (ANOVA, p < 0.05). RLA, relative luciferase activity.
LNCaP cells in a concentration-dependent manner, albeit less potently (supplemental Fig. 3b).

To exclude the possibility that Foxo1 inhibits AR transactivation by decreasing the protein level of the receptor, the AR protein level was examined by immunoblotting under the same conditions used for the reporter assays. As shown in supplemental Fig. 4, a–c, cotransfection of Foxo1 with AR into LNCaP cells did not alter the AR protein level in either the presence or absence of DHT. Similarly, the AR mRNA expression level in lentiviral Foxo1-infected LNCaP cells was not significantly altered (supplemental Fig. 4d). Hence, these data indicate that Foxo1 suppresses the specific activity of the receptor.

**Physical Interaction between Foxo1 and AR**—Next, we examined whether there is a direct physical interaction between AR and Foxo1 by using communoprecipitation assays. LNCaP cells cotransfected with FLAG-Foxo1 and pCMV-hAR together with PTEN and grown in serum-free medium were treated with IGF1 in serum-free medium for 24 h, before being lysed and analyzed by luciferase assays. Letters above the bars show statistical groups (ANOVA, p < 0.05). The ascending order of the groups is as follows: F, A, C, G, E, H, B, and D. RLA, relative luciferase activity.

The interaction was further confirmed by modified mammalian one-hybrid assays (29). COS7 cells were cotransfected with an MMTV reporter and AR together with wild-type Foxo1,
pACT-Foxo1, or both, and then treated with DHT or two antiandrogens, namely hydroxyflutamide and casodex. As shown in Fig. 3b, FLAG-Foxo1 inhibited DHT-induced AR transactivation as expected. However, pACT-Foxo1 (with a viral VP16 activation domain fused to the Foxo1 N terminus) enhanced the liganded AR function, and the coexistence of FLAG-Foxo1 and pACT-Foxo1 neutralized the luciferase activity. Thus, although Foxo1 was suppressive toward AR, VP16-Foxo1 became an artificial coactivator for AR, and this remarkable shift strongly suggests that there is a direct interaction between AR and Foxo1. Interestingly, the antiandrogens casodex and hydroxyflutamide did not produce the same phenomenon, suggesting that only an agonist can induce the interaction.

Further clarification of the individual domains of AR responsible for the interaction with Foxo1 was carried out by mammalian two-hybrid assays, in which the AR N terminus (amino acids 1–660) and C terminus (amino acids 615–919) were individually fused to the DNA-binding domain of GAL4 to produce pBIND-AR-N and pBIND-AR-C, respectively. As a control, the full-length Foxo1 cDNA was fused to the VP16 activation domain in either the forward (pACT-Foxo1-F) or reverse orientation (pACT-Foxo1-R). In comparison with pACT-Foxo1-R, pACT-Foxo1-F did not alter the activity of the pG5-LUC reporter coexpression with pBIND-AR-N in either the presence or absence of the ligand, suggesting a lack of interaction between the AR N terminus and Foxo1 (Fig. 3c). In the case of pBIND-AR-C coexpression, pACT-Foxo1-F, but not pACT-Foxo1-R, significantly stimulated the reporter in the presence of DHT (Fig. 3d). These data indicate that the AR C terminus interacts with Foxo1 in a hormone-dependent manner.

Subcellular Interactions between Foxo1 and AR in Living Cells—The intracellular distribution of EYFP-Foxo1 in living COS7 cells was visualized using laser confocal scanning microscopy. Consistent with previous observations (17), EYFP-Foxo1 in serum-starved COS7 cells was predominantly located in the nucleus in a homogeneous manner, with relatively weak diffuse fluorescence also visible in the cytoplasm (Fig. 4a). However, the nuclear accumulation of Foxo1 varied among the examined cells (supplemental Fig. 6, a–c). This variation was presumably because of differing IGF1/insulin signaling tones among the different cells, because challenge with IGF1 caused 100% of the EYFP-Foxo1-expressing cells to show complete cytoplasmic fluorescence (Fig. 4b). As expected, EGFP-AR predominantly showed diffuse fluorescence in the cytosol with very weak nuclear fluorescence in the absence of the ligand (Fig. 4c), whereas DHT induced complete nuclear translocation and typical subnuclear foci formation (Fig. 4d). When EYFP-Foxo1 and EGFP-AR were coexpressed, both proteins showed their original distribution patterns in cells treated with the control solvents (IGF1−/DHT−; Fig. 4f). Among the cells treated with DHT alone (IGF1−/DHT+; Fig. 4g), the distribution of EYFP-Foxo1 remained almost unchanged, whereas EGFP-AR exhibited incomplete nuclear translocation and significantly impaired subnuclear compartmentalization (foci formation) and was diffuse in both the nucleus and cytoplasm. Although a subgroup of cells showed relatively complete nuclear translocation, their foci formation was apparently impaired (supplemental Fig. 6d). In cells treated with IGF1 alone (IGF1+/DHT−; Fig. 4i), EYFP-Foxo1 was completely exported to the cytoplasm, whereas EGFP-AR maintained its original distribution, namely predominant diffuse cytoplasmic localization with weak nuclear fluorescence. In comparison with IGF1−/DHT+ cells (Fig. 4g), EGFP-AR in cells treated with both ligands (IGF1+/DHT+; Fig. 4j) showed more complete nuclear translocation and, importantly, also exhibited significantly improved subnuclear compartmentalization.

To quantitatively evaluate the effects of IGF1/Foxo1 on the subnuclear distribution of liganded AR, the intranuclear distribution patterns of EGFP-AR in the cell populations, shown representatively in Fig. 4, d, g, and j, were subjected to line scan analyses using the LSM software as described previously (22). In Fig. 4, e, h, and k show the line scan data of d, g, and j, respectively. In total, 20 cells from each group were subjected to the line scan analyses. The fluorescence intensity of the cells in Fig. 4d was quite heterogeneous (heterogeneity index (HI) = 53.12 ± 10.53), whereas cotransfection of Foxo1 (cells in Fig. 4g) decreased the HI to 17.83 ± 9.46, indicating that the AR distribution in these cells was quite constant. The HI value recovered to 35.16 ± 18.89 in cells further treated with IGF1 (cells in Fig. 4j), suggesting that the Foxo1-inhibited subnuclear reorganization of liganded AR was partially rescued by IGF1.

3T3-L1 cells contain a relatively high quantity of endogenous AR, which undergoes typical subnuclear reorganization and forms fine foci in the presence of DHT (23). To address whether Foxo1 also suppresses the foci formation of endogenous AR, EYFP-Foxo1 was expressed in 3T3-L1 cells, and the endogenous AR distributions in the presence and absence of DHT were studied by immunofluorescence staining. As shown in supplemental Fig. 7, DHT-induced AR foci were significantly suppressed by Foxo1 overexpression. Specifically, the HI of liganded nuclear AR was 66.26 ± 15.77 (n = 20) in the absence of Foxo1 but reduced to 38.25 ± 8.91 (n = 20, p < 0.01) by Foxo1.

The distribution of EYFP-Foxo1 in cells coexpressing EGFP-AR and treated with both ligands (IGF1+/DHT+) was striking in that a substantial amount of Foxo1 remained in the nucleus (Fig. 4j) and supplemental Fig. 6e). This distribution is in great contrast to that in the cells in Fig. 4, d, g, and i, where IGF1 induced complete nuclear export of Foxo1 in all cells. These observations suggest that liganded AR sequestered part of the Foxo1 in the nucleus, even in the presence of IGF1. Furthermore, the nuclear Foxo1 in these cells was heterogeneous and colocalized with ligand-bound AR (Fig. 4j) and supplemental Fig. 6e), suggesting an interaction between the two proteins.

In contrast to EYFP-Foxo1, EYFP-Foxo1-3A resided in the nucleus in a reticular manner regardless of the IGF1 availability. Furthermore, it disrupted DHT-induced AR foci formation, and importantly, the disruption was not rescued by IGF1 (supplemental Fig. 8).

Interaction of Foxo1 and AR within the Human PSA Promoter—Next, ChIP assays were carried out to determine whether Foxo1 and AR associate with the chromatin of an AR target gene promoter in vivo. LNCaP cells cotransfected with pcDNA-FLAG-Foxo1 and pCMV-hAR were subjected to serum starvation and challenged with IGF1, insulin, or PBS as a control in the presence of DHT or the solvent (ethanol).
Foxo1 Represses AR
Genomic DNA corresponding to the androgen-response elements ARE I/II and ARE III, which are located in the promoter and enhancer of the PSA gene, respectively, was remarkably enriched in a DHT-dependent manner, as evaluated using an anti-FLAG antibody (Fig. 5b). Weak bands were detectable in the absence of any ligand, whereas treatment of the cells with IGF1 or insulin either abolished or significantly reduced the occupancy of the DNA by Foxo1, regardless of the presence or absence of DHT. There was very weak Foxo1 occupancy of the DNA in a region between the enhancer and the promoter, but no enrichment was detected in the presence of DHT, thus demonstrating specificity (Fig. 5b). Again, the weak bands vanished in the presence of IGF1 or insulin. The association of Foxo1 with the chromatinized PSA promoter was specific, because DNA from the GAPDH gene was not precipitated (Fig. 5b).

Thus, agonist-bound AR induces specific associations of Foxo1 with chromatin AREs on the PSA promoter, demonstrating that Foxo1 and AR form a ligand-dependent complex on chromatin. Furthermore, IGF1 and insulin are able to remove Foxo1 from chromatinized DNA.

The ChIP assays further revealed that Foxo1 interfered with ligand-induced recruitment of AR to the PSA promoter in LNCaP cells. The cells were transfected with pCMV-hAR together with pcDNA-FLAG–Foxo1-3A or pcDNA–FLAG and treated with the ligand as described above. Chromatin-transcription factor complexes were precipitated with an anti-AR antibody and combined with real time PCR analysis to quantify the relative number of immunoprecipitated DNA copies to the input control for each sample as described previously (24). Small amounts of the DNA segments corresponding to PSA ARE I/II and ARE III were detected in the absence of DHT, and DHT induced significant enrichment of these amounts (Fig. 5c). IGF1 or insulin increased the AR-DNA interaction under each circumstance (presence or absence of DHT; Fig. 5c). Overexpression of the nonphosphorylatable Foxo1-3A significantly reduced the relative copy numbers of PSA promoter DNA at both positions, especially in the presence of DHT (Fig. 5c), suggesting that the association between the PSA promoter and liganded AR was impaired. Furthermore, Foxo1-3A also substantially abolished the responsiveness to IGF1 and insulin (Fig. 5c), indicating that intact and phosphorylatable Foxo1 is vital for IGF1 or insulin to enhance the liganded AR-DNA interaction.

Activation of AR Stimulates IGF1R Expression in LNCaP Cells—We further found that IGF1R expression was up-regulated in LNCaP cells in the presence of DHT. As shown in Fig. 6a, DHT treatment for 24 h dose-dependently increased the IGF1R mRNA levels in serum-starved LNCaP cells, and 10^{-8} M DHT boosted the mRNA level by 17-fold. This stimulation of IGF1R was relatively specific, because comparable concentrations of DHT did not increase the levels of epidermal growth factor receptor (supplemental Fig. 9a) and Foxo1 mRNAs (supplemental Fig. 9b). An autoregulation process may occur, because the up-regulated IGF1R is expected to enhance IGF1–PI3K-Akt signaling, thereby resulting in a further gain-of-function of AR via phosphorylation of the repressive Foxo1.

DISCUSSION

Foxo1 Suppression and IGF1-related Mechanism of Refractory PC—IGF1 is capable of reactivating AR in low or absent androgen environments and therefore represents one of the suggested multiple mechanisms by which PC cells progress to the androgen-insensitive stage (7). However, the mechanisms by which IGF1 and other growth factors regulate AR-mediated transcription in PC cells remain unclear.

IGF1, via binding to its receptor IGF1R, activates intracellular signaling pathways that favor proliferation and cell survival. One of the most important pathways that becomes activated is the PI3K/Akt pathway (30), because activation of this pathway by IGF1, as well as other molecules, has been implicated in PC. Specifically, increases in IGF1R expression (11, 31, 32) and loss of the tumor suppressor gene PTEN (27, 28) elicit increased activity of the PI3K/Akt pathway and greatly contribute to tumor progression of PC.

Foxo1, which is endogenously expressed in PC cells, is functionally inhibited by phosphorylation at Ser-253, Ser-316, and Thr-24 in response to IGF1 or insulin through PI3K/Akt kinase (17). Overexpression of Foxo1 in PC cells leads to apoptosis (33). Our present results have revealed that Foxo1 directly interacts with and suppresses the transactivation of AR, whereas IGF1 signaling ameliorates this suppression and results in AR gain-of-function. Thus, considering the reported mechanism that β-catenin may mediate the stimulatory effect of IGF1 signaling on AR (34), Foxo1 may represent an alternative explanation.

Because insulin induces PI3K-Akt signaling and the resultant modification of Foxo1 (17), similar to IGF1, and also enhances AR transactivation in PTEN-expressing LNCaP cells, it is of great value to further elucidate whether insulin signaling enhances AR function via a similar mechanism. Regarding this point, it is noteworthy that the syndrome of insulin resistance, which is characterized by an increased insulin level and abdom-

FIGURE 4. Subcellular interaction between Foxo1 and AR in living COS7 cells. The expressions of chimeric fluorescent proteins were observed in living COS7 cells using a Zeiss LSM 510 META laser confocal microscope as described under “Experimental Procedures.” a and b, COS7 cells were transfected with 0.5 μg/dish of EYFP-Foxo1 and then treated with PBS (a) or 100 ng/ml IGF1 (b). c and d, COS7 cells expressing EGFP-AR were exposed to ethanol (c) or 10^{-8} M DHT (d), respectively. g, i, and j, COS7 cells coexpressing EGFP-AR and EYFP-Foxo1 were exposed to DHT and/or IGF1 as indicated in the individual panels. The fluorescence signals for green fluorescent protein (GFP) and yellow fluorescent protein (YFP) and the merged images are shown separately. e, h, and k, line scan analyses. A straight line was drawn through the target cell, and the fluorescence intensities along the line were recorded. The mean and S.D. values of the fluorescence intensity signals for the segment of interest (nucleus, avoiding the nucleoli) were calculated. The heterogeneity of the fluorescence intensity along the segment of interest was evaluated by the parameter of the HI, which was calculated using the following formula: HI = 100 x S.D./mean. A fluorescence intensity fluctuation graph, which clearly demonstrated the heterogeneity, was then created by plotting the intensity against the distance along the line. The lines obtained by line scan analyses are shown for representative cells. The fluorescence intensity fluctuation graphs of the representative cells are shown in e, h, and k and relate to the cells in d, g, and j, respectively. The x axis represents the distance along each line, whereas the y axis shows the fluorescence intensity. The bar within each graph marks the segment of the line for which the HI analysis was performed, and the corresponding intensity and HI values are indicated at the top of each graph. Scale bars, 10 μm.
Obesity, is also associated with an increased incidence of PC and development of a more aggressive form of the disease (14, 15), although the mechanism remains unclear. Nevertheless, the association between the risk of PC and the serum insulin level remains a controversial topic (35). There is even a lack of consensus over the association between the circulating level of IGF1 and the PC risk (30). With regard to this concern, our present results suggest that, in contrast to systemic circulating growth factors, there is also potential importance for the local bioavailability of growth factors and their cross-talk with hormones. Ligand-bound AR, which is functionally enhanced by IGF1-PI3K-Akt signaling-mediated dissociation of the repressor Foxo1, in turn stimulates the expression of IGF1R and presumably results in increased tension of IGF1 signaling, thereby leading to further functional augmentation of the receptor itself. Consistently, androgen withdrawal leads to a decrease in PI3K-Akt signaling (36, 37). Thus, as depicted in Fig. 6b, a mutually stimulatory feedback circuit (androgen-AR increases IGF1 signaling tension, whereas IGF1 signaling enhances AR transactivation), which works in an autocrine/intracrine manner in the local cellular environment of PC, may play important roles in up-regulation of AR function and PC progression.

AR Subnuclear Compartmentalization and Transactivation—Ligand-induced subnuclear compartmentalization (foci formation) is closely correlated with the transcriptional activation function of nuclear receptors (19–20, 22). DHT induces 250–400 fine and well separated subnuclear AR foci (19, 20), and the process is accompanied by the recruitment of coactivators such as SRC-1, TIF2, and CBP (20). AR bound to antiandrogens, such as hydroxyflutamide, does not form any foci (19), and cofactors that repress AR function readily disrupt DHT-induced foci formation by the receptor (18, 38). Our recent study further proved that the intranuclear complete/distinct foci formation of an agonist-bound steroid hormone receptor is an indicator of its transcriptional activation status (23).

Regarding this point, Foxo1 is able to disrupt AR nuclear translocation, and more importantly, the subnuclear foci formation induced by DHT is mechanistically significant. Although the mechanism remains unclear, it can be speculated that Foxo1 may compete with coactivators, such as SRC-1, TIF2, and CBP (20). AR bound to antiandrogens, such as hydroxyflutamide, does not form any foci (19), and cofactors that repress AR function readily disrupt DHT-induced foci formation by the receptor (18, 38). Our recent study further proved that the intranuclear complete/distinct foci formation of an agonist-bound steroid hormone receptor is an indicator of its transcriptional activation status (23).

FIGURE 5. Interaction of Foxo1 and AR within the human PSA promoter. a, schematic diagram of the human PSA gene promoter region. The positions of the putative AREs are indicated, and the transcription start site is designated 1. Pairs of arrows indicate the PCR-amplified regions. b, occupancy of the PSA gene-regulatory regions by Foxo1. Soluble chromatin was prepared from FLAG-Foxo1-expressing LNCaP cells treated with the indicated ligands (10−8 M DHT; 100 ng/ml IGF1; 10 μg/ml insulin) for 30 min and then immunoprecipitated with an anti-FLAG antibody or normal mouse IgG as a control. Enrichment of ARE-containing DNA sequences in the immunoprecipitated DNA pool, indicating association of Foxo1 with the PSA promoter within intact chromatin, was visualized by PCR. The lower panels show the PCR-amplified PSA promoter bands from the input controls. c, effects of IGF1/insulin and Foxo1 on the occupancy of the PSA gene-regulatory regions by AR. Additional real time PCR was performed to quantify the amount of immunoprecipitated PSA promoter DNA copy numbers from cells under various treatments relative to their corresponding input controls.
Foxo1 and conserved from mice (459LKELL463) to humans (452LKELL466) (21). The role, if any, of this motif in mediating with a consensus sequence of L with IGF1R mRNA expression. Triplicate results are expressed as the mean ± S.D. Letters above the bars show statistical groups (ANOVA, p < 0.05). b, proposed model for the cross-talk between IGF1/insulin signaling and AR. The local bioavailability of IGF1 (and/or insulin), which is probably abnormally high in PC, can be delivered from remote sites of production through the circulation or produced locally. Following ligand binding to IGF1R or insulin receptor, the tyrosine activity is activated, and this stimulates signaling pathways through intracellular networks that regulate cell proliferation and cell survival. One of the key downstream networks is the PI3K-Akt system. Akt, or a similar phosphatidylinositol 3,4,5-trisphosphate (PIP3)-dependent kinase, translocates to the nucleus and phosphorylates Ser-253, Ser-316, and Thr-24 of Foxo1. Phosphorylated Foxo1 is exported to the cytoplasm, where it has the potential to bind 14-3-3 protein and become retained in the cytoplasm. Foxo1 interacts with the C-terminal region of AR in the presence of a ligand (androgens such as DHT) and interferes with the ligand-induced subnuclear compartmentalization of AR, as well as the liganded receptor-target gene promoter interaction, thereby shutting down the AR transactivation. Modification of Foxo1 by the PI3K-Akt system, which is activated by IGF1 and/or insulin, weakens the Foxo1-AR interaction and ameliorates the inhibitory effects of Foxo1 on AR. Ligand-bound AR, in turn, stimulates the expression of IGF1R, resulting in enhanced IGF1 signaling tension. Moreover, liganded AR may also strengthen the net outcome of the IGF1-PI3K-Akt-Foxo1 axis. The mutual inhibition between the two potential Akt sites Thr-24 and Ser-316, Thr-24 is phosphorylated by an unknown kinase that is specifically induced by insulin receptor. This type of site-specific phosphorylation of Foxo1, and the corresponding profiles of kinases downstream of IGF1 and insulin receptor in the LNCaP cellular environment, may provide a potential explanation for the different effects of IGF1 and insulin on the Foxo1-AR interaction as well as AR function.

It is noteworthy that another group also investigated the Foxo1-AR interaction recently (40). Consistent with our data, full-length AR, as well as its C-terminal ligand-binding domain, was found to interact with Foxo1 in an androgen-dependent manner, although the authors also reported a ligand-independent interaction between the N-terminal A/B region of the receptor and Foxo1, which we did not observe in our mammalian two-hybrid assays. Through direct interactions, ligand-bound AR was found to be able to inhibit DNA binding, as well as the transactivation activity of Foxo1, and impair the ability of Foxo1 to induce prostate cell apoptosis and cell cycle arrest (40). Liganded AR is also reportedly able to induce Foxo1 protein proteolysis and therefore ameliorate Foxo1-related apoptosis of PC cells (41). These studies, in combination with our present findings, suggest that AR and Foxo1 are mutually suppressive in terms of functional regulation. Besides the above-mentioned IGF1R up-regulation, inhibition of Foxo1 provides an additional way in which liganded AR may enhance the IGF1/insulin-PI3K-Akt-Foxo1 axis. The mutual inhibition between Foxo1 and AR in the context of IGF1 signaling may play an important role in AR function and prostate cell apoptosis, as well as in PC etiology and progression (Fig. 6b).

Taken together, the present results have identified Foxo1, a molecule functionally inhibited by IGF1/insulin-PI3K-Akt signaling, as a novel corepressor for AR. The results further suggest that positive feedback between the growth factor and androgen in the local cellular environment may play important roles in the regulation of AR transactivation. These findings provide a new mechanism by which IGF1, and also potentially

**Foxo1-AR Interaction and Its Structural Basis and Regulation**

The direct interaction between Foxo1 and AR is ligand (DHT)-dependent and relies on the C terminus of the receptor, which is functionally ligand-dependent (20). Nuclear receptor cofactors bind to AR via peptide motifs called nuclear receptor boxes with a consensus sequence of LXXLL (L, leucine; X, any amino acid) (39). These boxes are present in the N terminus of Foxo1 and conserved from mice (459LKELL463) to humans (452LKELL466) (21). The role, if any, of this motif in mediating the Foxo1-AR interaction remains to be determined. The interaction is probably weakened, but not completely abolished, by IGF1, because IGF1 reduced the anti-AR antibody-immuno-precipitated Foxo1 levels and improved the Foxo1-disrupted AR subnuclear compartmentalization. Liganded AR also recruited Foxo1 to the chromatin of AR target gene promoters, where Foxo1, in turn, interfered with AR-DNA interactions, mirroring its disturbance of AR subnuclear compartmentalization. IGF1 or insulin, however, reduced the DNA occupancy by Foxo1 but consistently enhanced that by AR. These IGF1/insulin regulations probably rely on Akt-mediated phosphorylation of Foxo1, because the Foxo1-3A-inhibited AR-DNA interaction as well as the AR foci formation was not recovered by IGF1/insulin. Thus, IGF1, and perhaps also insulin, appears able to regulate the Foxo1-AR interaction via PI3K/Akt phosphorylation of Foxo1. It is important to note that the effects of IGF1 and insulin on AR function are similar but not identical. IGF1 is generally more potent and efficient at enhancing AR transactivation, ameliorating the DHT-induced Foxo1-AR interaction and rescuing the Foxo1-interrupted AR subnuclear compartmentalization (data not shown), although the difference was not that obvious in the ChIP assays. This phenomenon is presumably because of differential phosphorylation patterns of Foxo1 in response to IGF1 and insulin signaling. Although both hormones bind to their cognate receptors and activate the PI3K-Akt kinase cascade, which phosphorylates Foxo1 at Thr-24, Ser-253, and Ser-316, previous studies on hepatocytes (17) revealed that the phosphorylation patterns differ. Although the signaling pathways triggered by IGF1R and insulin receptor both phosphorylate the Akt site at Ser-253, which is a prerequisite for phosphorylation of the other two potential Akt sites Thr-24 and Ser-316, Thr-24 is phosphorylated by an unknown kinase that is specifically induced by insulin receptor. This type of signaling may provide a potential explanation for the different effects of IGF1 and insulin on the Foxo1-AR interaction as well as AR function.

**FIGURE 6. Stimulatory feedback circuit between IGF1/insulin signaling and AR transactivation.** a, DHT up-regulates IGF1R mRNA expression. LNCaP cells were serum-starved and then incubated with increasing concentrations of DHT for 24 h. Next, total RNA was extracted and reverse-transcribed into cDNAs, before the IGF1R mRNA expression levels were analyzed by real-time PCR as described under “Experimental Procedures.” The relative mRNA amounts were normalized to the corresponding abundance of GAPDH mRNA. Triplicate results are expressed as the mean ± S.D. Letters above the bars show statistical groups (ANOVA, p < 0.05). b, proposed model for the cross-talk between IGF1/insulin signaling and AR. The local bioavailability of IGF1 (and/or insulin), which is probably abnormally high in PC, can be delivered from remote sites of production through the circulation or produced locally. Following ligand binding to IGF1R or insulin receptor, the tyrosine activity is activated, and this stimulates signaling pathways through intracellular networks that regulate cell proliferation and cell survival. One of the key downstream networks is the PI3K-Akt system. Akt, or a similar phosphatidylinositol 3,4,5-trisphosphate (PIP3)-dependent kinase, translocates to the nucleus and phosphorylates Ser-253, Ser-316, and Thr-24 of Foxo1. Phosphorylated Foxo1 is exported to the cytoplasm, where it has the potential to bind 14-3-3 protein and become retained in the cytoplasm. Foxo1 interacts with the C-terminal region of AR in the presence of a ligand (androgens such as DHT) and interferes with the ligand-induced subnuclear compartmentalization of AR, as well as the liganded receptor-target gene promoter interaction, thereby shutting down the AR transactivation. Modification of Foxo1 by the PI3K-Akt system, which is activated by IGF1 and/or insulin, weakens the Foxo1-AR interaction and ameliorates the inhibitory effects of Foxo1 on AR. Ligand-bound AR, in turn, stimulates the expression of IGF1R, resulting in enhanced IGF1 signaling tension. Moreover, liganded AR may also strengthen the net outcome of the IGF1-PI3K-Akt-Foxo1 axis by inducing biodegradation and/or direct functional inhibition of Foxo1, which results in further amelioration of Foxo1-related apoptosis of PC cells. These mutually inhibitory interactions between AR and Foxo1 functioning in the context of a positive feedback circuit between AR and IGF1 signaling and working in an autocrine/intracrine manner in the local cellular environment may play important roles in AR function modulation, prostate cell apoptosis, and also PC etiology and progression.
insulin, enhances AR transactivation and may shed new light on our understanding of the progression of PC to the androgen-insensitive stage. Pharmacological strategies that reduce IGF1/insulin signaling, in combination with antiandrogen therapies, are expected to have clinical benefits in fighting PC.

REFERENCES
1. Brinkmann, A. O., and Trapman, J. (2004) Adv. Pharmacol. 47, 317–341
2. Greenlee, R. T., Murray, T., Bolden, S., and Wingo, P. A. (2000) CA-Cancer J. Clin. 50, 7–33
3. Santos, A. F., Huang, H., and Tindall, D. J. (2004) Steroids 69, 79–85
4. Zegarra-Moro, O. L., Schmidt, L. J., Huang, H., and Tindall, D. J. (2002) Cancer Res. 62, 1008–1013
5. Chen, C. D., Welsbie, D. S., Tran, C., Baek, S. H., Chen, R., Vessella, R., Rosenfeld, M. G., and Sawyers, C. L. (2004) Nat. Med. 10, 33–39
6. Buchanan, G., Irvine, R. A., Coetzee, G. A., and Tilley, W. D. (2001) Cancer Metastasis Rev. 20, 207–223
7. Grossmann, M. E., Huang, H., and Tindall, D. J. (2001) J. Natl. Cancer Inst. 93, 1687–1697
8. Pollak, M., Beamer, W., and Zhang, J. C. (1998) Cancer Metastasis Rev. 17, 383–390
9. Wolk, A., Mantzoros, C. S., Andersson, S. O., Bergstrom, R., Signorelli, L. B., Lagiou, P., Adami, H. O., and Trichopoulos, D. (1998) J. Natl. Cancer Inst. 90, 911–915
10. Burfeind, P., Chernicky, C. L., Rininsland, F., and Ilan, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7263–7268
11. Nickerson, T., Chang, F., Lorimer, D., Smeekens, S. P., Sawyers, C. L., and Pollak, M. (2001) Cancer Res. 61, 6276–6280
12. Cutil, Z., Hobisch, A., Cronauer, M. V., Radmayr, C., Trapman, J., Hittmair, A., Bartsh, G., and Klocker, H. (1994) Cancer Res. 54, 5474–5478
13. Orio, F. Jr., Terouanne, B., Georget, V., Lumbroso, S., Avances, C., Stiatka, C., and Sultan, C. (2002) Mol. Cell. Endocrinol. 198, 105–114
14. Amling, C. L. (2005) Curr. Opin. Urol. 15, 167–171
15. Hsing, A. W., Gao, Y. T., Chua, S., Jr., Deng, J., and Stanczyk, F. Z. (2003) J. Natl. Cancer Inst. 95, 67–71
16. Kops, G. J., and Burgering, B. M. (1999) J. Mol. Med. 77, 656–665
17. Nakae, J., Barr, V., and Accili, D. (2000) EMBO J. 19, 989–996
18. Chen, G., Nomura, M., Morinaga, H., Matsubara, E., Okabe, T., Goto, K., Yanase, T., Zheng, H., Lu, J., and Nawata, H. (2005) J. Biol. Chem. 280, 36355–36363
19. Tomura, A., Goto, K., Morinaga, H., Nomura, M., Okabe, T., Yanase, T., Takayanagi, R., and Nawata, H. (2001) J. Biol. Chem. 276, 28395–28401
20. Saitoh, M., Takayanagi, R., Goto, K., Fukamizu, A., Tomura, A., Yanase, T., and Nawata, H. (2002) Mol. Endocrinol. 16, 694–706
21. Nakae, J., Cao, Y., Daitoku, H., Fukamizu, A., Ogawa, W., Yano, Y., and Hayashi, Y. (2006) J. Clin. Investig. 116, 2473–2483
22. Fan, W., Yanase, T., Wu, Y., Kawate, H., Saitoh, M., Oba, K., Nomura, M., Okabe, T., Goto, K., Yanagisawa, J., Kato, S., Takayanagi, R., and Nawata, H. (2004) Mol. Endocrinol. 18, 127–141
23. Wu, Y., Kawate, H., Ohnaka, K., Nawata, H., and Takayanagi, R. (2006) Mol. Cell. Biol. 26, 6633–6655
24. Fan, W., Yanase, T., Morinaga, H., Mu, Y. M., Nomura, M., Okabe, T., Goto, K., Harada, N., and Nawata, H. (2005) Endocrinology 146, 85–92
25. Fan, W., Yanase, T., Nomura, M., Okabe, T., Goto, K., Sato, T., Kawano, H., Kato, S., and Nawata, H. (2005) Diabetes 54, 1000–1008
26. Pfaffl, M. W. (2001) Nucl. Acids Res. 29, e45
27. Xin, L., Teitell, M. A., Lawson, D. A., Kwon, A., Mellinghoff, I. K., and Witte, O. N. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 7789–7794
28. McMenamin, M. E., Souza, P., Perera, S., Kostura, L. M., and Sellers, W. R. (1999) Cancer Res. 59, 4291–4296
29. Liao, G., Chen, L. Y., Zhang, A., Godavarthy, A., Xie, F., Ghosh, J. C., Li, H., and Chen, J. D. (2003) J. Biol. Chem. 278, 5052–5061
30. Pollak, M. N., Schernhammer, E. S., and Hankinson, S. E. (2004) Nat. Rev. Cancer 4, 505–518
31. Hellawell, G. O., Turner, G. D., Davies, D. R., Poulsom, R., Brewster, S. F., and Macaulay, V. M. (2002) Cancer Res. 62, 2942–2950
32. Grezmi, M., Hemmerlein, B., Thelen, P., Schwess, S., and Burfeind, P. (2004) J. Pathol. 202, 50–59
33. Modur, V., Nagarajan, R., Evers, B. M., and Milbrandt, J. (2002) J. Biol. Chem. 277, 47928–47937
34. Verras, M., and Sun, Z. (2005) Mol. Endocrinol. 19, 391–398
35. Statin, P., and Kaaks, R. (2003) J. Natl. Cancer Inst. 95, 1086–1087
36. Perna, S., Maninis, M., Teodori, L., Cotonio, G., De Valse, S., and Morel, L. (2004) J. Biol. Chem. 279, 14579–14586
37. Castoria, G., Lombardi, M., Barone, M. V., Bilancio, A., Di Domenico, M., De Falco, A., Varricchio, L., Bottero, D., Nanayakkara, M., Migliaccio, A., and Auricchio, F. (2004) Steroids 69, 517–522
38. Tao, R. H., Kawate, H., Wu, Y., Ohnaka, K., Ishizuka, M., Inoue, A., Hagihara, H., and Takayanagi, R. (2006) Mol. Cell. Endocrinol. 247, 150–165
39. McMenarn, E. M., Rose, D., Flynn, S. E., Westin, S., Mullen, T. M., Krones, A., Inostroza, J., Torchja, J., Noile, R. T., Assa-Munt, N., Milburn, M. V., Glass, C. K., and Rosenfeld, M. G. (1998) Genes Dev. 12, 3357–3368
40. Li, P., Lee, H., Guo, S., Unterman, T. G., Jenster, G., and Bai, W. (2003) Mol. Cell. Biol. 23, 104–118
41. Huang, H., Muddiman, D. C., and Tindall, D. J. (2004) J. Biol. Chem. 279, 13866–13877

Foxo1 Represses AR