The Androgen Receptor Negatively Regulates the Expression of c-Met: Implications for a Novel Mechanism of Prostate Cancer Progression

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Abstract

The precise molecular mechanisms by which prostate cancer cells progress from androgen-sensitive to androgen-insensitive status still remain largely unclear. The hepatocyte growth factor/scatter factor (HGF/SF) plays a critical role in the regulation of cell growth, cell motility, morphogenesis, and angiogenesis. The aberrant expression of HGF/SF and its receptor, c-Met, often correlates with poor prognosis in a variety of human malignancies, including prostate cancer. Here, we investigate a potential link between androgen signaling and c-Met expression in prostate cancer cells. First, we showed that the androgen receptor (AR) represses the expression of c-Met in a ligand-dependent manner. Using different c-Met promoter/reporter constructs, we identified that Sp1 induces the transcription of c-Met and that AR can repress the Sp1-induced transcription in prostate cancer cells. Moreover, the data from electrophoretic mobility shift assay showed that AR interferes with the interaction between Sp1 and the functional Sp1 binding site within the c-Met promoter. Furthermore, we tested the effect of AR on c-Met expression in an androgen-insensitive prostate cancer cell line, CWR22Rv1. Finally, the repressive role of androgen signaling on c-Met expression was confirmed in prostate cancer xenografts. The above data indicate a dual role of AR in transcriptional regulation. Although the current androgen ablation therapy can repress the expression of growth-promoting genes that are activated by the AR, it may also attenuate the repressive role of AR on c-Met expression. Therefore, the therapeutic strategies to inhibit the activation of the HGF/c-Met pathway may be of benefit when combined with current androgen ablation treatment. [Cancer Res 2007;67(3):967–75]

Introduction

The effects of androgens, which are mediated mostly through the androgen receptor (AR), are important for the growth and survival of prostate cells (1, 2). Therefore, androgen ablation therapy has been frequently used for the majority of advanced prostate cancer patients (3). However, most patients develop androgen-insensitive prostate cancer within 2 to 3 years after initiation of therapy, for which there is currently no effective treatment. Although substantial effort has been devoted toward understanding the regulatory process by which prostate cancer cells progress from androgen-sensitive to androgen-insensitive status, the precise molecular mechanisms that control this conversion still remain largely unclear.

The hepatocyte growth factor/scatter factor (HGF/SF) is a multifunctional growth factor that plays a critical role in the regulation of cell growth, cell motility, morphogenesis, and angiogenesis (4). HGF/SF exerts its effects through its only known receptor, c-Met, a transmembrane receptor tyrosine kinase (5). The signaling pathway mediated through HGF/SF and c-Met is involved in the angiogenesis and metastasis of a wide variety of human carcinomas. The aberrant expression of HGF/SF and c-Met often correlates with poor prognosis in cancer patients (6). The c-Met signaling pathway can be activated through either paracrine or autocrine mechanism in tumor cells (7). Overexpression of c-Met can also activate the receptor-mediated pathway in a ligand-independent manner (8). Moreover, activating mutations of c-Met have been found in some types of cancers and metastatic lesions (9). It is believed that the effect of HGF/SF and c-Met in tumorigenicity and malignant progression is mainly mediated through induction of cell cycle progression, tumor cell migration, and tumor angiogenesis, and by inhibition of apoptosis (6).

HGF/SF and c-Met malignant functions are mediated through a network of signal transduction pathways and transcriptional events. Activation of c-Met by HGF/SF results in the autophosphorylation of specific tyrosine residues in its intracellular region that further induces the recruitment of scaffolding proteins, such as Gab1 and growth factor receptor binding protein 2, which lead to the activation of Ras and extracellular signal-regulated kinase/mitogen-activated protein kinase (10, 11). This changes the expression of cell cycle regulatory genes, such as *pRb*, *cdk6*, and *p27*, as well as extracellular matrix proteinases, such as matrix metalloproteinases and urokinase-type plasminogen activator (10, 12). Active c-Met also directly binds to phosphoinositide 3-kinase at phosphotyrosines 1,349 (Y-1349VHV) and 1,356 (Y-1356VNV; ref. 13).

A paracrine mechanism for HGF/SF stimulation of c-Met has been largely implicated in the progression of prostate cancer (14). It has been shown that HGF derived from prostate stroma significantly increases the proliferation, motility, and invasion of malignant epithelial cells through the c-Met protein (15, 16). It has been observed that c-Met is highly expressed in the AR-negative prostate cancer cell lines, such as DU145 and PC-3, but slightly expressed in the AR-positive prostate cancer cell lines, such as LNCaP, LAPC-4, CWR22, and LuCaP (16, 17). In the Dunning rat model, the expression of c-Met is much higher in the androgen-insensitive and high metastatic potential HI-F subline than in the androgen-sensitive and low metastatic potential G subline (16). Moreover, it has been observed that c-Met is highly expressed in

basal and intermediate cells in the prostate epithelium but not in AR-positive luminal cells (18–20). Furthermore, an increase of c-Met expression was shown in castrated rats (16, 21). Finally, up-regulation of c-Met expression is observed in prostate cancer tissues, particularly in metastatic tumor samples (17, 20, 22). Based on the above observations, we hypothesized that androgen signaling regulates the expression of c-Met in prostate cancer cells. In this study, we provide multiple lines of evidence to show that the AR negatively regulates the transcription of c-Met through interfering with Sp1 transcription factor. Our results provide a novel insight into the progression of prostate cancer, which should significantly affect future prostate cancer therapeutic strategies.

Materials and Methods

Cell culture and transfections. The human prostate cell line DU145 was maintained in DMEM supplemented with 5% fetal bovine serum (FBS, HyClone, Denver, CO). An AR-positive prostate cancer cell line, LNCaP, was maintained in T-medium (Life Technologies, Gaithersburg, MD) with 5% FBS. Transient transfections were done using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) as described previously (23). For androgen induction, cells were cultured for 16 to 24 h in medium with charcoal-stripped FBS (HyClone) in the presence or absence of 1 or 10 nmol/L of dihydrotestosterone (DHT).

Plasmid construction. The β-galactosidase reporter plasmid driven by cytomegalovirus promoter was generated as described previously (24). The human c-Met promoter-luciferase constructs were generously provided by Dr. Youhua Liu (Department of Pathology, University of Pittsburgh, Pittsburgh, PA; ref. 25). A 21mer sequence (5′-GGTGTCACTATGGAGCTCT CA-3′, amino acids 568–575) of the human AR cDNA was used to generate the AR short hairpin RNA (shRNA) vector into the pBS/U6 vector (26). The AR shRNA lentiviral or adenoviral constructs were subsequently generated from the pBS/U6 plasmid and the viruses were produced as previously described (27).

Reverse transcription-PCR analyses. Total RNA (1 μg) was used to do reverse transcription-PCR (RT-PCR). The following primers were used: human c-Met forward, 5′-GTTTCCCAATTTCTGACC-3′, and reverse, 5′-TATATCAAAGGTGTTTAC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward (388–403 bp), 5′-CCATGGAGAAGGCTGGGG-3′, and reverse (582–563 bp), 5′-CAAAGTTGTCATGGATGACC-3′. Expression levels of c-Met mRNA were quantified using quantitative fluorescent real-time PCR. Briefly, RNA samples were first reverse-transcribed using random hexamers as described by the manufacturer (PE Applied Biosystems, Foster City, CA). PCR assays were done with TaqMan PCR reagent kits in the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems). The levels of c-Met or prostate-specific antigen (PSA) mRNA were normalized by coamplification of GAPDH mRNA as described by the manufacturer (PE Applied Biosystems).

Luciferase and β-galactosidase assays. Luciferase and β-galactosidase activities were measured as previously described. The relative light units (RLU) from individual transfection were normalized using β-galactosidase activity in the same samples. Individual transfection experiments were done in triplicate and the results are reported as mean RLU (luciferase/β-galactosidase; ±SD).

Western blotting. To prepare the whole-cell lysates, cells were washed with PBS and resuspended in radioimmunoprecipitation assay (RIPA) buffer [1% NP40, 0.1% SDS, 50 mmol/L NaF, 0.2 mmol/L Na_3VO_4, 0.5 mmol/L DTT, 150 mmol/L NaCl, 2 mmol/L EDTA, 10 mmol/L sodium phosphate buffer (pH 7.2)]. Proteins were boiled in SDS-sample buffer, resolved on a 10% SDS-PAGE, and transferred onto a nitrocellulose membrane. The membranes were then probed with a polyclonal anti-AR and a polyclonal anti–c-Met antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were detected using the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL).

Nuclear extracts and gel mobility shift assay. Nuclear extracts were prepared as described previously (28). The protein concentration in the extracts was determined by the BCA assay (Pierce, Rockford, IL). *In vitro* translated AR proteins were generated using the TNT-coupled reticulocyte lysate system (Promega, Madison, WI). The binding analysis was done using the following double-stranded oligonucleotides: c-Met 5′-CTTGTC-GTGGGCGGGGCAGAGGCGGGAGGAAAC-3′ (wt/wt) 5′-CTTGTCGTG-TTCATGGCAGAGGCGGGAGGAAAC-3′ (mut/wt) 5′-CTTGTCGTGG-GCGGGGCAGAGTTCATAGGAAAC-3′ (wt/mut) 5′-CTTGTCGTGTTCATGG-CAGAGTTCATAGGAAAC-3′ (mut/mut). Sequences of the primers used for amplifying the 0.2 c-Met fragment are 5′-GCGAGAAAACTTCTCCACCTAG-3′ and 5′-ACCAGACTGAGGCGCTCGCCTG-3′. Double-stranded oligonucleotides were labeled with T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and were used as probes. *In vitro* DNA binding was done by incubating the nuclear extract in a buffer containing 20 mmol/L HEPES (pH 7.9), 100 mmol/L KCl, 5 mmol/L $MgCl_2$, 0.1 mmol/L EDTA, 12% glycerol, 4 mmol/L DTT, and 1 μg poly(deoxyinosinic-deoxycytidylic acid), from Amersham Biosciences (Piscataway, NJ), for 30 min on ice. An anti-Sp1 antibody or anti-AR antibodies were added in the above reactions used (Santa Cruz Biotechnology) for supershift assays. When necessary, an excess of unlabeled double-stranded oligonucleotides was incubated with nuclear extracts before the addition of the labeled DNA probe. The reaction mixtures were analyzed on a 4% nondenaturing, native polyacrylamide gel (29:1 of acrylamide/bisacrylamide) using 0.25× Tris-borate EDTA buffer at 160 V for 3 h. Gels were then dried and visualized by autoradiography.

Northern blotting. Total RNA was isolated using an RNAwiz kit (Ambion, Austin, TX). Twenty micrograms of RNA were fractionated on a 1% agarose-formaldehyde gel, transferred to Hybond-N nylon membranes (Amersham Biosciences), and hybridized with a DNA fragment (amino acids 1–300) derived from the human c-Met gene. Hybridization was done overnight at 42°C in ULTRA hybridization buffer, according to the manufacturer's instructions (Ambion). The blots were stripped and rehybridized with a human GAPDH probe (29).

Xenografting. LNCaP cells were suspended in unpolymerized rat tail collagen (30). Twenty 8-week-old male nonobese diabetic-severe combined immunodeficient (NOD-SCID) mice bred through the BC Cancer Research Centre (BC Cancer Agency, Vancouver, BC, Canada) were used in the experiments. Resultant collagen gels were grafted (2×10^6 cells per gel) under renal capsules (one graft per kidney) of NOD-SCID mice that were then supplemented with testosterone via s.c. implanted testosterone capsules (10 mg/mouse) as reported earlier (30, 31). After 2 months, half of the animals were castrated for 1 week and then sacrificed with the rest of the animals. The grafts were harvested, measured, and fixed for histopathologic and immunohistochemical analyses.

Immunohistochemistry. Graft tissues were fixed in 10% neutral buffered formalin, processed through alcohols, and embedded in paraffin. Sections were cut at 4-μm intervals, dewaxed in Histoclear (National Diagnostic, Atlanta, GA), and hydrated in graded alcohol solutions. Endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol for 30 min followed by washing with PBS (pH 7.4), and then incubated with the blocking solution (ImmunoVision Technology, Spingdale, AR) for 30 min. The adjacent sections were then incubated with primary antibodies overnight at 4°C or with nonimmune mouse or rabbit IgG (Zymed Corp, So. San Francisco, CA). The rabbit anti–c-Met (Santa Cruz Biotechnology), the mouse anti-AR (Santa Cruz Biotechnology), or the mouse anti-human PSA antibody (DAKO, Carpinteria, CA) was used for immunohistochemical staining at a dilution of 1:200, 1:100, or 1:50, respectively. Following incubation, sections were washed, incubated with either a biotinylated anti-rabbit or anti-mouse antibody (Vector Laboratories, Burlingame, CA), and then incubated with avidin-biotin complex (Vector Laboratories). The samples were visualized using 3,3′-diaminobenzidine in PBS and 0.03% H_2O_2. All sections used for immunohistochemistry were lightly counterstained with 5% (w/v) Harris hematoxylin.

Results

The androgen signaling pathway negatively regulates c-Met expression. To assess the potential role of AR on the transcription

of c-Met, we first examined the level of c-Met transcript in the AR-positive prostate cancer cell line, LNCaP, cultured either in the presence or absence of androgens. Using RT-PCR, we showed that the transcript of c-Met is much higher in cells cultured in the absence of DHT than the cells cultured in the presence of 10 nmol/L of DHT (Fig. 1*A*). We then further confirmed the negative effect of DHT in regulating the expression of the *c-Met* gene by real-time PCR. As shown in Fig. 1*B*, levels of c-Met transcript are gradually reduced by DHT in a dose-dependent manner. At 10 nmol/L, the level of c-Met transcript is reduced nearly four times less than the samples in the absence of DHT. As a control, the level of PSA transcript is up-regulated in the same cells in a ligand-dependent manner. The above data provide the first line of evidence showing that the expression of c-Met is negatively regulated by androgens in AR-positive prostate cancer cells.

Next, we directly investigated the involvement of AR in the regulation of c-Met expression by carrying out knockdown experiments using shRNA interference. Depletion of endogenous AR through transduction of AR shRNA adenoviruses in LNCaP cells attenuated the repressive effect of AR on c-Met expression. As shown in Fig. 1*C*, c-Met mRNA levels, assessed by RT-PCR, were elevated in LNCaP cells after 8 or 16 h of infection with AR shRNA adenoviruses in both the presence and absence of DHT. As observed above, depletion of DHT also attenuated the repressive effect of the c-Met transcript in either untransduced cells or cells transduced with the control viruses. We also confirmed the above-mentioned effect of AR on c-Met by using real-time PCR. Knockdown of AR expression by the AR shRNA adenoviruses

induced an ∼ 3-fold increase in the expression of c-Met in LNCaP cells (Fig. 1*D*). To confirm the specificity and effectiveness of the AR shRNA adenoviruses used in the above experiments, we also examined the expression of *PSA*, an AR downstream target gene, in the same samples. As shown in the results, an inverse correlation between the expression of *PSA* and *c-Met* genes was observed in all of the above-mentioned experiments. These results suggest that the repressive effect of androgens on c-Met expression is mediated through the AR.

To further evaluate the AR role in the regulation of c-Met expression, we tested whether changing AR expression can regulate the levels of c-Met expression in an AR-positive but androgen-insensitive cell line, CWR22Rv1. As reported previously, two different sizes of the AR protein were present in CWR22Rv1 cells (32). Infection of these cells with AR shRNA adenoviruses down-regulated the expression of both AR transcript and protein (Fig. 1*G*). In addition, the expression of PSA mRNA was also significantly reduced by the AR shRNA virus infection (Fig. 1*E*). However, only a slight increase in the c-Met transcript (Fig. 1*E* and *F*) and protein levels (Fig. 1*G*) was observed in CWR22Rv1 cells infected with the AR shRNA viruses compared with the control samples. The above results indicate that although AR may still play a critical role in the regulation of PSA expression, the repressive effect of AR on c-Met expression is clearly reduced in CWR22Rv1 cells. Understanding the different roles of AR on c-Met signaling in both androgen-sensitive and androgen-insensitive prostate cancer cells may help us to explore the potential mechanism by which tumor cells progress from androgen-sensitive to androgen-insensitive or androgen-independent status.

Figure 1. AR signaling pathway negatively regulates c-Met expression in prostate cancer cells. *A,* total RNA (200 ng) isolated from LNCaP cells treated for 24 h in the presence or absence of 10 nmol/L DHT was analyzed by RT-PCR for *c-Met* expression. *B,* the RNA samples were isolated from LNCaP cells in the presence or absence of DHT and were analyzed by RT-PCR. *C,* LNCaP cells were infected with AR shRNA or control shRNA adenoviruses for 8 or 16 h and cultured in the presence or absence of DHT. Total RNA samples were then isolated and used for detecting *c-Met* expression by RT-PCR. The above RNA samples (30 μg) were also analyzed by Northern blotting to detect PSA expression. The expression of GAPDH by RT-PCR was used as a loading control. *D,* total RNA samples were isolated from LNCaP cells infected with either the AR shRNA or the control adenoviruses, in the presence of or absence of DHT (10 nmol/L), and were analyzed by RT-PCR to detect the expression of PSA and c-Met. *E,* CWR22Rv1 cells were infected with the AR shRNA or the control adenoviruses and incubated either in the presence of 10 nmol/L DHT or in the absence of DHT. Total RNA samples were isolated and analyzed by RT-PCR for c-Met expression. *F,* polyadenylate RNA samples were isolated from the above total RNA samples and 2 μg were used in Northern blot experiments. *G,* CWR22Rv1 cells were infected with AR shRNA adenoviruses and then cultured in the presence of 10 nmol/L DHT for 24 h as described above. Total cell lysates were analyzed by Western blotting with c-Met, AR, and tubulin antibodies.
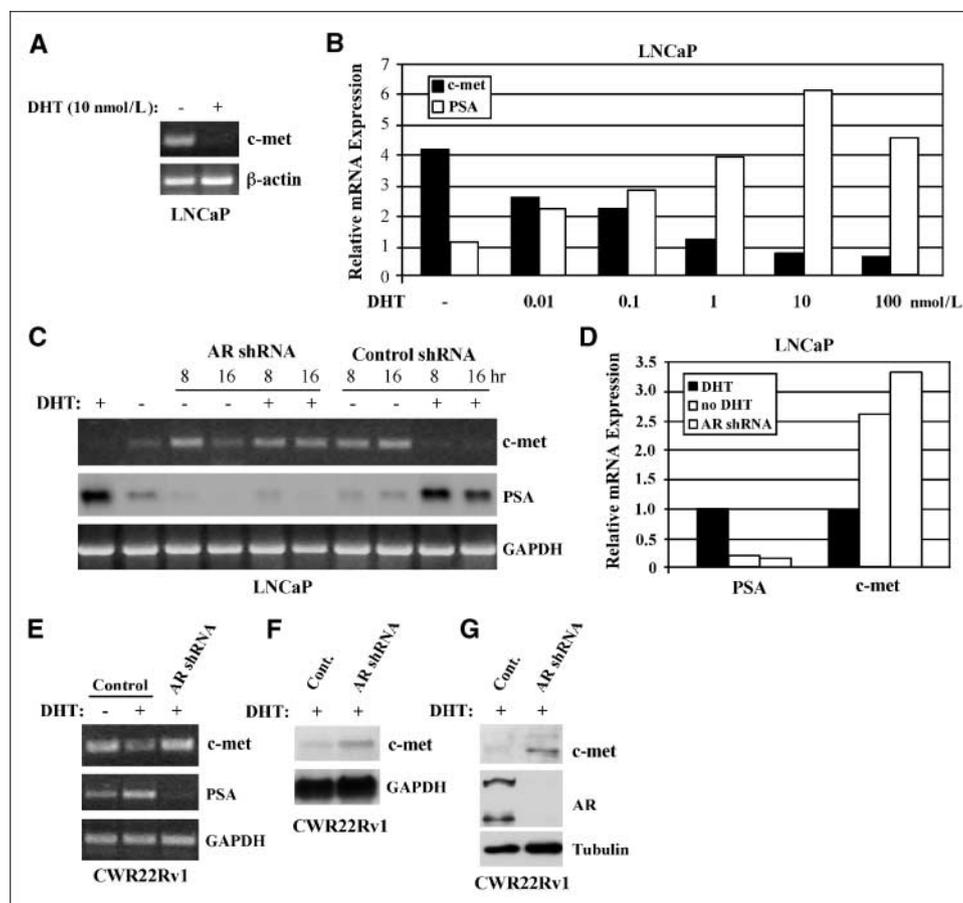
The AR negatively regulates the c-Met promoter. Given the fact that the AR is a ligand-dependent transcription factor, we examined whether the repressive effect of AR on c-Met expression is mediated directly through the promoter region of the *c-Met* gene. To examine the promoter activity of c-Met, we first transfected different truncated promoter/reporter constructs in the AR-negative prostate cancer cell line DU145. The reporter construct containing a proximate 200-bp promoter fragment (0.2 met-luc) seems similarly active as the construct with the 2.6 large fragment (2.6 met-luc). This is consistent with previous studies and shows that a functional promoter region exists within the ∼200-bp proximate fragment of the transcriptional start site of *c-Met* (33). Intriguingly, coexpression of the AR with the c-Met promoter/reporter constructs significantly reduced the activity of the reporter constructs that contain the proximate promoter region (Fig. 2*B*). A dose-dependent repressive effect of the AR was observed in the samples cotransfected with 0.2 c-Met luc. Repeat of the above experiments in the absence or presence of androgens further showed that the repressive effect of the AR on the c-Met promoter/reporter constructs is androgen-dependent (Fig. 2*C*). To evaluate the repressive effect of endogenous AR on the c-Met promoter, we transfected the c-Met promoter/reporter constructs into LNCaP cells. Interestingly, the 0.2 met-luc construct seems much more active than the other promoter/reporter constructs in the absence of androgens (Fig. 2*D*). Addition of androgens in the above experiments clearly reduced the activity of the c-Met promoter/reporter, demonstrating that endogenous AR negatively regulates the activity of the c-Met promoter in a ligand-dependent manner. Taken together, the above data indicate that the repressive effect of AR on c-Met expression is directly mediated through the c-Met promoter.

AR represses Sp1-induced c-Met promoter activity. Several functional Sp1 binding sites have been identified in the proximate promoter region of c-Met (Fig. 2*A*). Involvement of Sp1 transcription factors through the above sites on c-Met transcription has also been documented in a previous study (34). Particularly, it has been shown that two of the Sp1 binding sites between bp −144 and −127 of the *c-Met* promoter are important in the regulation of *c-Met* transcription (34). To confirm the role of Sp1 in the regulation of the promoter of c-Met, we coexpressed the Sp1 protein with the 0.2 met-luc reporter in LNCaP cells. One and half–fold induction of promoter activity was observed in cells with overexpressed Sp1 protein in the absence of DHT in the AR-positive prostate cancer cell line, LNCaP (Fig. 2*E*). However, the induction by Sp1 was gradually reduced in cells when different concentrations of DHT were added. Knockdown of the AR expression by the specific AR shRNA adenoviruses attenuated the repressive effect of the AR on the c-Met promoter. The above data suggest that AR represses the Sp1-induced c-Met promoter activity.

AR interferes with Sp1 binding to the promoter region of c-Met. To further explore the regulatory mechanisms by which AR represses Sp1-induced c-Met promoter activity, we examined whether the AR affects the DNA-binding activity of Sp1 to the c-Met promoter. As reported previously, several consensus sequences of Sp1 binding sites have been identified in the proximate promoter region of *c-Met* (Fig. 3*A*). To detect the interaction between Sp1 and the c-Met promoter region, we did electrophoretic mobility shift assay (EMSA) with nuclear extracts isolated from DU145 cells and the c-Met proximate promoter fragment (0.2 met) as the probe, which encompasses the region from bp −223 to +60. Two major DNA-protein complexes were detected (Fig. 3*B*). The complexes can be specifically competed out by the cold 0.2 c-Met fragment and also react with the monoclonal antibody of Sp1 to form a slower-migrating supershift complex. These results show that Sp1 can bind to the promoter region of c-Met and form DNA-protein complexes (Fig. 3*B*). Using four double-stranded oligonucleotides containing DNA consensus sequences of either wild-type or mutated Sp1 binding sites (Fig. 3*A*), we further determined the precise sites for Sp1 binding. As shown in Fig. 3*C*, although the data from EMSA showed that both of the Sp1 binding sites located between bp −152 to −120 contribute to the binding, the distal site is more effective than the proximal site because the double-stranded oligonucleotides containing the mutated distal site seems less competitive than the ones with the mutated proximal site.

To test the direct effect of AR on Sp1 binding to the c-Met promoter region, we repeated the EMSA assay using *in vitro* translated AR protein. The addition of *in vitro* synthesized AR protein reduces the activity of Sp1 binding to the c-Met promoter fragment (Fig. 3*D, lanes 3* and *4*). As a control, addition of the same amounts of reticulocyte lysate showed no effect on the DNA-binding activity of Sp1 (Fig. 3*D, lanes 5* and *6*). These results provide a direct line of evidence to show that AR interferes with the activity of Sp1 binding to the c-Met promoter region, which may be a molecular mechanism for AR to repress c-Met expression.

Castration induces the expression of c-Met expression in LNCaP cell xenografts. To further determine the role of androgens in the regulation of c-Met expression *in vivo*, we examined the expression of c-Met in LNCaP cell xenografts with or without castration in the male SCID mouse hosts. Poorly differentiated tumor masses were observed in both testosterone-supplemented intact (Fig. 4*A*) and castrated (Fig. 4*B*) hosts after ∼8 weeks of tumor cell injection. Results of immunohistochemistry showed the strong expression of both AR (Fig. 4*C*) and PSA (Fig. 4*E*), but weak expression of c-Met (Fig. 4*G*), in LNCaP xenografts isolated from the testosterone-supplemented mouse hosts. Intriguingly, in the castrated animals, increased expression of c-Met was detected in LNCaP tumors (Fig. 4*H*). In contrast, the expression of AR (Fig. 4*D*) and PSA (Fig. 4*F*) was decreased in these samples. The above data provide an additional line of evidence to show the link between androgen signaling and c-Met expression *in vivo*.

Discussion

The critical roles of the HGF receptor, c-Met, have been largely implicated in tumorigenesis, and particularly in tumor progression. Up-regulation of c-Met expression has been frequently found in advanced, metastatic, and refractory prostate cancers (16). An inverse correlation between the expression of AR and c-Met has been observed in prostate epithelium and prostate cancer cell lines (16, 17), implying a potential link between androgen signaling and c-Met expression in prostate cells. In this study, we confirmed the repressive role of androgens on c-Met transcription in the AR-positive prostate cancer cell line LNCaP. A clear induction of c-Met transcription was observed in LNCaP cells cultured in the absence of androgens. Addition of androgens into the culture medium repressed the above induction in a dose-dependent manner. We also observed the increase of c-Met expression in cells treated with low concentrations of androgens, which is relevant to the fact that residual androgens are always detected in prostate

Figure 2. AR negatively regulates c-Met promoter activity. *A,* schematic representation of the *c-Met* promoter truncation constructs used in this study. *B,* DU145 cells were transiently transfected with 100 ng of the different *c-Met* promoter-luciferase constructs and the pcDNA3-AR expression construct. After 16 h, cells were incubated in DMEM with 5% charcoal-stripped FBS and 10 nmol/L DHT for another 24 h. Luciferase and β-galactosidase activities were measured and reported as RLU. *C,* 5 ng of pcDNA3-AR expression vector and 100 ng of the 0.2, 1.6, and 2.6 *c-Met* promoter-luciferase constructs were transfected into DU145 cells. Sixteen hours after transfection, the indicated amounts of DHT were added and the cells were incubated for another 24 h. Luciferase and β-galactosidase activities were measured. *D,* LNCaP cells were transiently transfected with 100 ng of different *c-Met* promoter-luciferase constructs as described in Materials and Methods. Cells were then incubated in T-medium with or without DHT. After 24 h, whole-cell lysates were prepared, and luciferase and β-galactosidase activities were measured. *E,* different plasmids were transiently transfected into LNCaP cells, and luciferase and β-galactosidase activities were measured from the above lysates.
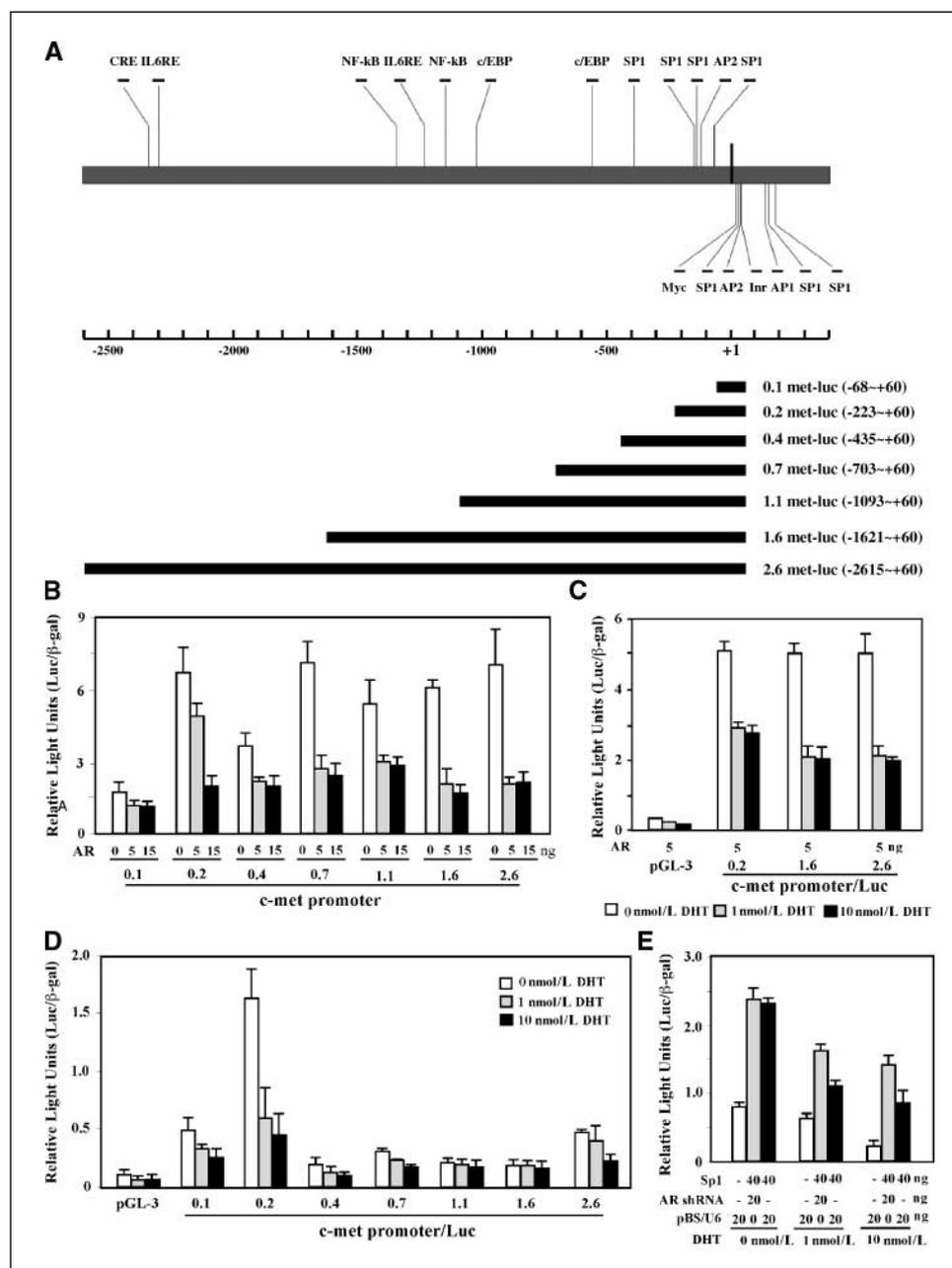
cancer patients during or after androgen ablation therapies (35). Using shRNA interference approach, we further showed a negative role for AR in the regulation of c-Met expression. Down-regulation of AR by the shRNA in LNCaP cells was coupled to an increase in c-Met expression levels. Intriguingly, elevation of c-Met in cells infected with the AR shRNA viruses was observed in cells cultured in both the presence and absence of androgens. This clearly indicated that the repressive effect of androgens on c-Met expression is solely mediated through the AR. Using an AR downstream target gene, *PSA*, as a control, we further showed an inverse effect of AR knockdown in prostate cancer cells, which provides the additional line of evidence to show the involvement of the AR in the regulation of c-Met expression.

Using different c-Met promoter/reporter constructs, we further tested the repressive effect of AR on c-Met transcription.

Transfection of seven c-Met promoter constructs that encompass ~ 2.6 kb of the c-Met promoter region (−2,612 to +60 bp) showed almost similar luciferase activity, except for the 0.1 construct, in DU145, an AR-negative prostate cancer cell line (25). Importantly, expression of AR in DU145 cells led to a significant decrease in the activity of c-Met promoter/reporter constructs. These repressive effects seem to be dose dependent and require androgens. A similar androgen-dependent repression through endogenous AR on the above c-Met promoter/reporter vectors was also observed in the AR-positive prostate cancer cells. The consistent repressive effect of AR on 2.6, 1.6, and 0.2 c-Met promoter/reporter constructs indicates that the proximal promoter of c-Met is the potential target region for AR. It has been previously shown that there are three functional Sp1 binding sites within the proximal region of the c-Met promoter (25). In human fibroblasts, two distal Sp1 sites
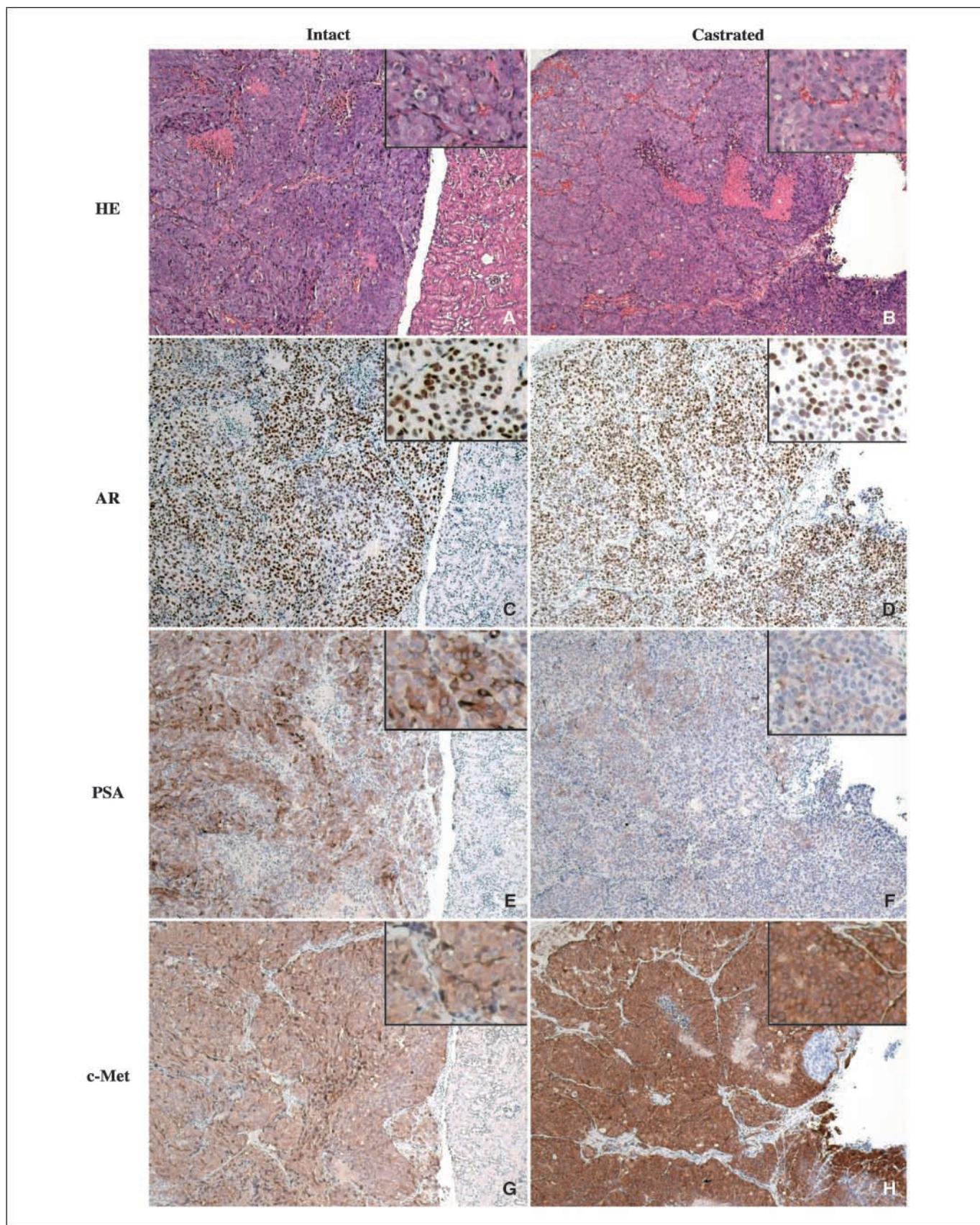
Figure 3. AR interferes with Sp1 binding ability to c-Met promoter. *A,* a schematic representation of four double-stranded oligonucleotides from the c-Met promoter region. *B,* EMSA analyses were carried out with DU145 nuclear extracts (10 μg/lane) and the ^{32}P-labeled 0.2 c-Met fragment. Different amounts of either the unlabeled 0.2 c-Met fragment or the unrelated DNA fragment were added into the reactions for competition assay. The anti-Sp1 antibody or normal rabbit IgG (0.5 or 1.5 μg) were also used for the supershift assay. *Arrows,* the specific DNA-Sp1 complexes and DNA-Sp1-antibody complexes. *C,* DU145 nuclear extracts were used in EMSA analysis with the ^{32}P-labeled 0.2 c-Met fragment. Different amounts of unlabeled double-stranded oligonucleotides from the c-Met promoter regions as shown in (*A*) were added into the reactions for competition assays. *D,* either 2 or 4 μL of *in vitro* synthesized AR proteins or control cell lysates were incubated with DU145 nuclear extracts and the 0.2 c-Met fragment and then analyzed by EMSA assays. *Arrows,* the specific DNA-Sp1 complexes.

located between bp −144 and −129 seem to be more active than other binding sites (34). In prostate cancer cells, we also showed that overexpression of Sp1 protein induces the transcription of the c-Met promoter. Moreover, introducing an Sp1 expression vector into AR-positive prostate cancer cells can attenuate the AR-repressive effect on c-Met promoter/reporter constructs, suggesting that AR may antagonize Sp1-mediated induction of c-Met expression.

Although nuclear hormone receptors in general are ligand-dependent transcriptional activators, transcriptional repression mediated by the receptors has also been reported (36). Several nuclear receptors, such as the glucocorticoid receptor, retinoic acid receptor, thyroid receptor, estrogen receptor, and AR, can repress transcription through interference with other transcription factors binding to their target promoters (37–39). Particularly, it has been shown that AR inhibits Sp1-induced transcription of the

rat *Lhβ* gene through a direct protein-protein interaction with Sp1 to block its binding to the promoter (40). To explore the regulatory mechanisms by which AR represses Sp1-induced c-Met activity, we did EMSA using the 0.2 c-Met promoter fragment as a probe. It seemed that Sp1 specifically binds to the 0.2 c-Met promoter fragment. By using double-stranded oligonucleotides containing either wild-type or mutated Sp1 binding sites encompassed between bp −144/−127, we further confirmed the specific binding site for Sp1 within the c-Met promoter region. Importantly, addition of the AR protein in the above system significantly interfered with the interaction between Sp1 and the 0.2 c-Met DNA fragment. These data suggest that AR represses Sp1-induced c-Met promoter activity by interfering with the binding of Sp1 to the c-Met promoter. Sequence analysis showed that there is no putative consensus sequence of the androgen response element within the 0.2 c-Met promoter. Using EMSA

Figure 4. Castration induces the expression of c-Met in LNCaP cell xenografts in SCID mice. LNCaP xenografts were established in SCID mice as described in Materials and Methods. Eight weeks after tumor cell injection, half of the mice were castrated. One week following castration, all mice were sacrificed and tumors were isolated. Tumor tissues were stained with H&E (*HE*; *A* and *B*), AR (*C* and *D*), PSA (*E* and *F*), or c-Met (*G* and *H*) antibody. *Insets,* high-power images.

with *in vitro* translated AR proteins, we were also unable to detect any interaction between AR and the 0.2 c-Met promoter fragment (data not shown). These lines of evidence indicate that the repression by AR of Sp1-mediated activation of the c-Met promoter may be through a protein-protein interaction as shown in the rat *Lhβ* promoter (40).

Although the mechanisms of how prostate cancer cells progress to androgen-insensitive states are currently unclear, it is believed that the tumor cells must either bypass or adapt the AR pathway to survive in a low-androgen microenvironment. AR expression has been observed in most androgen-insensitive prostate cancer cells (41). Multiple lines of evidence have shown that the AR may still be transcriptionally active in these androgen-insensitive cells. Based on the above observations, we further assessed the repressive role of AR in the androgen-insensitive but AR-positive prostate cancer cell CWR22Rv1. Depletion of androgens in those cells only slightly affected the transcription of PSA and c-Met, which is consistent with the previous observation that CWR22Rv1 cells are androgen-insensitive. However, knockdown of AR expression by shRNA in the above cells showed more pronounced reduction in PSA transcription. This suggests that the AR is still transcriptionally active in those androgen-insensitive cells. Interestingly, we only observed a slight effect on c-Met expression by the AR shRNA in CWR22Rv1 cells. In addition, a higher level of c-Met expression in CWR22Rv.1 cell line than its parental line CWR22 had been reported (42). In transient transfection experiments, we also observed that increasing expression of Sp1 proteins ectopically in prostate cancer cells can attenuate the repressive effect of AR on c-Met expression (data not shown). This compelling evidence may explain the increased cellular level of c-Met in androgen-insensitive but AR-positive prostate cancer cells during tumor progression.

Our findings that the AR represses Sp1-induced c-Met transcription should shed new light on the future therapeutic implications for prostate cancer treatment. Current androgen ablation therapy mainly targets the growth-promoting effects of the AR. Although the precise targets of AR activation in the growth of prostate cancer cells are currently unclear, they are believed to be critical for cellular proliferation and to be transcriptionally activated by the AR in a ligand-dependent manner. Thus, androgen ablation represses the expression of these genes, resulting in inhibition of the growth of primary prostate cancer cells. Our data demonstrating that the AR represses Sp1-induced c-Met transcription suggest a dual regulatory role for AR that can act either as a transcriptional activator or repressor in prostate cancer cells. The repressive role of AR on c-Met expression could lead to the inhibition of cell proliferation. Consistent with this idea, previous studies have shown that androgens are able to induce the expression of proliferative shutoff genes in prostate cancer cells, which promotes cell quiescence and cycle arrest (43, 44). Importantly, as we have shown in this study, the transcriptional repression of c-Met by AR can directly contribute to prostate cancer progression. Because the up-regulation of c-Met is linked to the progression of prostate cancer, it is conceivable that inhibition of AR activity through androgen ablation may increase the expression of c-Met, which might directly contribute to androgen insensitivity and more aggressive phenotypes of prostate cancer. In this regard, the therapeutic strategy to inhibit the activation of HGF/c-Met pathway should be considered and included during standard androgen ablation therapy. Further investigation of the AR-negative role on c-Met expression *in vivo* and establishment of an animal model to test an alternative therapeutic strategy would be extremely important in the field of prostate cancer research.

Acknowledgments

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